Poly(C) Synthesis by Class I and Class II CCA-Adding Enzymes†

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Received November 26, 2001; Revised Manuscript Received February 11, 2002

ABSTRACT: The CCA-adding enzymes [ATP(CTP):tRNA nucleotidyl transferases], which catalyze synthesis of the conserved CCA sequence to the tRNA 3′ end, are divided into two classes. Recent studies show that the class II *Escherichia coli* CCA-adding enzyme synthesizes poly(C) when incubated with CTP alone, but switches to synthesize CCA when incubated with both CTP and ATP. Because the poly(C) activity can shed important light on the mechanism of the untemplated synthesis of CCA, it is important to determine if this activity is also present in the class I CCA enzymes, which differ from the class II enzymes by significant sequence divergence. We show here that two members of the class I family, the archaeal *Sulfolobus shibatae* and *Methanococcus jannaschii* CCA-adding enzymes, are also capable of poly(C) synthesis. These two class I enzymes catalyze poly(C) synthesis and display a response of kinetic parameters to the presence of ATP similar to that of the class II *E. coli* enzyme. Thus, despite extensive sequence diversification, members of both classes employ common strategies of nucleotide addition, suggesting conservation of a mechanism in the development of specificity for CCA. For the *E. coli* enzyme, discrimination of poly(C) from CCA synthesis in the intact tRNA and in the acceptor-TΨC domain is achieved by the same kinetic strategy, and a mutation that preferentially affects addition of A76 but not poly(C) has been identified. Additionally, we show that enzymes of both classes exhibit a processing activity that removes nucleotides in the 3′ to 5′ direction to as far as position 74.

The tRNA CCA-adding enzyme, which catalyzes the stepwise synthesis and repair of the CCA sequence at the tRNA 3′ end, is present in organisms from all major phylogenetic lineages (*1*). The CCA sequence is the amino acid attachment site for tRNA aminoacylation and is used as a primer for initiation of replication in retroviruses (*2*). The importance of the CCA sequence emphasizes the significance of the CCA-adding enzyme. In organisms of eukarya and many archaea that do not encode the CCA sequence in tRNA genes, the CCA-adding enzyme is essential (*3*). In organisms of eubacteria that do encode the CCA sequence in tRNA genes, the enzyme is responsible for repairing damaged 3′ ends. Although the repair function is not essential (*4*), eubacterial organisms that lack the CCA-adding enzyme suffer from a significantly reduced growth rate. Presumably, they compensate for defective tRNA 3′ ends by increasing the level of *de novo* synthesis of full-length tRNA or the activities of functionally compensating enzymes (*5*).

The CCA-adding enzyme is a member of the nucleotidyl transferase superfamily (*6*, *7*), which can be divided into two classes of distinct amino acid sequence characteristics (*8*). The archaeal CCA-adding enzymes belong to class I, which includes eukaryotic poly(A) polymerase (PAP), terminal deoxy transferase (TdT), kanamycin nucleotidyl transferase, and DNA polymerase β. The eubacterial and eukaryotic CCA-adding enzymes belong to class II, which includes eubacterial PAP (*8*). In both classes, the CCA-adding enzyme is most closely related to PAP in sequence (*7*, *8*). Members of the nucleotidyl transferase family, although diverse, share the conserved DXD motif at the catalytic site. In the crystal structures of three members of the superfamily [PAP (*9*, *10*), kanamycin nucleotidyl transferase (*11*), and the DNA polymerase β enzyme (*12*−*14*)], the DXD motif coordinates with divalent metal ions to establish a metal-mediated catalysis.

An interesting feature of the CCA-adding enzyme is the synthesis of CCA without a nucleic acid template. The precise synthesis of CCA distinguishes this enzyme from other untemplated nucleic acid polymerases, such as PAP and TdT. The mechanism for the specificity of the CCA-adding enzyme is not well understood, although several models have emerged from recent studies. One model, based on studies of the class I archaeal *Sulfolobus shibatae* enzyme, suggests enzyme−RNA collaborative templating (*15*), whereby the nucleotide specificity of the catalytic site is jointly determined by the enzyme and a tRNA 3′ end. The binding of a tRNA, lacking the 3′ end, to the enzyme creates the site for addition of C74. The 3′ end then refolds with the enzyme to create the site for addition of C75. The CC terminus refolds with the enzyme to create the site for addition of A76. This "one-nucleotide-binding site" model suggests that the CTP- and ATP-binding sites overlap at the

† This work was supported by grants from the NIH (GM56662 to Y.-M.H. and 1R15GM57685 to D.L.T.) and the American Cancer Society (PRG-99-347-01-GMC to Y.-M.H.).

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10.1021/bi0120953 CCC: $22.00
Published on Web 03/15/2002

catalytic site. In the dimeric *S. shibatae* enzyme, this model can be modified into a "scrunching−shuttling" model (*16*), where the 3′ end scrunches on one subunit for synthesis of CC and shuttles to the other for addition of A76.

A second model, based on the poly(C) synthesis activity of the class II rabbit liver and *Escherichia coli* enzymes (*17*, *18*), suggests that these enzymes possess an ATP-binding site and that occupation of the ATP site can regulate the extent of poly(C) synthesis (*18*). Recent studies show that the *E. coli* enzyme alone, when isolated from cell lysate, exists in an ATP-bound form (*19*). Removal of the bound ATP allows the enzyme to synthesize CCC or poly(C), whereas addition of ATP switches the enzyme specificity to synthesize CCA (*18*, *19*). The existence of an ATP-bound form in the absence of an RNA substrate and the inhibition of poly(C) synthesis by ATP support a distinct binding site of ATP. Kinetic studies show that the poly(C) synthesis is two-phased, where the addition of C74 and C75 occurs in the first and faster phase while addition of further Cs occurs in the second and slower phase (*17*, *19*). The two-phase kinetics suggest conformational changes of the enzyme−tRNA complex after addition of CC.

The poly(C) activity may shed important new light on the mechanism of CCA synthesis. For example, the synthesis of poly(C) by the rabbit liver and *E. coli* enzymes clearly indicates that the complex of an enzyme and the CC end does not necessarily specify A76. This is in contrast to the one-site model, which suggests synthesis of only CCA after the CC end. Also, the isolation of the *E. coli* enzyme with a tightly bound ATP suggests a pre-existing ATP-binding site, in contrast to the one-site model, which suggests that the ATP site is created after the enzyme forms a complex with the CC end. However, the poly(C) activity is so far only reported for the class II rabbit liver and *E. coli* enzymes, and this raises the question of whether the class I *S. shibatae* enzyme might have this activity. Because the two classes of enzymes differ substantially in primary sequences, the possibility exists that they operate by different mechanisms of CCA synthesis. Here, we show that the poly(C) activity of the class II *E. coli* enzyme is shared in common with two members of the class I family, the archaeal *S. shibatae* and *Methanococcus jannaschii* (*20*) CCA-adding enzymes. In addition, the kinetic basis for discrimination of poly(C) from CCA synthesis by the two classes is the same. Further, a mutation in the class II *E. coli* enzyme has been isolated, which recapitulates features of a previously isolated mutation in the class I *S. shibatae* enzyme. These studies emphasize the similarity of the two classes and suggest a common mechanism for specifying synthesis of CCA.

MATERIALS AND METHODS

CCA-Adding Enzymes. The gene for the wild-type *E. coli* CCA-adding enzyme was cloned into the expression vector pET-22b(+) (Novagen) between the *Nde*I and *Bam*HI sites, and the TGA termination codon was changed to the CTA leucine codon for expression with a C-terminal His tag (*18*). The gene for the D93A mutant was constructed according to the QuickChange (Stratagene) protocol. The gene for the wild-type *M. jannaschii* enzyme was amplified from cloned genomic DNA (pAMJFQ90, American Type Culture Collection, Rockville, MD) by PCR and was cloned into the *Bam*HI and *Kpn*I sites of the plasmid pQE32 (Qiagen) for expression with an N-terminal His tag (*20*). The gene for the wild-type *S. shibatae* enzyme was cloned into the *Nde*I and *Eco*RI sites of the plasmid pET-17xb (Novagen) for expression with an N-terminal His tag, and was a gift from A. M. Weiner (University of Washington, Seattle, WA) (*8*). Recombinant *E. coli* and *S. shibatae* enzymes were expressed in *E. coli* strain BL21(DE3), while the recombinant *M. jannaschii* enzyme was expressed in SG13009/REP4. Purification of His-tagged CCA-adding enzymes was carried out with the Co^{2+}-chelated TALON resin (ClonTech) according to the manufacturer's instructions. The purified enzymes were stored in 0.1 M glycine (pH 9.0), 10 mM DTT, and 40% glycerol at −20 °C.

Substrates for the CCA-Adding Enzyme. Minihelices were synthesized by the Nucleic Acid Facility of the University of Pennsylvania (Philadelphia, PA). Reconstituted $tRNA^{Ala}$ substrates were prepared by annealing the large fragment (57-mer) with a small fragment (19-mer) in equal molar amounts, heating at 70 °C for 3 min, and slowly cooling at 37 °C for 20 min. The large fragment was prepared by T7 transcription of the gene for *E. coli* $tRNA^{Ala}$ restricted with *Taq*I, while the small fragment was prepared by chemical synthesis (Dharmacon). Labeling of minihelices at the 5′ end was achieved by T4 polynucleotide kinase (5 units, New England Biolabs) and [γ-^{32}P]ATP (3000 Ci/mmol, NEN) at 37 °C for 15 min. After heat inactivation of the kinase enzyme, the labeled minihelices were separated from free ATP by a denaturing 12% polyacrylamide (PAGE)/7 M urea gel, eluted from the gel by crushing and soaking in 0.125 M ammonium acetate, 0.125 mM EDTA, and 0.025% SDS, and precipitated with ethanol.

Assays for Synthesis of CCA and Poly(C). The addition of CCA to minihelices and to reconstituted tRNAs was assayed as described previously (*18*). The *E. coli* enzyme was assayed at 37 °C, while the *M. jannaschii* and *S. shibatae* enzymes were assayed at 70 °C. The addition of poly(C) was assayed with 50 μM CTP and no ATP under the same buffer conditions. Aliquots were removed from an assay reaction mixture at various time points and mixed with an equal volume of a denaturing dye containing 89 mM Tris-HCl (pH 8.1), 89 mM boric acid, 2 mM EDTA, 7 M urea, bromophenol blue (0.1%), and xylene cyanol (0.1%). The mixture was separated by electrophoresis on a denaturing 15% PAGE/7 M urea gel, and amounts of product and substrate were quantified by a phosphorimager (Molecular Dynamics). Kinetic parameters K_m and k_{cat} were calculated from the Lineweaver−Burk plot of a series of reactions over a range of substrate concentrations.

Inhibition Studies. AMPCPP (Sigma) as an inhibitor was added to reaction mixtures that monitored the addition of ATP or CTP to the 5′-labeled Val-34C substrate, for synthesis of CCA or CCC, respectively. The assay conditions for synthesis of CCA and CCC were as described in the footnotes of Table 1, except that the ATP concentration and the CTP concentration were 150 and 150 μM, respectively. The residual activity of synthesis of CCA or CCC was measured in response to increasing concentrations of the inhibitor.

Table 1: Kinetic Parameters of Addition of One Nucleotide to the CC Terminus of the Val-34C Minihelix[a]

	CTP			ATP			
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	CTP/ATP
E. coli	332 ± 31	0.73 ± 0.14	2.2×10^3	144 ± 39	15.9 ± 2.9	1.1×10^5	0.02
M. jannaschii	430 ± 31	1.2 ± 0.14	2.8×10^3	287 ± 3	11.5 ± 1.1	4.0×10^4	0.07
S. shibatae	515 ± 5	0.16 ± 0.01	3.1×10^2	500 ± 7	2.1 ± 0.1	4.2×10^3	0.07

[a] Kinetics for CTP addition were measured using the Val-34C substrate (50 μM, 1×10^5 cpm) with 100−500 μM CTP and the *E. coli* (2 μM), *M. jannaschii* (3 μM), and *S. shibatae* (3 μM) enzymes. Kinetics for ATP addition were measured using Val-34C (50 μM, 1×10^5 cpm) with varying concentrations of ATP and the *E. coli* (0.5 μM, 50−250 μM ATP), *M. jannaschii* (0.5 μM, 50−500 μM ATP), and *S. shibatae* (1.5 μM, 100−1000 μM ATP) enzymes. The RNA was labeled at the 5′ end, and the fraction of RNA that was shifted to one nucleotide longer was calculated as a measure of initial velocity. For CTP addition, time points were taken between 0 and 80 min and between 0 and120 min for the *E. coli* and the two archaeal enzymes, respectively. For ATP addition, time points were taken between 0 and 40 min, between 0 and 60 min, and between 0 and 120 min for the *E. coli*, *M. jannaschii*, and *S. shibatate* enzymes, respectively. Initial rates of the time course were used to determine kinetic parameters.

RESULTS

Poly(C) Addition on Minihelices. The minihelix domain of *E. coli* tRNA^{Val}, consisting of the acceptor stem and the TΨC stem−loop portion of the full-length tRNA (Figure 1A), was used as a substrate to test poly(C) addition by CCA-adding enzymes. The minihelix domain was attractive because it can be chemically synthesized with precise 3′ ends for investigation of CCA-adding enzymes. This is clearly an advantage over the full-length tRNA substrates, which are usually prepared enzymatically by T7 RNA polymerase and as such suffer from an inherent property of the polymerase that generates heterogeneous 3′ ends (*21*). Recent studies have shown that minihelices terminating in different positions of the NCCA 3′ end are efficient substrates for the *E. coli* CCA-adding enzyme, displaying kinetic parameters similar to those of the full-length tRNA (*18*, *22*, *23*). Also, the backbone of minihelices requires ribose only at the 3′ terminus and can accommodate deoxy in the rest of the molecule (*23*). In this study, deoxy minihelices were prepared, containing ribose moiety at positions 73−76 to provide consistency for the entire 3′ end (*18*).

To test the poly(C) activity, the concentration of a CCA-adding enzyme was at least 15-fold lower than that of the RNA substrate and at least 200-fold lower than that of the nucleotides. Under these conditions, the enzyme acted as a catalyst and the initial rate of the reaction was measured over a time course. Three minihelices were tested for poly(C) activity, Val-34C (a 34-mer that terminated with a C at position 75), Val-35C (a 35-mer that terminated with a C at position 76), and Val-36C (a 36-mer that terminated with a C at position 77) (Figure 1A). Under the same condition that was used for CCA addition, except for the omission of ATP, all three minihelices were substrates for poly(C) addition by the *E. coli* CCA-adding enzyme (Figure 1B, the *Ec* lanes). Specifically, the enzyme extended the CC terminus of Val-34C to CCC in the presence of [α-^{32}P]CTP. The labeled product was a 35-mer and was separated from the starting 34-mer (Figure 1B, 5′-end-labeled materials in lanes marked with "−") by one nucleotide on a denaturing PAGE. The enzyme extended the Val-35C and Val-36C substrates to a 36-mer and a 37-mer, respectively, generating CCCC and CCCCC at the 3′ ends, respectively. Extension to minor products of longer lengths was also detected, demonstrating the feature of poly(C). These results confirmed recent studies that, in the absence of ATP, the *E. coli* CCA-adding enzyme catalyzes synthesis of poly(C) (*18*).

Both the *M. jannaschii* and *S. shibatae* CCA-adding enzymes are of hyperthermophilic archaeal origin (*8*, *20*), and thus, they were assayed at 70 °C under conditions otherwise identical to those for the *E. coli* enzyme. Both enzymes showed the poly(C) activity with minihelices (Figure 1B, the *Mj* and *Ss* lanes, respectively). With Val-34C, both catalyzed addition of one C to the substrate to generate a 35-mer. With Val-35C and Val-36C, both catalyzed multiple additions of C to generate poly(C) as a ladder of discrete bands near the region of the starting materials.

In addition to poly(C) synthesis, all three enzymes (*Ec*, *Mj*, and *Ss*) generated labeled products identical in size to, or shorter than, the starting materials. The labeled product of the same length indicated an exchange reaction between the terminal C and [α-^{32}P]CTP, whereas products of shorter lengths indicated processing of the 3′ end and resynthesis of Cs with [α-^{32}P]CTP. With the Val-34C substrate, the *M. jannaschii* enzyme synthesized a labeled 34-mer, indicating an exchange activity. This activity was not detected for the *E. coli* or *S. shibatae* enzyme. However, with Val-35C and Val-36C, which contained abnormal numbers of Cs at the 3′ end, the exchange and processing activity was evident with all three. For example, with Val-36C, all three enzymes generated 34- and 33-mers. Interestingly, the smallest product among various processing reactions was the 33-mer, which corresponded to the minihelix ending with ^{32}P-labeled C74. Thus, the processing and exchange activity terminated prior to A73 in the minihelix framework. The specific termination of processing at position 74 argued against contamination of the three enzymes by nonspecific nucleases. Also, the processing activity was absent from the *E. coli* or *S. shibatae* enzyme with the Val-34C substrate. To further confirm the absence of nonspecific nucleases, a 5′-^{32}P-labeled Val-34C was incubated with all three enzymes without CTP and, after incubation for 45 min, examined by denaturing PAGE. The labeled substrate remained essentially the same as in the control without a CCA enzyme[1] (Figure 1C). If nonspecific nucleases were present, the substrate would have been degraded to generate 33- and 32-mers and fragments of smaller sizes.

Poly(C) Addition on Reconstituted tRNAs. Reconstituted tRNAs with different 3′ ends were prepared to further test the poly(C) activity of the three CCA-adding enzymes to

[1] Abbreviation: CCA enzymes, CCA-adding enzymes.

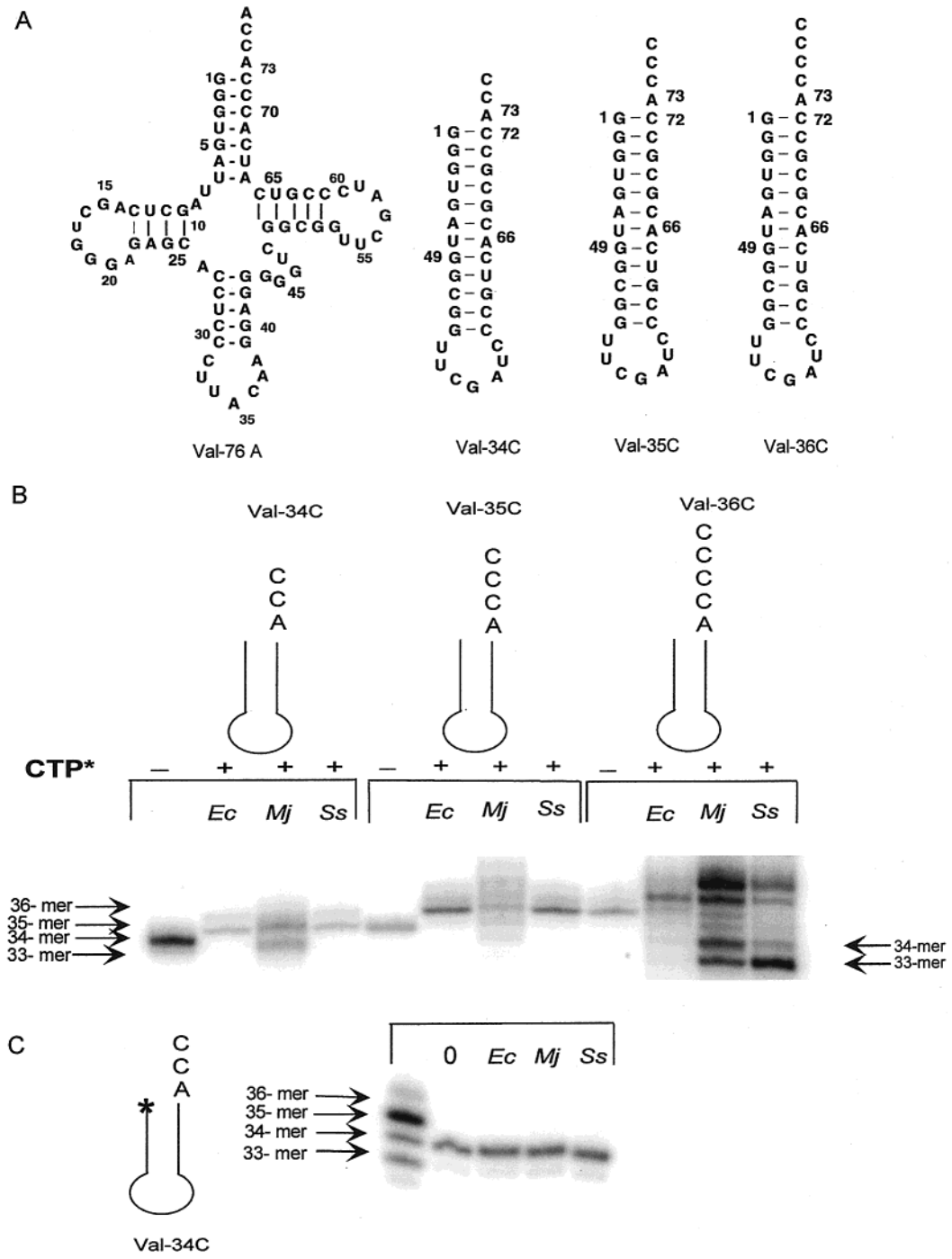

FIGURE 1: (A) Sequences and secondary structures of tRNA and minihelices used in the study. Val-76A, full-length tRNA of *E. coli* $tRNA^{Val}$, ending with A76. Val-34C, 34-mer of the minihelix domain (the acceptor−TΨC stem−loop portion) of *E. coli* $tRNA^{Val}$, ending with C75. Val-35C, 35-mer of the minihelix domain of $tRNA^{Val}$, ending with C76. Val-36C, 36-mer of the minihelix domain of $tRNA^{Val}$, ending with C77. Numbering on tRNA and minihelices is based on standard features of yeast $tRNA^{Phe}$. (B) Addition of poly(C) from [α-^{32}P]CTP (50 μM) to Val-34C, Val-35C, and Val-36C (20 μM) by the *E. coli* (*Ec*, 2 μM), *M. jannaschii* (*Mj*, 0.75 μM), and *S. shibatae* (*Ss*, 3 μM) CCA-adding enzymes. Reaction mixtures were incubated for 30 min at 37 °C for the *E. coli* enzyme and at 70 °C for the *M. jannaschii* and *S. shibatae* enzymes, and analyzed with a 15% PAGE/7 M urea gel, which was developed by a phosphorimager (Molecular Dynamics). Lanes without [α-^{32}P]CTP contained substrate RNAs (34-, 35-, and 36-mers, respectively) labeled at the 5′ end with [γ-^{32}P]-ATP and T4 polynucleotide kinase. (C) The 5′-labeled Val-34 (20 μM) incubated with no enzyme (0), *E. coli* (2 μM), *M. jannaschii* (0.75 μM), and *S. shibatae* (3 μM) enzymes for 30 min at 37 (*Ec*) or 70 °C (*Mj* and *Ss*) and then examined with a 15% PAGE/7 M urea gel.

ensure that this activity was not due to an artifact of using the isolated acceptor stem domain. Reconstitution was achieved by annealing two fragments of a tRNA that can complement each other through hydrogen bonding interactions to re-establish the structure of the native enzyme, containing all subdomains of the tRNA tertiary structure. The sequence framework of *E. coli* $tRNA^{Ala}$ was chosen for reconstitution (Figure 2A), as cleavage at the *Taq*I site in the gene can provide the templates to synthesize a large RNA (positions 1−57) and a small RNA (positions 58−76) fragments (Figure 2B). The large fragment (57-mer) was prepared by runoff transcription of the tRNA gene restricted at the *Taq*I site near position 57, while the small fragment (18- or 19-mer) was prepared by chemical synthesis to ensure

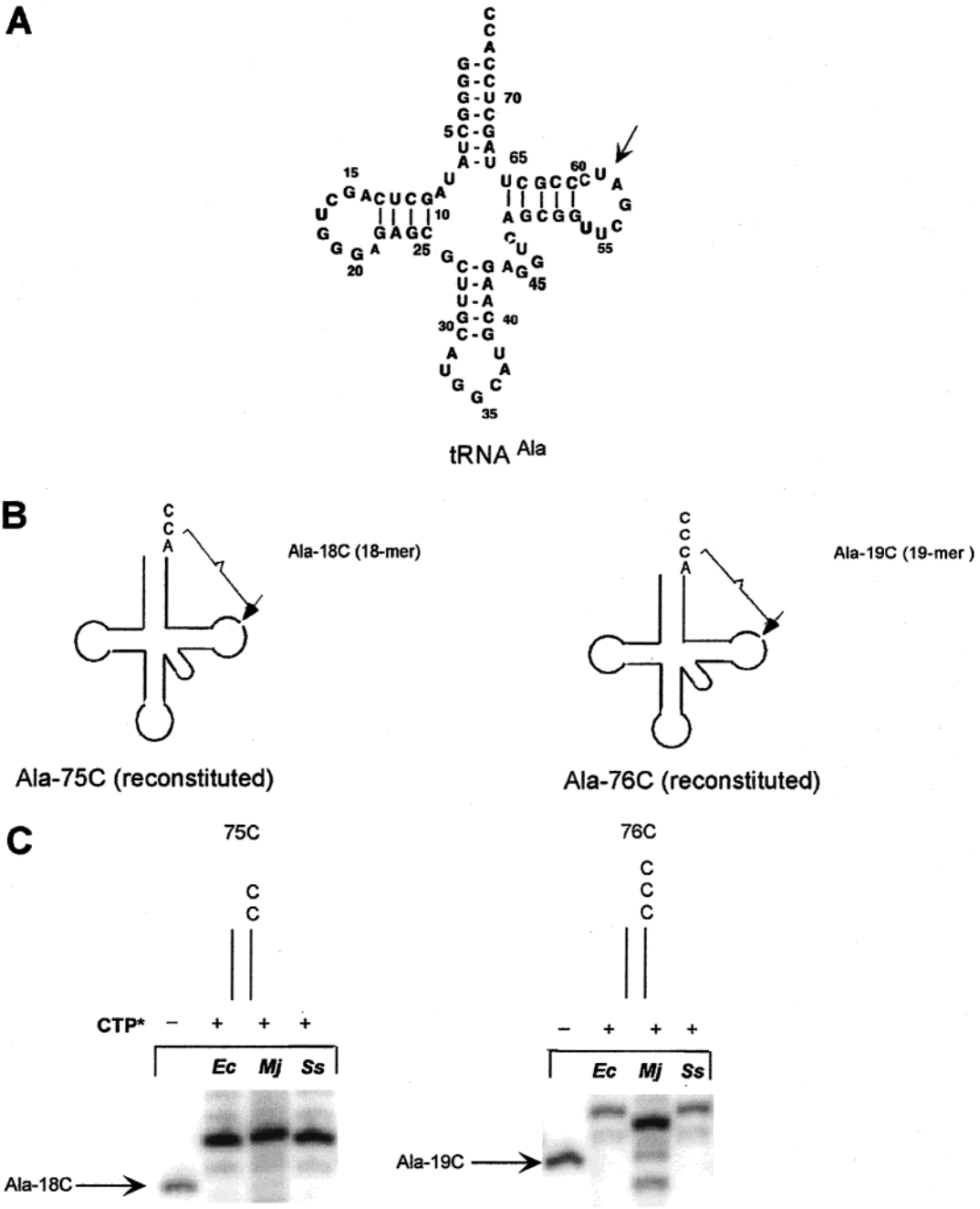

FIGURE 2: (A) Sequence and cloverleaf structure of *E. coli* tRNA^{Ala} (CC). The arrow indicates the site where the full-length tRNA is separated into two fragments, the 57-mer and the 18- or 19-mer. (B) Creation of reconstituted constructs of *E. coli* tRNA^{Ala}, containing variations on the 3′ end. Ala-75C consisted of a 57-mer (nucleotides 1−57) and an 18-mer (Ala-18C) that terminated with the CC sequence. Ala-76C consisted of the same 57-mer as Ala-75C but with a 19-mer (Ala-19C) that terminated with the CCC sequence. (C) Addition of poly(C) from [α-^{32}P]CTP (50 μM) to Ala-75C (20 μM) and Ala-76C (20 μM) by the *E. coli* (*Ec*, 2 μM), *M. jannaschii* (*Mj*, 0.75 μM), and *S. shibatae* (*Ss*, 3 μM) CCA-adding enzymes. Reaction mixtures were incubated for 30 min and analyzed with a 15% PAGE/7 M urea gel, which was developed by phosphorimaging (Molecular Dynamics). Lanes without [α-^{32}P]CTP contained Ala-18C labeled at the 5′ end.

a precise 3′ terminus. A reconstituted *E. coli* tRNA^{Ala}, composed of these two fragments, was recently shown to be an efficient substrate for CCA synthesis by the *E. coli* enzyme (*18*).

Two reconstituted tRNAs were prepared and tested (Figure 2B). The Ala-75C tRNA carried the CC terminus, while the Ala-76C tRNA carried the CCC terminus. Both were substrates for addition of poly(C), and this was observed with all three enzymes. Addition of poly(C) was examined by denaturing PAGE analysis of the small fragment, which was compared to markers of 5′-labeled starting materials (Ala-18C for Ala-75C or Ala-19C for Ala-76C). The large fragment in the reconstituted tRNA was not the substrate for extension and was thus not labeled. With both the Ala-75C and Ala-76C substrates, the major extension product for all three enzymes was addition of one C to the small fragment, while products of multiple additions were also evident. In this analysis (Figure 2C), the markers Ala-18C and Ala-19C were labeled with ^{32}P at the 5′ end, and due to a higher charge/mass ratio, they migrated faster than the unphosphorylated Ala-18C and Ala-19 used in the reconstitution experiment. The effect of the charge/mass ratio is

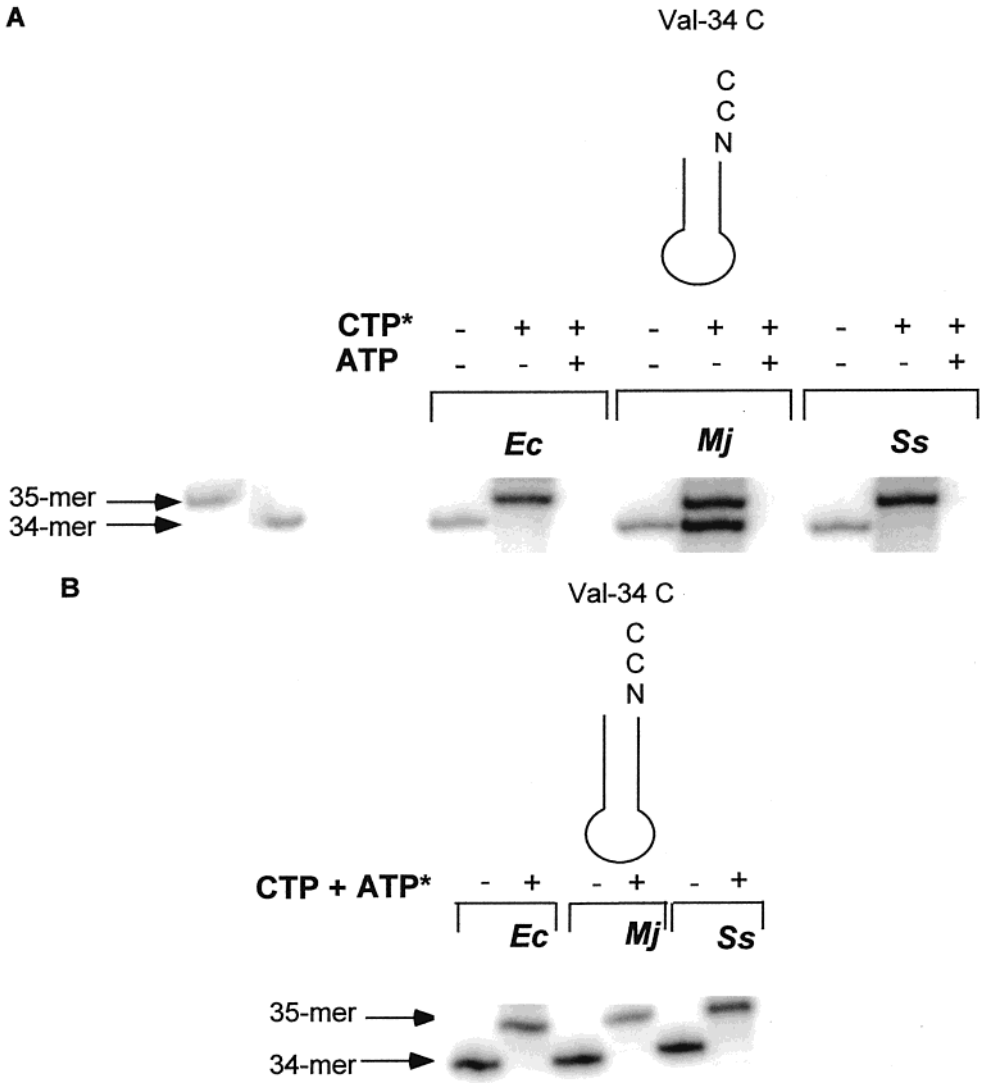

FIGURE 3: (A) Inhibition of C addition to the CC terminus of Val-34C by ATP of the *E. coli* (*Ec*, 1 μM), *M. jannaschii* (*Mj*, 0.5 μM), and *S. shibatae* (*Ss*, 1.5 μM) enzymes: left, a marker of Val-34C labeled at the 5′ end; center, 12 μM Val-34C incubated with 50 μM [α-^{32}P]-CTP; and right, 12 μM Val-34C incubated with 50 μM CTP and 100, 500, and 1000 μM ATP for the *E. coli*, *M. jannaschii*, and *S. shibatae* enzymes, respectively. (B) Extension of the CC terminus of Val-34C by ATP of the *E. coli* (0.5 μM), *M. jannaschii* (0.1 μM), and *S. shibatae* (5 μM) enzymes, respectively: left, a marker of Val-34C labeled at the 5′ end; and right, 12 μM Val-34C incubated with 50 μM CTP and 50 μM [α-^{32}P]ATP. Reaction mixtures were incubated for 40 (A) or 30 min (B), analyzed with a 15% PAGE/7 M urea gel, and examined by phosphorimaging (Molecular Dynamics).

more dramatic for smaller sizes of Ala-18C and Ala-19C than for larger sizes of Val-34C and derivatives, as shown in Figure 1.

The processing and exchange activity was detected with the Ala-76C substrate, which terminated with the abnormal CCC (Figure 2C). This is clearly seen with the *M. jannaschii* enzyme. However, the processing and exchange activity was reduced with Ala-75C, which terminated with the normal CC. These results reproduced the pattern with the minihelix substrates, showing that the abnormal CCC end is a better substrate than the normal CC for processing. The reproduction provides evidence that the processing activity was not an artifact of minihelices but was also present with reconstituted tRNA.

Effect of ATP and AMPCPP on Poly(C) Synthesis. Recent studies showed that ATP inhibits poly(C) synthesis of the *E. coli* enzyme. When incubated with ATP and CTP, the *E. coli* enzyme arrests synthesis of CCC and switches to synthesize CCA (*18*, *19*). The inhibitory effect of ATP on synthesis of poly(C) was verified with the Val-34C substrate (Figure 3A, lanes *Ec*). In the presence of CTP alone, the *E. coli* enzyme catalyzed addition of one C to the CC terminus to synthesize CCC, but this addition was blocked by the presence of ATP. Separate experiments with [α-^{32}P]ATP and unlabeled CTP confirmed that the enzyme instead incorporated the labeled ATP to synthesize CCA (Figure 3B, lanes *Ec*).

The inhibitory effect of ATP on poly(C) synthesis was also observed with the *M. jannaschii* and *S. shibatae* enzymes. Both extended the CC terminus of Val-34C with one C when incubated with CTP alone, although the *M. jannaschii* enzyme also exhibited a substantial exchange activity (Figure 3A, lanes *Mj* and *Sc*). Upon addition of ATP to the incubation mixture, the two enzymes arrested the addition with C and switched to synthesize CCA (panels A and B of Figure 3, lanes *Mj* and *Ss*). However, the inhibitory effect of ATP on these two enzymes was distinguished from that on the *E. coli* enzyme by two features. First, the

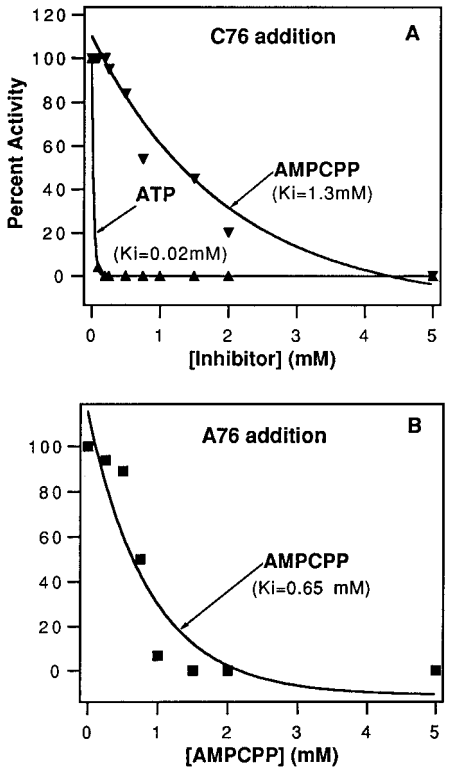

FIGURE 4: (A) Inhibition of addition of C76 to Val-34C by ATP and AMPCPP, where the concentration of the *E. coli* enzyme was 1 μM, the concentration of the 5′-labeled Val-34C was 12 μM, and the concentration of CTP was 150 μM. (B) Inhibition of addition of A76 or C76 to Val-34C by AMPCPP, where the concentration of the *E. coli* enzyme was 1 μM, the concentration of the 5′-labeled Val-34C was 12 μM, and the concentration of ATP or CTP was 150 μM.

concentration of ATP required to achieve complete inhibition was higher. While the *E. coli* enzyme required 100 μM ATP to completely arrest synthesis of CCC, the *M. jannaschii* and *S. shibatae* enzymes required 500 μM and 1 mM ATP, respectively. Second, while the effect of ATP on the *E. coli* enzyme was instantaneous, that on the two archaeal enzymes required a preincubation at 37 °C for 10 min. Without the preincubation, the two archaeal enzymes maintained a weak activity of synthesis of CCC (not shown).

To determine if hydrolysis of ATP is required for inhibition of poly(C), the nonhydrolyzable analogue AMPCPP was tested. AMPCPP had a much weaker inhibitory effect than ATP on poly(C) synthesis. The substrate Val-34C containing the CC end was 5′-labeled with ^{32}P, and the synthesis of the CCC end by the *E. coli* enzyme in the presence of ATP or AMPCPP was monitored. While ATP effectively inhibited the reaction with an apparent K_i (concentration of the inhibitor that reaches 50% inhibition) of 20 μM, AMPCPP inhibited the reaction with an apparent K_i of 1.3 mM (Figure 4A). The 65-fold difference in the inhibitory effect demonstrates the importance of the α-phosphate of ATP. Further, AMPCPP was not an efficient competitor of ATP in the synthesis of CCA from the Val-34C substrate. The apparent K_i for inhibition of ATP addition was 650 μM (Figure 4B), which is 4.5-fold higher than the K_m for ATP addition to the same substrate (144 μM) (see Table 1; also ref *23*). These results show that the enzyme does not recognize AMPCPP in the same way as ATP for inhibition of poly(C), or for synthesis of CCA.

Effect of Mutations on Poly(C) Synthesis. Recent studies of the *S. shibatae* enzyme show that mutations of D53 and D55, located at the DXD motif, eliminated addition of C75 and A76, whereas mutation of D106 selectively impaired addition of A76 but not C75 (*24*). Thus, D53 and D55 are interpreted as being in the catalytic site, while D106 may be a component of the ATP site that contributes to the ATP adding specificity. Guided by a multiple-sequence alignment, we mutated D21, D23, and D93 in the *E. coli* enzyme (*20*) (which correspond to D53, D55, and D106, respectively, of the *S. shibatae* enzyme) to alanine and examined the effects of mutation. The mutant *E. coli* enzyme harboring D21A/D23A in the DXD motif was inactive for addition of C75 to Val-33C and inactive for addition of A76 to Val-34C (Figure 5A,B), as expected. The mutant enzyme harboring D93A was active for addition of C75 (Figure 5A) at a level similar to that of the wild type, but severely impaired for addition of A76 (Figure 5B, <0.001-fold of that of the wild type). This reproduces the phenotype of the D106A mutation of the *S. shibatae* enzyme. The parallel effects of mutations in the class I *S. shibatae* and class II *E. coli* enzymes provide independent support for the sequence alignment (*20*).

The D93A mutant of the *E. coli* enzyme was active for poly(C) synthesis, and it catalyzed addition of C76 to Val-34C in the absence of ATP (Figure 5C). Although this C76 addition activity is 0.13-fold of that of the wild type, it is significantly greater than the A76 addition activity. Analysis of the relative activity of addition of C75 compared to that of addition of C76 showed that the wild type and the D93A mutant are comparable. The wild-type enzyme suffered a decrease of 15-fold from addition of C75 to addition of C76 (*19*), and the mutant suffered a 30-fold decrease (Figure 5A,C). Thus, to a first approximation, the mutant has maintained the relative activities of the wild type of the synthesis of C75 and C76. In the presence of ATP, the D93A mutant differed from the wild type in its ability to continue the synthesis of poly(C) (Figure 5D). At the concentration of ATP (0.2 mM) that effectively terminated poly(C) synthesis of the wild type, the mutant maintained poly(C) synthesis. Because the mutant was unable to catalyze addition of A76, it presumably failed to bind ATP, which may explain the lack of sensitivity of poly(C) synthesis to ATP. The continued synthesis of poly(C), despite a defect in the ATP binding site, suggests that the donor CTP has its own binding site.

Kinetics of Synthesis of Poly(C). The kinetic parameters of poly(C) synthesis were determined for the three wild-type enzymes (*Ec*, *Mj*, and *Ss*) to gain insights into possible differences from those of CCA synthesis. The Val-34C minihelix, containing the natural CC end, was used as a substrate, and conditions of kinetics were such that only one C was added to the substrate. The substrate was labeled at the 5′ end, and the fraction that was converted to product was analyzed by denaturing PAGE and quantified by a phosphorimager. Initial rates of addition over a range of CTP concentrations were collected under steady-state Michaelis−Menten conditions. These rates were used to determine the parameters K_m and k_{cat} and to derive k_{cat}/K_m for addition of C76. In parallel, kinetic parameters K_m, k_{cat}, and k_{cat}/K_m were determined for addition of A76 over a range of ATP concentrations. The values for addition of A76, which have been published (*8*, *23*), allowed comparison to those for

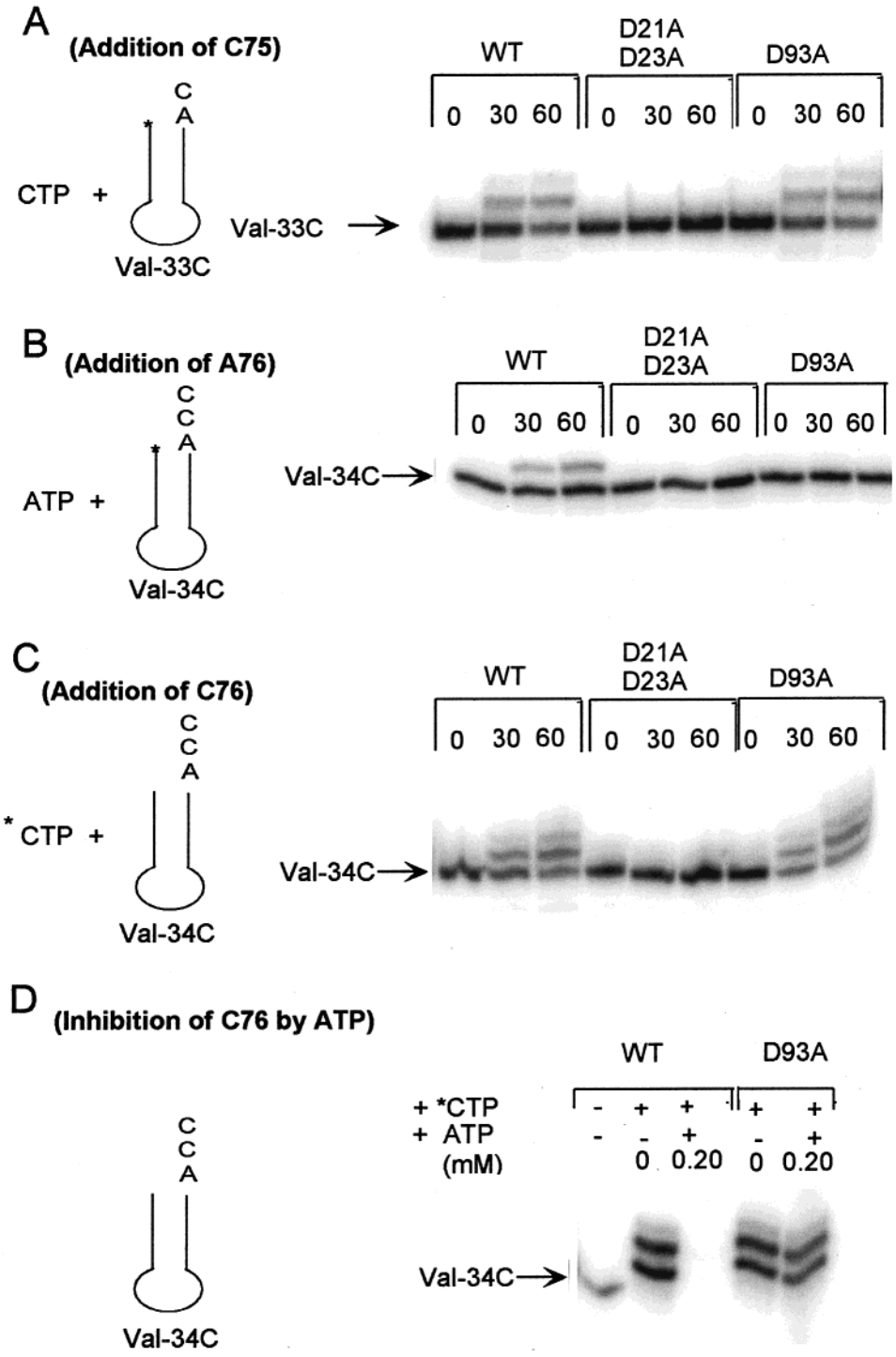

FIGURE 5: (A) Addition of C75 (0.4 mM) to 5′-labeled Val-33C (30 μM) by the wild type (WT, 2 μM) and D21A/D23A (5 μM) and D93A (5 μM) mutants of the *E. coli* enzyme over the indicated times. (B) Addition of A76 (0.2 mM) to 5′-labeled Val-34C (30 μM) by the WT (0.5 μM) and D21A/D23A (15 μM) and D93A (15 μM) mutants of the *E. coli* enzyme over indicated times. (C) Addition of C76 (0.4 mM) to 5′-labeled Va-34C (30 μM) by the WT (2 μM) and D21A/D23A and D93A mutants (15 μM each) of the *E. coli* enzyme. (D) Inhibitory effect of ATP (0 and 0.2 mM) on the 40 min synthesis of C76 ([CTP] = 0.2 mM) onto Val-34C (30 μM) by the WT (1.5 μM) and D21A/D23A and D93A mutants (15 μM each) of the *E. coli* enzyme. The labeled marker Val-35C is provided to indicate the position of addition of one C to Val-34C.

addition of C76 and provided internal controls to eliminate sample variations and to verify that reaction conditions were appropriate.

Table 1 summarizes kinetic analysis for all three enzymes. For the *E. coli* enzyme, the apparent K_m for A addition to the CC terminus (144 μM) was in good agreement with that determined recently (175 μM) on a similar substrate (*23*). The apparent K_m for C addition (332 μM) was higher than the K_m for the normal addition of C75 (25 μM). However, the apparent K_m for C-addition was comparable to that for A addition, and it is the k_{cat} that served to differentiate the two additions. The k_{cat} for C addition (0.73 min^{-1}) was smaller than that for A addition (15.9 min^{-1}) by 22-fold. The overall k_{cat}/K_m ratio for the C to A addition (2.2×10^3 vs 1.1×10^5 min^{-1} M^{-1}) is 0.02. Similarly, the *M. jannaschii* and *S. shibatae* enzymes use k_{cat} as the primary discriminating

Table 2: Kinetic Parameters of the RNA Substrate for Nucleotide Addition to the CC Terminus by the *E. coli* CCA-Adding Enzyme[a]

	CTP addition			ATP addition			
RNA	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	CTP/ATP
Val-34C	366 ± 5	0.07 ± 0.02	2.0×10^2	263 ± 10	1.3 ± 0.1	5×10^3	0.04
Ala-75C	145 ± 6	0.08 ± 0.06	5.5×10^2	54 ± 4	1.1 ± 0.1	2×10^4	0.03

[a] The minihelix Val-34C was assayed in the range of 50−500 μM by 0.5 μM *E. coli* enzyme, and the reconstituted Ala-75C was assayed in the range of 25−150 μM by 0.5 μM enzyme. CTP and ATP concentrations were 1.5 mM each. Time points for Val-34C and Ala-75C were taken at 0−120 and 0−60 min, respectively.

factor to distinguish C addition from A addition. For the *M. jannaschii* enzyme, the difference in K_m between C and A addition is less than 2-fold, while the difference in k_{cat} is 10-fold, which accounts for the overall k_{cat}/K_m ratio for C addition to A addition of 0.07. For the *S. shibatae* enzyme, the K_m values for C and A addition are comparable, while the k_{cat} values differ by 13-fold, which accounts for the overall k_{cat}/K_m ratio for C addition to A addition of 0.07. This is identical to that of the *M. jannaschii* enzyme and close to that of the *E. coli* enzyme (0.02).

Kinetic parameters associated with the RNA substrate for addition to the CC terminus were also determined to provide an independent evaluation of discrimination of CTP from ATP as the donor nucleotide to position 76. Only the parameters for the *E. coli* enzyme were determined, because this enzyme is the most discriminative against the C addition among the three enzymes that were tested (Table 1). Two RNA substrates were assayed, the Val-34C minihelix and the Ala-75C reconstituted tRNA, to compare the efficiency for the acceptor stem domain with that of the complete tRNA structure. If the enzyme discriminates C addition from A addition by recognition of the acceptor−T stem−loop domain, then the discriminatory factor for the minihelix domain should be similar to that of the tRNA. For assessment of C addition, the assay contained a saturating concentration of CTP (10-fold excess over K_m and spiked with [α-^{32}P]-CTP) and varying concentrations of an RNA substrate. For assessment of A addition, the assay contained a saturating concentration of ATP (10-fold excess over K_m and spiked with [α-^{32}P]ATP) and varying concentrations of an RNA substrate. Incorporation of the label into RNA was monitored by precipitation on filter pads in 5% trichloroacetic acid. Separate analysis by PAGE confirmed that only one nucleotide was incorporated into each substrate under the assay condition (not shown).

Table 2 summarizes kinetic parameters for the two RNA substrates. For the Val-34C substrate, the K_m increased 1.4-fold from A addition to C addition, while the k_{cat} decreased 18-fold. Thus, the driving force for discrimination against C addition is again at k_{cat}. The overall k_{cat}/K_m ratio of C addition to A addition is 0.04. The same pattern is observed for the Ala-75C substrate, where the K_m increased 2.7-fold from A addition to C addition, while the k_{cat} decreased 14-fold. The overall k_{cat}/K_m ratio of C addition to A addition is 0.03. Thus, the two RNA substrates use the same interplay of kinetic parameters to discriminate C addition from A addition. This suggests that the acceptor−TΨC stem domain common to the two RNA substrates is the primary recognition site for C addition and A addition.

DISCUSSION

Poly(C) Synthesis of the Class I CCA-Adding Enzymes. The superfamily of nucleotidyl transferases is divided into two classes based on amino acid sequences around the active site that contains the DXD motif (*7*, *8*). The active site of the class II enzymes is located in a highly conserved amino-terminal domain of 25 kDa, whereas the active site of the class I enzymes is located in different sequence motifs (*8*). Additionally, the active site α-helix of the class II enzymes is extended by four amino acids compared to that of the class I enzymes. Although the function of the extension is not clear from inspection of crystal structures of members of the superfamily, the sequence of the extension is highly conserved and thus evolutionarily significant. Also, members of the two classes are from phylogenetically distant organisms (such as the class II *E. coli* CCA enzyme and the class I archaeal enzymes of this study), and thus, a comparative analysis of these two classes of enzymes can provide insights into the evolutionary relationship of their development.

Recent studies show that the class II *E. coli* CCA enzyme exhibits a poly(C) synthesis activity that is sensitive to ATP (*18*, *19*). In the absence of ATP, this activity catalyzes multiple rounds of addition of C to the tRNA 3′ end in minihelices and in reconstituted tRNA (*18*). However, the presence of ATP attenuates the poly(C) activity such that the addition of A is preferable to that of C (*18*). The preference for A is dominant at position 76, but diminishes as the number of Cs increases (*18*). Results here show that the poly(C) activity of the *E. coli* enzyme can now be extended to two members of the class I enzymes, the archaeal *M. jannaschii* and *S. shibatae* CCA enzymes. The activity observed with the class I enzymes recapitulates major features of the class II enzymes, although differences also exist in the minimal inhibitory ATP concentrations and whether preincubation is required. These differences reflect the evolutionary divergence of class I from class II enzymes. However, because ATP regularly exists at 3−4 mM under physiological conditions (*25*, *26*), which is above the minimal concentrations required to inhibit addition of C76, these three enzymes should maintain the fidelity of CCA synthesis *in vivo*.

Discrimination of Synthesis of Poly(C) or CCC from CCA. Kinetic analysis of the poly(C) activity and comparison with the CCA activity suggest that the two classes of CCA-adding enzymes use a common mechanism to discriminate addition of C76 from addition of A76. First, the K_m for C addition is comparable (within 2-fold) to that of A addition (Table 1) but significantly higher than K_m values for C addition at positions 74 and 75 for the *E. coli* (35 μM) (*23*), *S. shibatae* (30 μM) (*8*), and *M. jannaschii* (38 μM) (unpublished results)

enzymes. The elevated K_m for addition of C76 suggests that the donor CTP is bound unfavorably. Second, the primary discrimination against C addition at position 76 from A addition is by k_{cat}, which differs between the two by at least 10-fold for all three enzymes (Table 1). The k_{cat} effect is also observed with the RNA substrate. For both the minihelix domain and the reconstituted tRNA, discrimination of addition of C76 from addition of A76 is by a large decrease in k_{cat} (13−18-fold) and a small increase in K_m (1.4−2.7-fold) (Table 2). The k_{cat} effect raises the possibility of alteration of the active site after synthesis of the CC end.

The possibility of alteration of the active site after synthesis of the CC end is supported by the difference between ATP and AMPCPP. ATP can prevent addition of C76 by acting as the preferred substrate for synthesis of CCA (Figure 3). The nonhydrolyzable analogue AMPCPP is 65-fold weaker than ATP for prevention of C76 (Figure 4A), and this emphasizes the fact that, as the analogue is not the normal substrate for synthesis of CCA, it does not readily prevent synthesis of CCC. However, at a sufficiently high concentration, the analogue is still able to completely inhibit synthesis of C76 (Figure 4A), even though it cannot be incorporated into the CC end. This suggests that binding of the analogue to the enzyme is sufficient to interfere with addition of C76. One interpretation is that binding of the analogue or ATP induces a conformational change in the active site that reduces the affinity of the enzyme for CTP and synthesis of C76.

A Model of Two Nucleotide-Binding Sites. The synthesis of C76 and poly(C) is an important determinant in evaluating different models of the untemplated synthesis of CCA. Although this activity is observed under nonphysiological conditions, where ATP is absent, the identification of the activity is important to our understanding of the mechanism of CCA synthesis. Because both classes of enzymes have the poly(C) activity and both rely on the same kinetic strategy to specify CCA, results here can provide insights into a unifying model for CCA synthesis. The addition of C76 and further Cs to the CC end implies that these enzymes are not obligated to specify A76, which does not agree with the enzyme−RNA collaborative templating model that predicts addition of only A76 to the CC end (*15*). The poly(C) activity also cannot be readily explained by the scrunching−shuttling model of the *S. shibatae* enzyme, which is a dimer and forms a tetramer with two tRNA molecules (*16*). The scrunching−shuttling model suggests that the tRNA 3′ end scrunches on one subunit of the dimer for addition of CC but that the CC end is long enough to shuttle to the other subunit for addition of A76. The two subunits are arranged such that one accommodates addition of only two Cs while the other provides only A. However, it is difficult to visualize how the CC terminus, which should enter the other subunit for addition of A76, can be added with C76 and further Cs.

The results here support the model of two nucleotide-binding sites (*18*), where a CTP site is separated from an ATP site. Previous studies that argued for the two-site model include selective inactivation of addition of A by periodate-oxidized ATP on the rabbit liver enzyme (*27*), and K_m values of CTP during addition of C74 and C75 distinct from the K_m of ATP during addition of A76. For example, the K_m values for CTP and ATP on minihelices are 25 and 175 μM, respectively (*23*), and those in natural tRNA are 15−200 μM and 0.2−3 mM, respectively (*1*, *28*). Here, the two-site model is supported by mutational analysis of the CCA and poly(C) activities. Specifically, mutations of Ds in the DXD motif eliminate addition of C75 and A76. This has been shown for the class I *S. shibatae* enzyme (*24*) and the class II *E. coli* enzyme (Figure 5A,B). It is likely that the CTP site is substantially overlapped with the single catalytic site at the conserved DXD motif. The ATP site is likely to be adjacent to D106 and D93 in the class I *S. shibatae* and class II *E. coli* enzymes, respectively, where mutation primarily eliminates addition of A76 but not C75 or C76 (Figure 5A−C). The ability of the D93A mutation of the *E. coli* enzyme to differentially affect addition of A76 from C76 is particularly enlightening. Not only is this mutation able to eliminate A76 addition, but it also alleviates the sensitivity of the C76 addition to the presence of ATP. This provides a strong argument for the separation of the ATP site from the CTP site. If the two sites were overlapping, one would expect that the D93A mutation would have a synergistic effect on addition of C76 and ATP, and the sensitivity to ATP. The D93 position is close to G70 in the *E. coli* enzyme, where a previously identified mutation (G70D) selectively inhibited addition of A76 (*29*).

To gain further insights into separation of the ATP site from the CTP site, a sequence alignment and structural modeling of the class I *M. jannaschii* and *S. shibatae* CCA enzymes superimposed onto the crystal structure of the bovine PAP enzyme was performed (Figure 6). Although mutational analysis has been extensively carried out in the *E. coli* CCA enzyme, this enzyme is a class II nucleotidyl transferase and does not align well with known crystal structures of PAP of bovine and *Saccharomyces cerevisiae*, which are members of the class I group of nucleotidyl transferases. However, a previous sequence alignment (*20*) has identified homologous residues between the *E. coli*, *M. jannaschii*, and *S. shibatae* CCA enzymes, and these residues can be further identified in the sequence of bovine PAP (Figure 6A). Specifically, the DXD motif of bovine PAP is located at positions 113−115, while the residue homologous to D93 of the *E. coli* CCA enzyme is D167 (Figure 6A). All three Ds are conserved among the bovine and yeast PAP enzymes, and *M. jannaschii* and *S. shibatae* CCA enzymes. In the crystal structure of bovine PAP (*9*), the DXD motif is at the beginning of the β_2 strand while D167 is located in the middle of the β_5 strand (Figure 6B). Although all three Ds are involved in coordination with metal ions, D167 is specifically also involved in H-bonding interaction with N6 of the adenine ring of ATP. Thus, D167 is separated from the DXD motif in distinct β strands and in distinct functional roles. This provides a plausible rationale for how their homologous residues in the CCA enzymes might represent distinct nucleotide-binding sites. Further analysis of the structures of PAP (*9*, *10*) shows that the active site is not properly positioned for the in-line attack of nucleotidyl transferase, and thus, conformational rearrangement is invoked between the enzyme, RNA, and the ATP nucleotide for poly(A) synthesis. The CCA enzyme is likely to adopt a strategy similar to that for PAP for synthesis of poly(C), but it must also differ from PAP by developing additional mechanisms for achieving the specificity for CCA.

A Processing Activity of the CCA Enzyme. Our studies suggest that both classes of CCA enzymes possess a

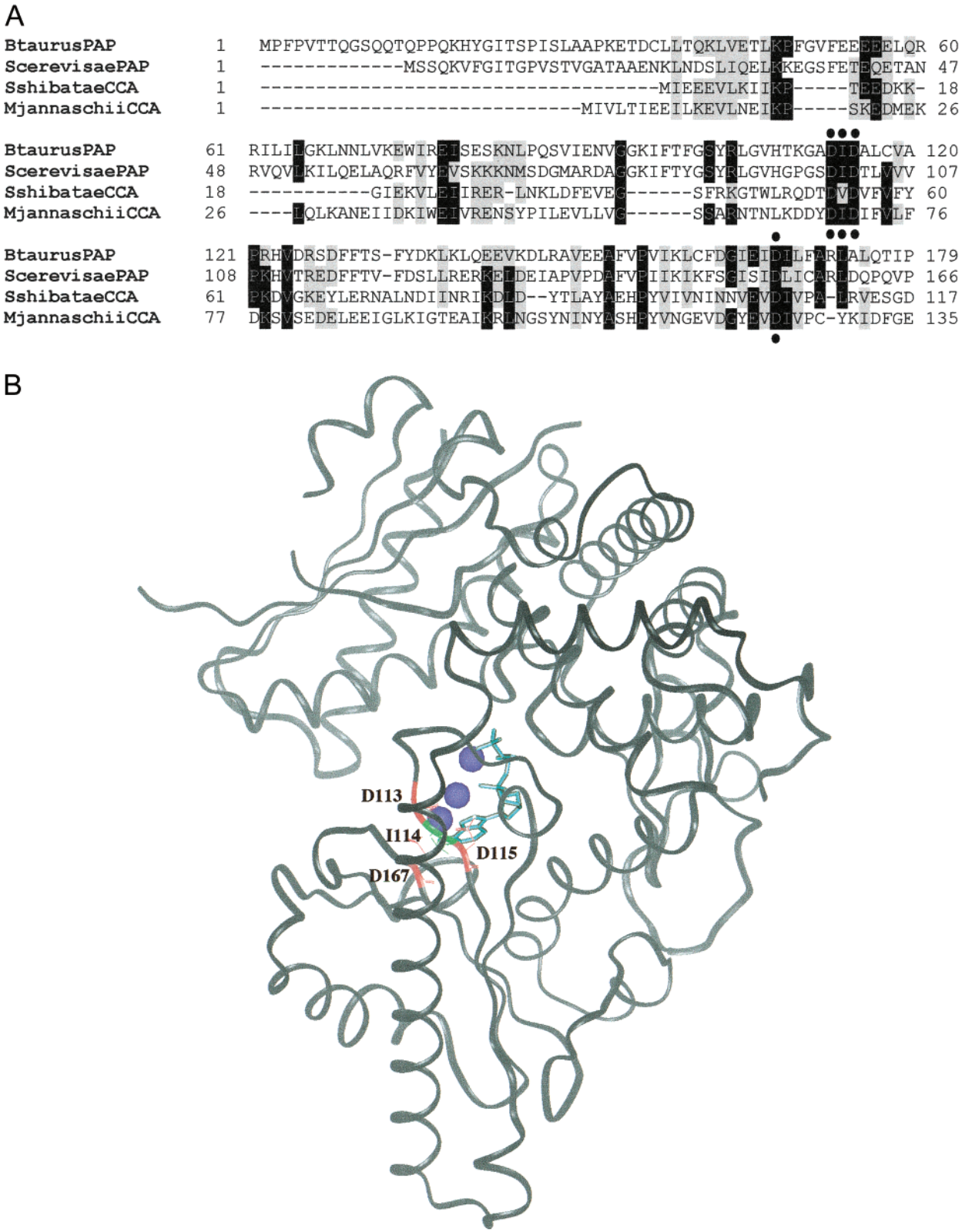

FIGURE 6: (A) Multiple-sequence alignment by CLUSTAL W of the bovine PAP, *S. cerevisiae* PAP, *S. shibatae* CCA, and *M. jannaschii* CCA enzymes. Conserved residues are shaded in black, while similar residues are shaded in gray. (B) Crystal structure of bovine PAP, displaying three bound metal ions (purple) complexed with ATP (cyan). The Ds of the DXD motif (D113-I114-D115) and D167 are highlighted in red to indicate their relationship in the active site.

processing activity of the tRNA 3′ end. In the presence of CTP and absence of ATP, these enzymes catalyze a processing and exchange activity from the 3′ end on minihelices and reconstituted tRNA. This activity is most active in the class I archaeal enzymes, and with the abnormal poly(C) ends rather than the normal CC end. The processing and exchange indicates a 3′ to 5′ exonuclease activity that hydrolyzes the terminal nucleotide and recouples CMP to the 3′ end. In the presence of labeled CTP, the activity incorporates the label into poly(C). The processing extends

as far as the C74 position, which is 3′ to A73 in *E. coli* $tRNA^{Val}$. Termination at A73 is observed with both minihelices (all deoxy except from position 73 and further, Figure 1B) and reconstituted tRNA (all ribose, Figure 2C). Thus, it is independent of the sugar−phosphate backbone. The 3′ to 5′ processing activity is reminiscent of the 3′ to 5′ exonuclease activity of the Klenow fragment of DNA polymerase and related enzymes (*30*−*32*), but is unusual for an RNA polymerase. The *E. coli* RNA polymerase, for example, enhances specificity by recruiting two cleavage factors, GreA and GreB, to hydrolyze incorrect sequences (*33*, *34*). No processing or exchange activity has been reported for PAP or TdT, the two other template-independent RNA polymerases. Further studies of the processing activity of the CCA enzymes may shed more light on the common origin in the development of DNA and RNA polymerases.

ACKNOWLEDGMENT

We thank Dr. Alan Weiner for providing the clone of the *S. shibatae* CCA enzyme, Drs. Caryn Evilia and Chun-Mei Zhang for assistance with the graphic work, and members of the Hou laboratory for discussion.

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BI0120953