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Unusual synthesis by the *Escherichia coli* CCA-adding enzyme

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ABSTRACT

The tRNA 3′ end contains the conserved CCA sequence at the 74–76 positions. The CCA sequence is synthesized and maintained by the CCA-adding enzymes. The specificity of the *Escherichia coli* enzyme at each of the 74–76 positions was investigated using synthetic minihelix substrates that contain permuted 3′ ends. Results here indicate that the enzyme has the ability to synthesize unusual 3′ ends. When incubated with CTP alone, the enzyme catalyzed the addition of C74, C75, C76, and multiple Cs. Although the addition of C74 and C75 was as expected, that of C76 and multiple Cs was not. In particular, the addition of C76 generated CCC, which would have conflicted with the biological role of the enzyme. However, the presence of ATP prevented the synthesis of CCC and completely switched the specificity to CCA. The presence of ATP also had an inhibitory effect on the synthesis of multiple Cs. Thus, the *E. coli* CCA enzyme can be a poly(C) polymerase but its synthesis of poly(C) is regulated by the presence of ATP. These features led to a model of CCA synthesis that is independent of a nucleic acid template. The synthesis of poly(C) by the CCA-adding enzyme is reminiscent of that of poly(A) by poly(A) polymerase and it provides a functional rationale for the close sequence relationship between these two enzymes in the family of nucleotidyltransferases.

Keywords: ATP-binding site; poly(A) polymerase; poly(C) polymerase; specificity; tRNA nucleotidyltransferase

INTRODUCTION

The CCA sequence at the 74–76 positions of the tRNA 3′ end is important for many biological functions. It provides the amino acid attachment site for tRNA to participate in protein synthesis. It serves as the primer for initiation of replication by retroviruses (Maizels & Weiner, 1994). It is also important in the transport of eucaryotic tRNA from nucleus to cytoplasm as a checkpoint for tRNA maturity (Lund & Dahlberg, 1998). The synthesis and maintenance of the CCA sequence are carried out by the CCA-adding enzyme [ATP(CTP):tRNA nucleotidyltransferase], which catalyzes the addition of CMP, CMP, and AMP one at a time from the respective nucleotide triphosphate (Deutscher, 1982). This enzyme also adds the CCA sequence to the 3′ end of viral RNAs that have the tRNA-like structures (Rao et al., 1989; Hegg et al., 1990; Giege, 1996). The CCA-adding enzyme is present in all three domains of life, the eubacteria, the eucarya, and the archaea. In organisms of eucarya and many archaea that do not encode the CCA sequence in tRNA genes, the CCA-adding enzyme is responsible for the synthesis of CCA and thus it is essential (Aebi et al., 1990). In organisms of eubacteria, such as *Escherichia coli*, that do encode the CCA sequence in tRNA genes, the CCA-adding enzyme is responsible for repairing damaged 3′ ends by resynthesizing the CCA sequence. The repair function of the CCA-adding enzyme is not essential (Zhu & Deutscher, 1987). *E. coli* strain that lacks the CCA enzyme is viable, albeit with a reduced growth rate.

The most intriguing feature of the CCA-adding enzyme is its specific synthesis of CCA without a nucleic acid template. This specificity is far greater than that of the poly(A) polymerase, which also lacks a nucleic acid template, but synthesizes products of heterogeneous lengths. The mechanism for the specificity of the CCA-adding enzyme is not well understood. An earlier model was proposed based on studies of the rabbit liver CCA-adding enzyme (Deutscher, 1972a). This model suggests that the enzyme contains a linear array of three nucleotide-binding sites, two for CTP and one for ATP, which served as a protein template to guide the synthesis of CCA. A recent model was proposed based on studies of the archaeal *Sulfolobus shibatae* CCA enzyme (Shi et al., 1998a). This model suggests a dynamic protein–RNA complex that determines the specificity of nucleotide addition. For example, the bind-

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ing of a tRNA, missing nt 74–76, to the enzyme would create the site for addition of C74. The 3′ end would refold with the enzyme to create the site for addition of C75. The CC terminus would refold with the enzyme to create the site for addition of A76. Once A76 was added, there would be no further nucleotide addition. This model was proposed based on biochemical evidence that the enzyme has one active site and that the tRNA substrate remains immobile on the enzyme throughout the addition of CCA (Shi et al., 1998a; Yue et al., 1998).

The apparent difference between the model of the rabbit liver enzyme and the model of the *S. shibatae* enzyme raises the question of the relatedness of these two enzymes. Sequence analysis of CCA-adding enzymes indeed has separated the archaeal enzymes as class I from the eucaryotic and eubacterial enzymes as class II (Yue et al., 1996). All of these enzymes are members of the family of nucleotidyltransferases, which also includes poly(A) polymerase, terminal deoxynucleotidyltransferase, DNA polymerase β, and kanamycin nucleotidyltransferase (Holm & Sander, 1995; Martin & Keller, 1996). The crystal structures of DNA polymerase β and kanamycin nucleotidyltransferase are known, and analysis of these structures shows a conserved DXD motif at the catalytic site (Sakon et al., 1993; Davies et al., 1994; Sawaya et al., 1994). The class I members of the nucleotidyltransferase family share the conserved DXD motif, but lack major homology (Yue et al., 1996). The class II members, in contrast, share a more extensive homology in the N-terminal domain that contains the DXD motif (Shanmugam et al., 1996). In both classes, the CCA-adding enzymes are most closely related to poly(A) polymerase in sequence (Masters et al., 1990; Cao & Sarkar, 1992; He et al., 1993).

To gain further insights into the separation of the two classes and to determine if this separation is the basis for the distinct features between the rabbit liver model and the *S. shibatae* model, the examination of an eubacterial CCA-adding enzyme would be important. This study focused on the *E. coli* CCA-adding enzyme as an example of the eubacterial enzymes and aimed to gain insights in two directions. First, as the *E. coli* enzyme partitions in the alignment with the eucaryotic enzymes, it provided an independent test of the two models. Second, mutant tRNAs that have altered 3′ ends have been isolated (O'Connor et al., 1993), such as *E. coli* $tRNA^{Val}$ variants ending with GCA, ACA, and *Salmonella typhimurium* $tRNA^{His}$ variants ending with UCA. The study of the *E. coli* enzyme could shed light on the origin of these natural variations.

Recent studies of the *E. coli* enzyme showed that it efficiently recognized synthetic minihelices that consist of just the acceptor and TΨC stems of a full-length tRNA (Shi et al., 1998b). This provided the rationale to use minihelices as substrates to examine enzyme specificity at each of the 74–76 positions. Minihelices were prepared chemically to ensure a clearly defined 3′ end. Results here showed that the *E. coli* CCA-adding enzyme catalyzed aberrant synthesis of 3′ ends both to minihelices and to a reconstituted tRNA. In the absence of ATP, the enzyme synthesized 3′ ends of C, CC, CCC, or poly(C) as if it were a poly(C) polymerase. Addition of ATP had a regulatory effect. It abolished the synthesis of CCC and inhibited the synthesis of poly(C). The poly(C) polymerase activity and the regulatory role of ATP led to a model of the untemplated synthesis of CCA. This model sheds light on the separation of two classes of the CCA-adding enzymes in evolution and their close sequence relationship with poly(A) polymerases.

RESULTS

Substrates for the *E. coli* CCA-adding enzyme

The minihelix domain of *E. coli* $tRNA^{Val}$, consisting of the acceptor stem and the TΨC stem-loop of the full-length tRNA, is a substrate for the *E. coli* CCA enzyme and is numbered as in tRNA (Fig. 1). This minihelix, when synthesized in the deoxy backbone except for the terminal ribose of A76, exhibits a K_m of 25 μM for CTP, 175 μM for ATP, and a k_{cat} of 0.3 s^{-1} (Shi et al., 1998b). These are in good agreement with those of natural tRNA for the previously characterized rabbit liver enzyme (K_m of 15–200 μM for CTP and 2–3 mM for ATP and a k_{cat} of 9.0 s^{-1}) (Deutscher, 1972a). In this study, a slightly enhanced rate of CTP and ATP addition was observed when nucleotides at the 3′ end of the minihelix (the 73–76 positions) were all in the ribose backbone. Thus, all minihelices derived from *E. coli* $tRNA^{Val}$ and from other tRNAs were synthesized with the 3′ end (the 73–76 positions) in the ribose form but the rest of the molecule in the deoxy form.

Minihelices were synthesized with permutation of the terminal nucleotide at the 3′ end. For example, the full-length minihelix is a 35-mer (Val-35N) and four minihelices were synthesized with A76 (Val-35A), C76 (Val-35C), G76 (Val-35G), and U76 (Val-35U), respectively. These four were to test the ability of the *E. coli* CCA-adding enzyme to discriminate at the 76 position. This test could provide new insight into the ability of the CCA-adding enzyme to terminate synthesis and to release product after it fills the 76 position. The precise termination of synthesis is the main feature that differentiates the CCA-adding enzyme from the poly(A) polymerase. Similarly, four minihelices that end at the 75 position were synthesized (Val-34N, where N = A, C, G, U) and these four were to test substrate recognition and the specificity of synthesis from the 75 position. Another four minihelices with permutations at the 74 position were synthesized (Val-33N, where N = A, C, G, U) to test the specificity at the 74 position. Because of the reported aberration of 3′ ends such as GCA, ACA, and UCA (O'Connor et al., 1993), the test of the 74 position was

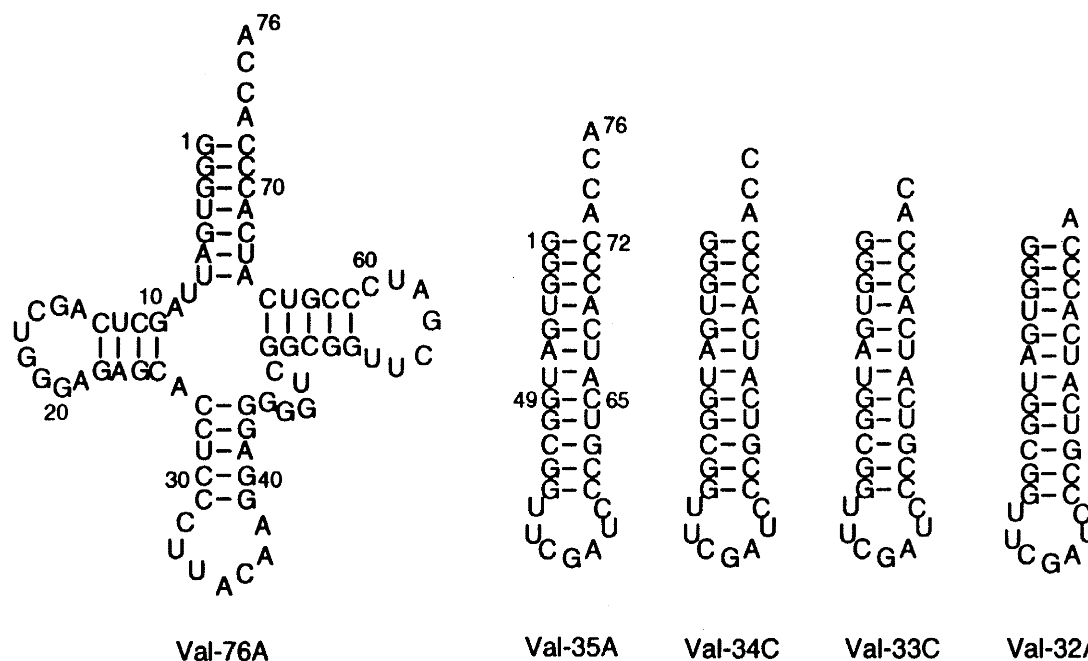


FIGURE 1. Sequence and cloverleaf structure of the 76 nt in *E. coli* tRNA$^{Val/UAC}$ that ends with A76 (Val-76A). The minihelix substrate Val-35A consists of the sequences in the acceptor stem and TΨC stem-loop. This minihelix is a 35-mer and ends with A76. The Val-34C substrate is a 34-mer and ends with C75. The Val-33C and Val-32A substrates, respectively, consist of 33-mer and 32-mer and end with C74 and A73. The numbering of the tRNA and of minihelices are the same as that described for yeast tRNA^{Phe} (Sprinzl et al., 1998).

of particular interest. It would determine if the enzyme would use G74, for example, as a substrate and extend it into the GCA sequence. Finally, a minihelix that terminates at the A73 position was synthesized (Val-32A). This minihelix served as a control for the addition of CTP.

The *E. coli* CCA-adding enzyme used in the study was expressed from a recombinant gene and contained a $(His)_6$-tag at the C terminus (see Materials and methods). The activity of the enzyme was indistinguishable from that of the native enzyme purified from *E. coli* but without the $(His)_6$-tag. The purified recombinant enzyme was incubated with the minihelix substrates at a catalytic amount under conditions as previously described (Shi et al., 1998b). A typical reaction contained 0.2 μM of the enzyme with 20 μM of the minihelix substrates that terminate at the 75 or 76 position (Val-34N, Val-35N), or 2.0 μM of the enzyme with 20 μM of substrates that terminate at the 73 or 74 position (Val-32N, Val-33N). The higher concentration of the enzyme for shorter 3′ ends was necessary to generate levels of products similar to those of the longer 3′ ends. Overall, the concentration of the enzyme was maintained at least 10-fold below that of the substrate so as to evaluate the specificity of the enzyme as a catalyst.

Specificity at the 76 position

The specificity at the 76 position was first investigated by incubating the Val-35N substrates with ATP only. If the enzyme precisely terminated at the 76 position, there would be no further addition of ATP to any of the permuted substrates. However, Figure 2A shows that ATP (labeled as α-^{32}P-ATP) was incorporated to the Val-35C substrate. The incorporation was detected as a 1-nt addition on a denaturing polyacrylamide gel that contained 7 M urea. The product was a 36-mer that comigrated with a marker of the same size. The signal of the incorporation, as quantified by a phosphorimager, was undetectable at time 0 but increased at the 15- and 30-min time points. The precise migration of the product and the time dependence indicate that the addition of one ATP to the Val-35C substrate was enzyme catalyzed. As a positive control, the minihelix substrate missing A76 (Val-34C) was run in parallel. This control was the normal substrate for the CCA-adding enzyme and it was extended with one ATP in the same time-dependent manner under the reaction conditions.

The addition of ATP was specific to the Val-35C substrate. No addition was observed with the Val-35A, Val-35G, and Val-35U substrates. Thus, although the enzyme was not expected to continue synthesis from position 76, it did so nonetheless with specificity. It only recognized C76 as a substrate. The addition of ATP to the Val-35C substrate most likely generated the product of CCCA. The exact sequence of the product can be only determined by sequence analysis of the RNA, which was extremely difficult at the 3′ end on a minihelix substrate. However, if the label came from an

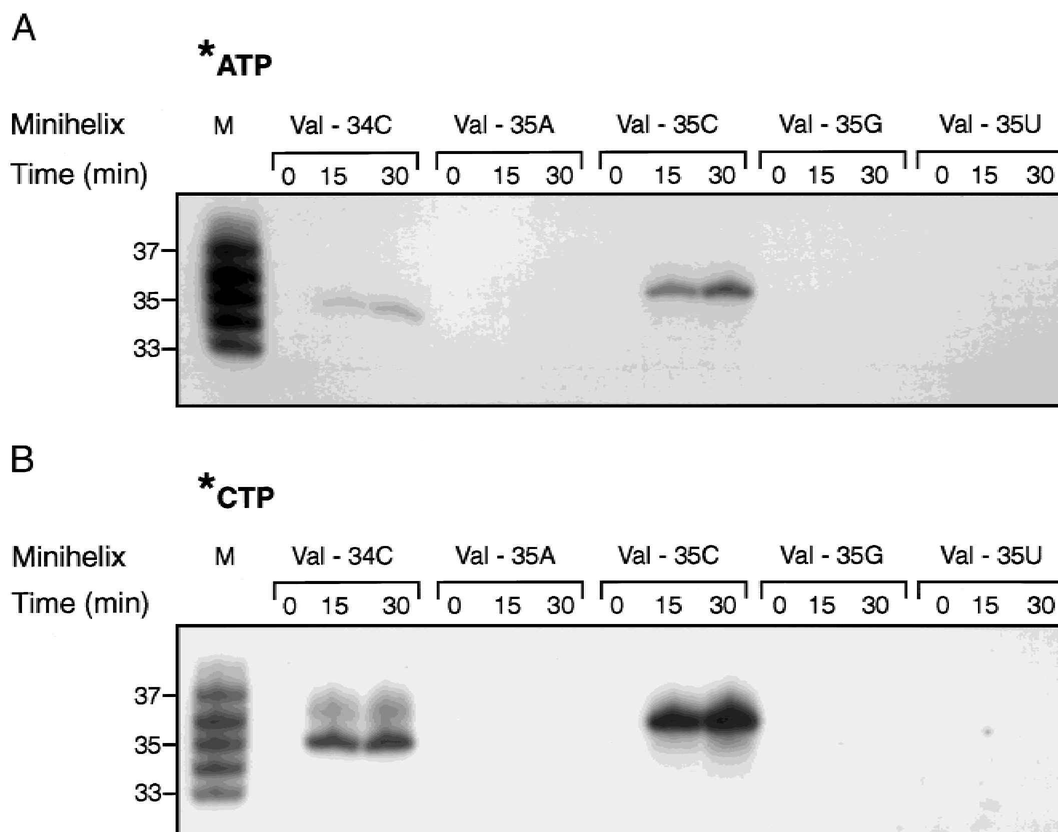


FIGURE 2. Minihelix substrates Val-35N (N = A, C, G, U) for addition of (**A**) α-^{32}P-ATP (shown as *ATP) or (**B**) α-^{32}P-CTP (shown as *CTP) by the *E. coli* CCA-adding enzyme. The Val-35N substrates and the Val-34C substrate as a control were 15 μM each in the reaction and aliquots at 0, 15, and 30 min were loaded on a 15% denaturing PAGE. M indicates the markers for the 33- to 37-mers of the valine minihelices.

enzyme-catalyzed exchange between α-^{32}P-ATP and nucleotides at the 3′ end, one would have observed labeled products that correspond to the 35-, 34-, and 33-mers. The absence of these products within the sensitivity of the detection suggests that the exchange did not occur.

The specificity at the 76 position was also investigated by incubating the Val-35N substrates with CTP only. Figure 2B shows an addition of one CTP to the Val-35C substrate but not to any others. This unexpected addition of CTP to the Val-35C substrate was also time dependent, indicating an enzyme-catalyzed reaction. The product of the addition is most likely CCCC, because there were no other labeled products of 35-mer or smaller. Also unexpected was the addition of one or two CTPs to the control substrate, Val-34C. Most likely, the main product was CCC and the second product was CCCC.

The addition of one ATP or CTP to the C76 position is not unique to the minihelix of *E. coli* tRNA^{Val}. Two other minihelices were synthesized and both were substrates for the aberrant addition. These two minihelices were based on the acceptor-TΨC stem domain of *E. coli* tRNA^{Ala} and tRNA^{Cys}, respectively. Figure 3 shows the addition of ATP to minihelices Ala-35C and Cys-35C and Figure 4C shows the addition of CTP to Ala-35C. In all cases, the addition was time dependent as observed with the Val-35C substrate. The gel in Figure 3 also contains the positive controls Ala-34C and Cys-34C. As expected, these two controls were substrates for the addition of one ATP. The migration of the product of Ala-34C and of Ala-35C was similar to those of Val-34C and Val-35C. However, the migration of the product of Cys-34C and of Cys-35C was much faster and this was reproducible even when the minihelix was prepared by two different sources (see Materials and methods). The sequence of *E. coli* tRNA^{Cys} contains U73, whereas those of *E. coli* tRNA^{Val} and tRNA^{Ala} contain A73. U73 can cause an unusual conformation at the tRNA 3′ end (Puglisi et al., 1994; Hou et al., 1998), which might not be completely denatured under the gel condition.

Addition of ATP and CTP to a reconstituted tRNA

The addition of one ATP or CTP to C76 was also observed in a reconstituted tRNA. Previous studies

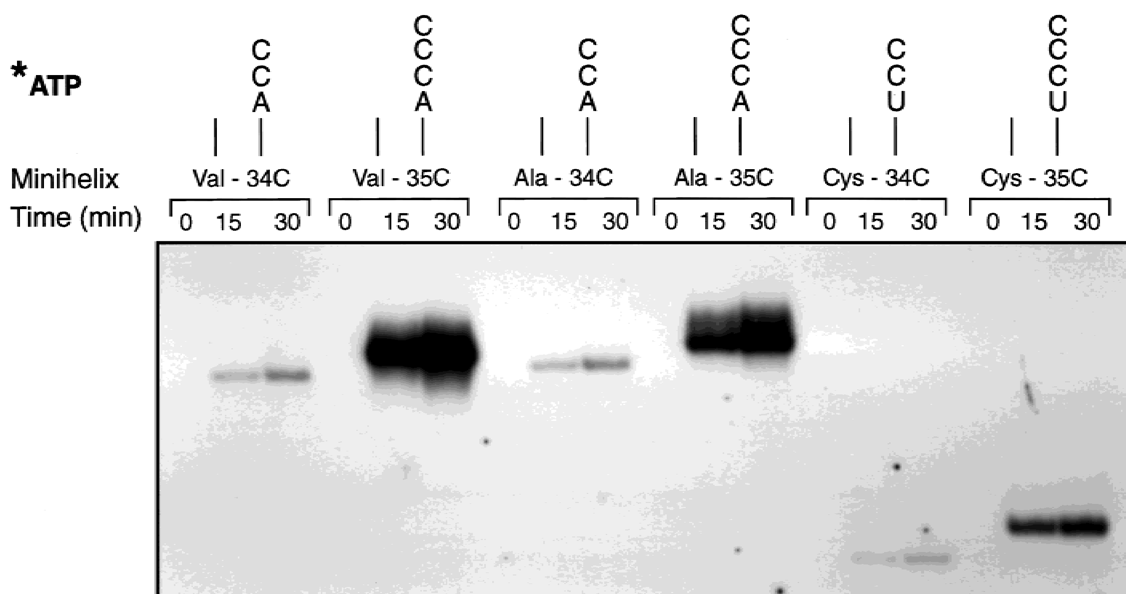


FIGURE 3. Addition of α-^{32}P-ATP (*ATP) to minihelix substrates Val-34C and Val-35C, Ala-34C and Ala-35C, and Cys-34C and Cys-35C by the CCA-adding enzyme. The products of 0, 15, and 30 min at 37 °C were analyzed by a 15% denaturing PAGE.

showed that splitting a tRNA in the TΨC loop (between positions 57 and 58) and reannealing the large fragment (nt 1–57) and the small fragment (nt 58–76) reconstituted the tRNA as a functional substrate for aminoacylation (Yap et al., 1995). The functional reconstitution suggested that the annealed fragments afforded sufficient secondary and tertiary interactions that maintained the overall tRNA structure and function. The reconstitution offered the advantage that the small fragment can be chemically synthesized with a precise 3′ end. Although the reconstituted tRNA had a break in the TΨC loop, this was probably not important as studies showed that the *E. coli* enzyme primarily recognized the acceptor stem of the minihelix domain (Shi et al., 1998b). Control experiments confirmed the prediction. The gene for *E. coli* $tRNA^{Ala}$ was restricted with *Taq*1 to generate the template for synthesis of the large RNA fragment of nt 1–57 by T7 RNA polymerase (Fig. 4A). The small RNA fragment of nt 58–76 was prepared by chemical synthesis and was composed of a complete ribose backbone. The large and small fragments (57-mer and 19-mer, respectively) were annealed to reconstitute the complete sequence. Aminoacylation showed that the reconstituted tRNA was a substrate for aminoacylation. Substitution of the 19-mer with an 18-mer that lacked A76 confirmed that the reconstituted tRNA was a substrate for the CCA enzyme (Fig. 4B).

The reconstituted tRNA was examined to investigate the erroneous addition of ATP and CTP to C76. This investigation was necessary to determine if nucleotides outside of the minihelix domain might prevent the aberrant addition as observed with the minihelix substrates. For example, G19 in the D loop of the full-length tRNA forms a tertiary interaction with C56 in the TΨC loop, which has been shown as important for the correct recognition of tRNA by the *E. coli* CCA-adding enzyme (Spacciapoli et al., 1989). However, results in Figure 4B show the erroneous addition of one ATP to tRNA-75C and tRNA-76C, and those in Figure 4C show the addition of one CTP. The small fragment in the tRNA-75C substrate contained CC and that in the tRNA-76C substrate contained CCC. Because the labeled nucleotide was added to the small fragment, it comigrated with the small fragment. The large fragment was not covalently linked to the small fragment and therefore was undetectable. Thus, the aberrant addition of ATP to the CCC end and of CTP to the CC and CCC ends were observed in the sequence and structure context of tRNA as well. Control experiments showed that the addition was dependent on the hybridization of the large and small fragments. The small fragment alone gave no signal of addition (not shown).

In the reconstituted tRNA substrates (tRNA-75C and tRNA-76C, Fig. 4), the aberrant addition had the same activity as the normal addition. However, the aberrant addition in the minihelix substrates (Val-35C, Ala-35C, and Cys-35C) was more active than the normal addition of ATP to the CC end (Val-34C, Ala-34C, and Cys-34C). The higher activity for the aberrant addition is an artifact of the minihelix substrates. The source for the artifact is unknown, but could arise from the lack of the tRNA moiety or from the deoxy backbone of the minihelix substrates.

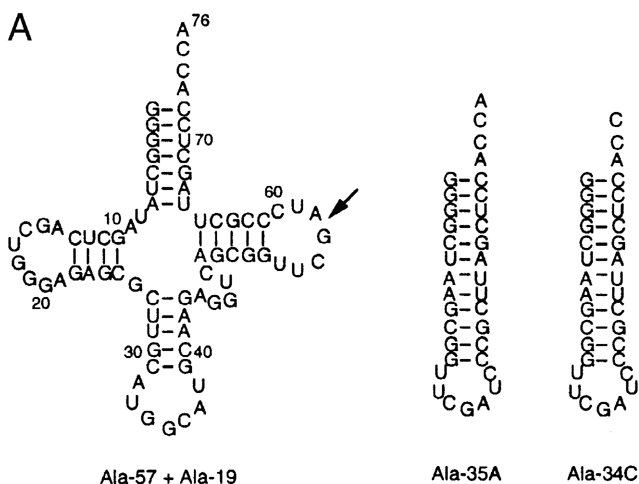
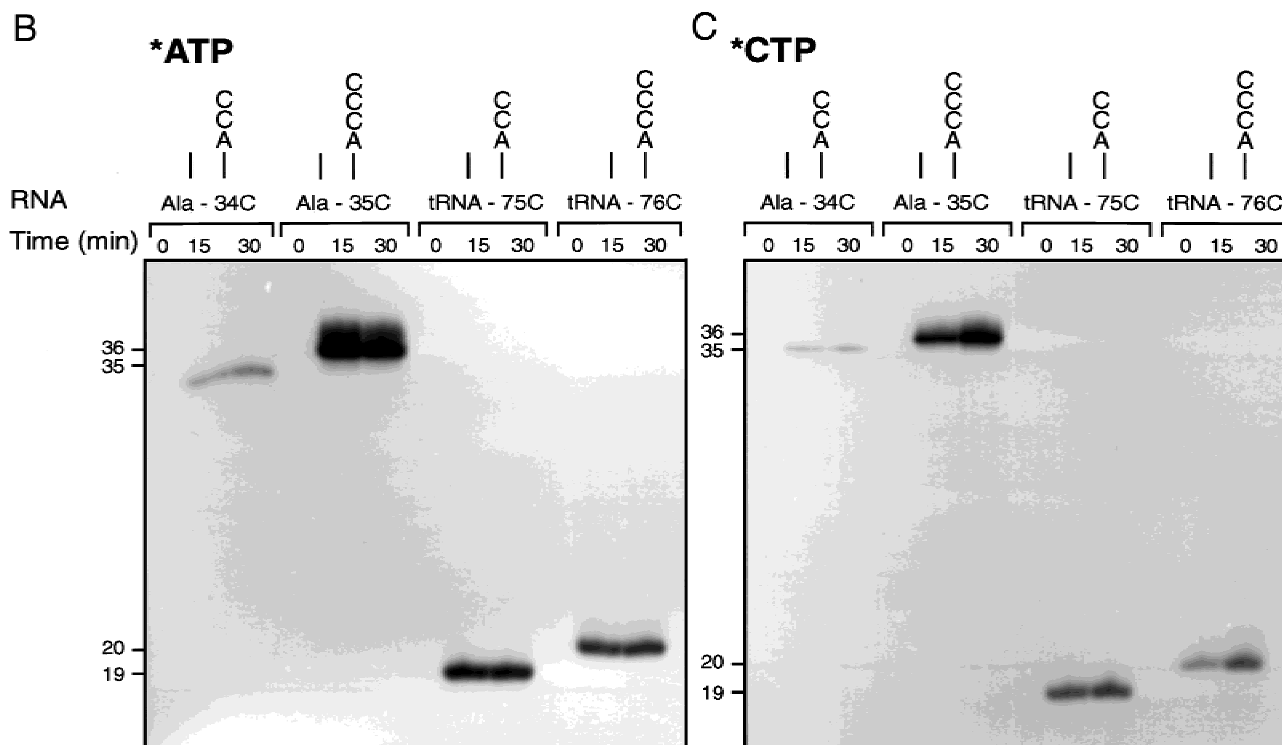


FIGURE 4. **A**: The sequence and cloverleaf structure of *E. coli* tRNA$^{Ala/GGC}$, reconstituted from a 57-mer and a 19-mer as shown by the arrow. Ala-35A and Ala-34C indicate the minihelix domain of the tRNA ending with A76 and C75, respectively. **B**: Addition of α-^{32}P-ATP (*ATP) to Ala-34C, Ala-35C, and to tRNA-75C (the reconstituted tRNA ending with C75) and tRNA-76C (the reconstituted tRNA ending with C76). Addition was initiated by the CCA-adding enzyme and was monitored at 0, 15, and 30 min. The migration of the 36-, 35-, 20-, and 19-mers is indicated on the left of the 15% denaturing gel. **C**: Addition of α-^{32}P-CTP (*CTP) to Ala-34C, Ala-35C, and to tRNA-75C and tRNA-76C monitored as described for **B**.



Specificity at the 75, 74, and 73 positions

The specificity at the 75 position of the minihelix substrates was investigated by incubating the Val-34N substrates with ATP alone or CTP alone (Fig. 5A). Figure 5A (left) shows that the enzyme recognized C75 as the substrate and extended it with one ATP. There was no activity with any of the other three substrates. The recognition and extension of C75 is the expected function of the CCA-adding enzyme. It has been observed for Val-34C, Ala-34C, Cys-34C, and tRNA-75C (Figs. 2A, 3, and 4B, respectively). Unexpected was the recognition and extension of C75 with CTP (Fig. 5A right), as shown for Val-34C (Fig. 2B), Ala-34C, and tRNA-75C (Fig. 4C). Figure 5A confirms this unexpected result with Val-34C under a slightly different reaction condition (90 min rather than 30 min; see below). However, Figure 5A indicates that this extension was only with C75 and no other substrates.

The specificity at the 74 position was investigated by incubating the Val-33N substrates with ATP or CTP alone. Figure 5B shows no addition of ATP to any of the Val-33N substrates, but the addition of one CTP to Val-33C. These observations were the expected specificity of the CCA-adding enzyme. A separate control indicated that, to the Val-32A substrate, which terminated with A73, the enzyme only added CTP but not ATP (see Fig. 6A). This established the expected specificity at the 73 position.

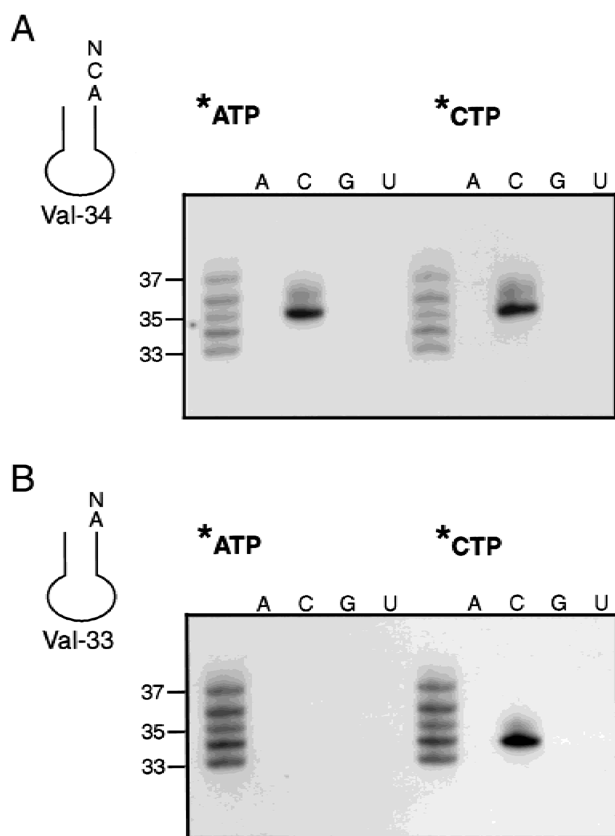


FIGURE 5. **A**: Gel analysis of addition of α-^{32}P-ATP (*ATP) and α-^{32}P-CTP (*CTP) to Val-34N (N = A, C, G, U) by the CCA-adding enzyme at 37 °C for 90 min. **B**: Gel analysis of addition of α-^{32}P-ATP (*ATP) and α-^{32}P-CTP (*CTP) to Val-33N (N = A, C, G, U) by the CCA-adding enzyme at 37 °C for 90 min.

The time point in the CCA-adding reaction for Figure 5A,B was 1.5 h and the enzyme concentration was raised to 2.0 μM (rather than 0.2 μM) relative to 20 μM of substrates. The gel was purposely overexposed to detect any trace signal of nucleotide addition. Under this condition, Figure 5A shows the addition of a second ATP or CTP to the Val-34C substrate. However, there was still no detectable addition of CTP to substrates other than Val-33C. This suggests that the enzyme would not use A74, G74, or U74 to synthesize ACA, GCA, or UCA. The second addition of ATP to C75 would generate the CCAA sequence. However, the level of synthesis of CCAA must not be significant, because incubation of the enzyme directly with CCA (Val-35A) and ATP gave no signal of the CCAA sequence (Fig. 2A).

Competition between ATP and CTP

Competition between ATP and CTP was investigated at each of the 74–76 positions. The *E. coli* enzyme was incubated with both nucleotides and either ATP or CTP was labeled. This was to determine if some of the aberrant synthesis could be prevented through competition between ATP and CTP. The reaction conditions were such that only the addition of the first nucleotide was examined. Figure 6A shows results of the competition. The Val-32A substrate was extended with CTP to a 33-mer in the presence of ATP. This extension was detected only when CTP, but not ATP, was labeled. The Val-33C substrate was extended in the presence of

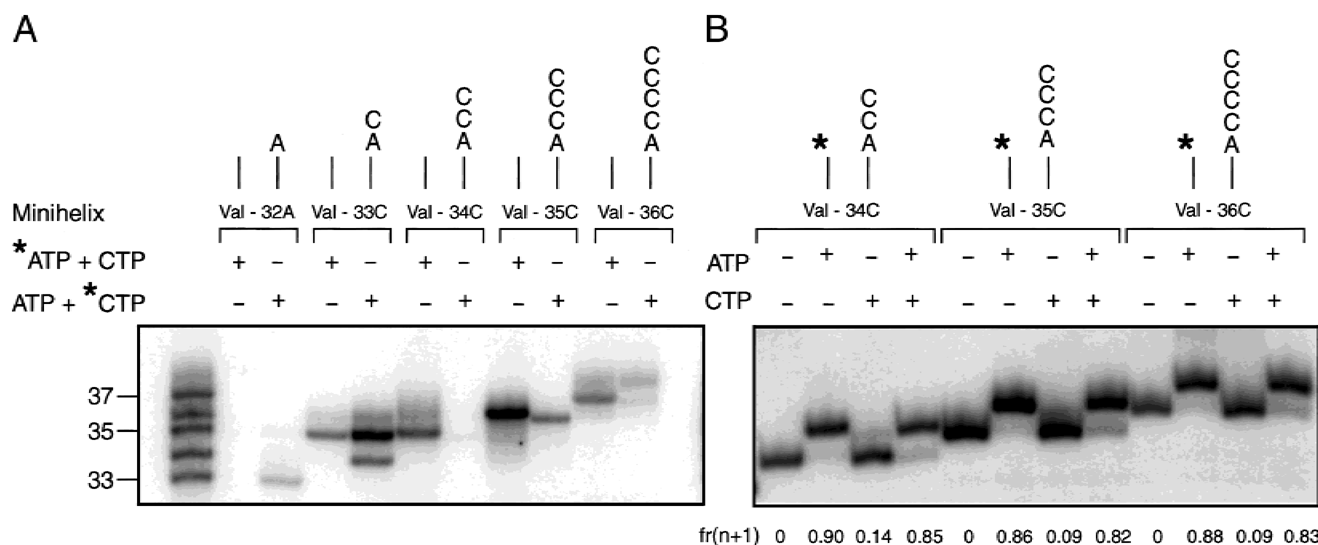


FIGURE 6. **A**: Competition between ATP and CTP in the addition to Val-32A, Val-33C, Val-34C, Val-35C, and Val-36C minihelices. Each substrate (20 μM) was incubated with an appropriate amount of the enzyme and with 50 μM ATP, 50 μM CTP, and 0.05 μM α-^{32}P-ATP or α-^{32}P-CTP. The products of each substrate in the reaction of 30 min at 37 °C were analyzed by a 15% denaturing PAGE. **B**: Gel analysis of the addition of a nucleotide to Val-34C, Val-35C, and Val-36C in the presence of 250 μM ATP, 250 μM CTP, or both at 250 μM. Each minihelix substrate was labeled at the 5′ end (indicated by *) and the fraction of the substrate (to the total intensity of each lane) that was converted to the n + 1 product (fr(n + 1)) was quantified and indicated below the gel. Quantification was by phosphorimager analysis. Each value was the average of three determinations and was corrected for the background, which came from trailing of bands on the gel.

both CTP and ATP. It was converted to a 35-mer when ATP was labeled but was converted to a 34-mer first, then to a 35-mer when CTP was labeled. Most likely, this substrate was extended with one CTP, then was quickly extended with one ATP to generate the normal CCA. The extension and specificity of the Val-32A and Val-33C substrates was as expected of the CCA-adding enzyme.

An effect of competition between ATP and CTP was noticed with the Val-34C substrate. This substrate was extended with one ATP in the presence of CTP, but it was not extended with CTP in the presence of ATP. Thus, whereas the normal addition of ATP was retained in the presence of CTP, the aberrant addition of CTP was prevented by the presence of ATP. The ATP and CTP concentrations in Figure 6A were 50 μM each. Additional experiments showed that ATP in the range of 25 to 300 μM completely suppressed the addition of CTP (at 50 μM, not shown). The ability of ATP to suppress CTP addition was also observed with the larger Val-35C substrate, but the inhibitory effect was not as dominant. Val-35C was extended with ATP in the presence of CTP and was extended with CTP in the presence of ATP. As the signal for ATP incorporation was fivefold higher than that for the CTP, this suggests that the enzyme had a preference for ATP, but that this preference was not enough to completely block the addition of CTP.

A larger substrate Val-36C, which contained four Cs at the 3′ end, was synthesized and tested. Val-36C was simply the product of the addition of CTP to Val-35C. Figure 6A shows that Val-36C was a substrate for the CCA-adding enzyme. It was extended with ATP when CTP was present and was extended with CTP when ATP was present. The signal for ATP addition was threefold higher than that for CTP addition, suggesting a preference for ATP. However, additional extensions of higher molecular weights were also detected, particularly when CTP was labeled.

Quantification of the ATP/CTP addition

Quantification of the ATP/CTP addition was performed with the Val-34C, Val-35C, and Val-36C substrates where aberrant additions were observed. This was to determine to what extent the aberrant addition could occur under normal physiological conditions. Two features were noted. First, each substrate was labeled at the 5′ end such that the synthesis of aberrant products could be precisely quantified. This purpose could not be effectively achieved with α-^{32}P-ATP or α-^{32}P-CTP, as specific activities of these labels were not accurately determined when supplied by the manufacturer. Second, each substrate was incubated with concentrations of ATP and CTP that mimic the physiological conditions (about two times the concentration of K_m) at 250 μM each. When experiments were performed with labeled ATP or CTP, the concentration of the labeled nucleotide was kept low (50 μM each) to give the sensitivity of detection.

Figure 6B shows the gel analysis and the results of quantification. For the Val-34C substrate, the normal addition of ATP was achieved by 90% of the substrate in the absence of CTP. However, the abnormal addition of CTP was achieved by 14% of the substrate in the absence of ATP. The value 14% was obtained after correction of the background and had a standard error of 10–20%. It suggests that the synthesis of CCC, in the absence of ATP, was significant. This level of synthesis was quantitatively similar to that of the normal addition of CTP to Val-33C or to Val-32A (not shown). When ATP and CTP were both present, the addition of 1 nt to Val-34C was achieved by 85% of the substrate and this could be entirely due to the addition of ATP, as Figure 6A suggested that ATP inhibited CTP-addition. For the Val-35C and Val-36C substrates, the addition of ATP or CTP alone proceeded to approximately the same extent as that with the Val-34C substrate and this suggests similar activities of the enzyme with these substrates. However, in the presence of both ATP and CTP, the products of Val-35C and Val-36C were heterogeneous. Based on the analysis of Figure 6A, the products of Val-35C were expected to consist of 83% extension by ATP and 17% extension by CTP to reflect the fivefold preference for ATP. The products of Val-36C were expected to consist of 75% extension by ATP and 25% extension by CTP to reflect the threefold preference for ATP.

The poly(C) polymerase activity

The ability of the CCA-adding enzyme to continue adding CTP is evident in several examples. First, the enzyme added CTP to Val-32A, Val-33C, Val-34C, Val-35C, and Val-36C (Fig. 6A, Fig. 2B for Val-34C). With some substrates, such as Val-34C, the addition of multiple Cs was detected directly (Figs. 2B and 5A). Each addition resulted in a product that became the substrate for the next addition. This progressively increased the number of Cs at the 3′ end and provided evidence for the poly(C) polymerase activity. Although this activity slowed down with longer 3′ ends, it was detectable by using higher concentrations of the enzyme. In fact, the activity of synthesis of poly(C) was successfully used to create the labeled markers for gel analysis in the study. Second, the enzyme generated heterogeneous products of higher molecular weight from the Val-36C substrate in the presence of labeled CTP and cold ATP (Fig. 6A). These products most likely consisted of multiple Cs but terminated with an A. The termination with an A is justified, as results presented

here suggest that the enzyme could not add CTP to a terminal A (Figs. 2B, 4B, and 5). Thus, until ATP was added, the enzyme essentially behaved as a poly(C) polymerase.

DISCUSSION

Specificity of the *E. coli* CCA-adding enzyme and the role of ATP

Table 1 summarizes the specificity of the *E. coli* enzyme. Analysis of Table 1 shows two main features. First, the enzyme is specific at the 74 and 75 positions. It only catalyzes the addition of CTP to a 3′ end of N73 or C74. Of importance is that the enzyme does not add CTP to A74, G74, or U74 and thus cannot synthesize the 3′ ends ACA, GCA, and UCA that were isolated from genetic studies (O'Connor et al., 1993). These anomalous 3′ ends must have resulted from additional tRNA processing enzymes that are independent of, or work in concert with, the CCA-adding enzyme. The nature of the additional processing enzymes remains to be identified. Second, starting at position 76, the enzyme can catalyze addition of ATP or CTP when incubated with either nucleotide alone. However, the addition of CTP is inhibited by the presence of ATP. Most interestingly, addition of CTP at position 76, which would generate an aberrant CCC end, is completely inhibited by ATP. Thus, ATP switches the preference of the enzyme at position 76 from CTP to ATP.

The continuous addition of CTP, in the absence of ATP, suggests that the enzyme can be a poly(C) polymerase. Under steady-state conditions (Fig. 6), the erroneous synthesis of CCC can reach 14% and that of CCCC and CCCCC can reach 9%. The ability of ATP to inhibit erroneous addition of CTP suggests the possibility that ATP is a regulator for the control of specificity. ATP as low as 25 μM is sufficient to inhibit erroneous synthesis of multiple Cs. Thus, under normal physiological conditions, where ATP is at 1–3 mM, the *E. coli* enzyme should only synthesize CCA. These results emphasize the ability of the *E. coli* enzyme to maintain the specificity of CCA over a wide range of ATP concentrations from 25 μM to 3 mM. This specificity is necessary, given the diverse cellular functions of CCA.

TABLE 1. Summary of the *E. coli* CCA-adding enzyme.

Position	ATP	CTP	ATP + CTP	Preference for ATP
74	−	+	CTP	None
75	−	+	CTP	None
76	+	+	ATP	100%
77	+	+	ATP and CTP	83%
78	+	+	ATP and CTP	75%

Although the poly(C) polymerase activity and the regulatory effect of ATP are observed with minihelices, these features can be extended to tRNA. The specificity of the enzyme to precisely synthesize C and CC with minihelix substrates is the same as that expected of the full-length tRNA. This suggests that the enzyme correctly recognizes minihelices and does not slip. The ability of the enzyme to synthesize CCC is observed with both minihelices and with a reconstituted tRNA. This suggests that the synthesis of CCC is not an artifact of minihelices.

A model for CCA synthesis for the *E. coli* enzyme

Based on the poly(C) polymerase activity and the regulatory effect of ATP, a model for CCA synthesis is proposed for the *E. coli* CCA-adding enzyme (Fig. 7A). In this model, the enzyme has one CTP site near the catalytic site and one ATP site that is the ATP donor site and that controls the enzyme conformation. When the ATP site is free, the enzyme is in the open form and it catalyzes the synthesis of poly(C) as a poly(C) polymerase. When the ATP site is bound, the enzyme is in the closed form and it catalyzes the synthesis of only CCA. A conformational change from the open to the closed forms is proposed to bring the ATP donor site to the catalytic site. Alternatively, the conformation change is to build a wall that blocks poly(C) polymerization. In either case, the ATP-bound site regulates the catalytic site to only accommodate C74 and C75. Once these two are added, the enzyme then catalyzes the addition of ATP to the 3′ end and the presence of a terminal A will signal termination and the release of product. The integration of the poly(C) polymerase activity and the regulatory effect of ATP thus can provide a basis for the untemplated synthesis of CCA.

Major features of the proposed model are amenable to experimental test. One feature is that the enzyme has a single catalytic site. Sequence analysis of members of the nucleotidyltransferase family has suggested that the catalytic site contains the conserved DXD motif (Holm & Sander, 1995). In the crystal structures of DNA polymerase β and kanamycin nucleotidyltransferase, the Ds in the DXD motif coordinate with two metal ions that are responsible for catalysis (Sakon et al., 1993; Davies et al., 1994; Sawaya et al., 1994). The significance of these Ds in the *S. shibatae* enzyme has been confirmed (Yue et al., 1998). Substitution of these D residues eliminated both the CTP- and ATP-adding activity, providing evidence that they are located at the catalytic site. Thus, both structural analysis and biochemical studies have supported the DXD motif as the active site. This DXD motif occurs once in the known sequences of CCA-adding enzymes.

A second feature is that ATP has an allosteric effect on the enzyme conformation to control specificity. This

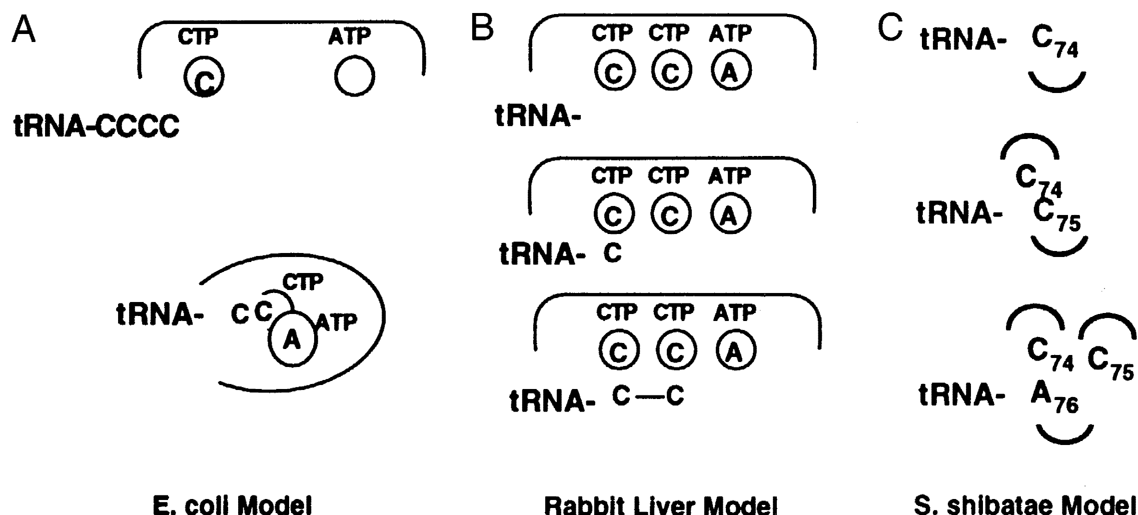


FIGURE 7. Models of CCA addition. **A**: The *E. coli* model suggests that the enzyme exists in the open form when the ATP site is open and it acts as a poly(C) polymerase. The enzyme exists in the closed form when the ATP site is bound and it catalyzes the addition of CCA. **B**: The rabbit liver model suggests that the enzyme uses two CTP sites and one ATP site that are linearly arranged to direct the entry of a tRNA 3′ end (Deutscher, 1972a). **C**: The *S. shibatae* model suggests that the enzyme and a 3′ end form a dynamic complex that refold with each nucleotide addition to determine the specificity of the next addition (Shi et al., 1998a).

effect can be tested, for example, by using ATP analogs that might initiate the conformational change but not ATP addition. Such an allosteric effect of the ATP site would provide a mechanistic parallel between the untemplated synthesis of CCA and the template-dependent synthesis by many DNA or RNA polymerases. For example, *E. coli* DNA polymerase I, the Klenow fragment, and T4 DNA polymerase show a conformational change upon nucleotide binding that is necessary for catalysis (Eger & Benkovic, 1992; Frey et al., 1995). This conformational change ensures the specific hydrogen bonds between the incoming nucleotides and the template strand. The T7 DNA helicase has a conformational change, induced by nucleotide binding, that increases the affinity of the enzyme to its template so as to more efficiently use the binding energy for synthesis of DNA (Yong & Romano, 1995). In the most recent crystal structure of T7 RNA polymerase, the conformational change is used to form a more stable elongation complex (Cheetham & Steitz, 1999).

If the allosteric effect is not supported by studies of ATP analogs, however, alternative interpretation of the ability of ATP to inhibit poly(C) synthesis must be invoked. For example, the preference for ATP addition may be achieved by an ATP site that has a dual specificity for both ATP and CTP. This ATP site of dual specificity will have a higher affinity for ATP than for CTP. As such, it will confer AMP addition when ATP is present, but will confer CMP addition when ATP is absent. The addition of CMP from an ATP site has been previously shown for the rabbit liver enzyme (Masiakowski & Deutscher, 1980). This alternative mechanism can be also tested for the *E. coli* enzyme.

Comparison among existing models

Comparison of the model for the *E. coli* enzyme with the models for the rabbit liver and the *S. shibatae* enzymes provides new insight into relationships of these enzymes. The major features of the *E. coli* enzyme are consistent with features of the rabbit liver enzyme (Fig. 7B). For example, the separation of the ATP site from the CTP site was implicated in kinetic studies of the rabbit liver enzyme. Specifically, the ATP site of the rabbit liver enzyme was more sensitive to salt than the CTP site, and the K_m for ATP addition was at least one order larger than that for CTP addition (Deutscher, 1972a). Also, the two CTP sites in the rabbit liver enzyme appeared to overlap and the erroneous synthesis of CCC, CCCC, and poly(C) was observed in the presence of Mg^{+2} (as shown for the *E. coli* enzyme) and Mn^{+2} (Deutscher, 1972b, 1973a, 1973b). Overall, the strong similarity between the *E. coli* and rabbit liver enzymes highlights the close relationship between the eubacterial and eucaryotic enzymes.

The *E. coli* model differs from the *S. shibatae* model in one major feature. While the *E. coli* model can explain the synthesis of CCC, CCCC, and poly(C), the *S. shibatae* model does not predict the synthesis of poly(C). The *S. shibatae* model suggests that the binding of the CC end to the enzyme only creates the site for ATP (Fig. 7C) (Shi et al., 1998a) and as such does not predict the synthesis of CCC, CCCC, or poly(C). Because the *E. coli* enzyme and the *S. shibatae* enzyme belong to different classes and do not exhibit obvious sequence similarity outside the active site, this major difference in their mechanism of sequence is not entirely

surprising. However, the determination of whether the *S. shibatae* enzyme can catalyze poly(C) addition will help to clarify this difference. On the other hand, the poly(C) polymerase activity of the *E. coli* model might suggest a similarity with some aspect of the *S. shibatae* model. Specifically, the *S. shibatae* enzyme can synthesize CCA even when it is crosslinked to a tRNA substrate. This suggests an overall fixed enzyme–RNA complex that "scrunches" the 3′ end without translocation of the enzyme relative to the rest of the tRNA substrate (Shi et al., 1998a). The poly(C) polymerase activity of the *E. coli* model implies a similar scrunching at the 3′ end. The synthesis of poly(C), beyond C74 and C75, suggests that the enzyme adjusts after each addition of C to accommodate the increased length of the 3′ end. The test of the *E. coli* enzyme, when crosslinked to a tRNA, to synthesize poly(C) would further support this notion. The scrunching is also observed in the recent crystal structure of T7 RNA polymerase for the enzyme to accommodate the template during synthesis of RNA (Cheetham & Steitz, 1999).

Relationships with poly(A) polymerase

In the class II family of nucleotidyltransferases, the extensive homology between the eubacterial CCA-adding enzymes and poly(A) polymerases is unparalleled by any other members. This homology was previously not understood, given the contrast in product synthesis between these two types of enzymes. Recent studies also indicate a functional link between the *E. coli* CCA-adding enzyme and poly(A) polymerase. Although elimination of the CCA-adding enzyme from an *E. coli* strain reduces the growth rate and leads to accumulation of defective tRNA 3′ ends, this defect can be partially rescued by overexpression of poly(A) polymerase (Reuven et al., 1997).

The demonstration that the CCA-adding enzyme can be a poly(C) polymerase now sheds light on its close sequence and functional relationship with poly(A) polymerase. Without ATP bound, the enzyme synthesizes poly(C) of undefined lengths at the tRNA 3′ end. This activity was also reported for the rabbit liver enzyme, including non-tRNA substrates such as the 5SRNA and rRNA (Deutscher, 1973b). Thus, the addition of poly(C) of undefined lengths to a wide range of substrates is analogous to the addition of poly(A) to the end of mRNAs by poly(A) polymerase. Except for the specificity for CTP of the CCA-adding enzyme and for ATP of the poly(A) polymerase, both enzymes can catalyze polymerization of homopolymers. This suggests a common origin in the evolution of these two enzymes and supports the hypothesis of a shared ancestor (Yue et al., 1996). The poly(A) polymerase would be the more primitive enzyme that only had the catalytic site. The development of the ATP site in the CCA-adding enzyme would have come later and it would serve to distinguish the CCA-adding enzyme from poly(A) polymerase.

The template-independent nature of the CCA-adding enzyme and poly(A) polymerase distinguishes these two enzymes from the template-dependent polymerases in the family of nucleotidyltransferases. Conceivably, some polymerases might have evolved a separate domain for template recognition. Recent results from domain swaps between a template-dependent member of the family of nucleotidyltransferases and a template-independent member have supported this idea (Quintana-Hau et al., 1995).

MATERIALS AND METHODS

Preparation and labeling of substrates

Minihelices were synthesized by the Nucleic Acid Facility of the University of Pennsylvania. The substrates Cys-35C and Cys-35A were synthesized by the Biotechnology Resources Facility (Howard Hughes Medical Institute Biopolymer Keck Foundation) at the Yale University School of Medicine. Minihelices of the correct size were separated from impurities by a 15% polyacrylamide gel in 7 M urea, extracted from gel materials, and eluted in 0.125 M ammonium acetate, 0.125 mM EDTA, and 0.025% SDS at room temperature overnight. The eluted minihelices were further purified by a C18 reverse-phase Sep-pak column (Waters) and resuspended in an appropriate concentration (Hamann & Hou, 1995). Minihelices (8 μM of gel purified materials) were labeled at the 5′ end by T4 polynucleotide kinase (5 U, New England Biolabs) and γ-^{32}P-ATP (3,000 Ci/mmol, NEN) in a 10-μL reaction at 37 °C for 15 min. After heat denaturation of the kinase enzyme, the labeled minihelices were separated from free ATP by a Centri-spin-20 column (Princeton Separation) and used directly.

The tRNA-75C and tRNA-76C substrates were prepared by reconstituting the large fragment (57-mer) and the small fragment (19-mer). The large fragment was prepared by T7 transcription of the *Taq*I-linearized plasmid pTFMA-Ala01 that encodes the gene of *E. coli* $tRNA^{Ala/GGC}$ (Hou et al., 1993, 1998). The small fragment was prepared by chemical synthesis through the facility at the University of Pennsylvania and purified as described for minihelices. The purified large and small fragments were annealed in equal molarity in 10 mM Tris-HCl and 20 mM $MgCl_2$ at 80 °C (3 min) and 37 °C (20 min) (Yap et al., 1995) to reconstitute the tRNA.

Purification of the recombinant *E. coli* CCA-adding enzyme

The construction of the plasmid pET22b-CCA, which encodes the *E. coli cca* gene under the control of the *lac* promoter, was as described. Transformation of pET22b-CCA into *E. coli* BL21 (DE3) for overexpression of the CCA-adding enzyme has been described (Shi et al., 1998b). The enzyme was purified by the Talon metal affinity resin according to instructions of the manufacturer (Clontech). The purified recombinant enzyme was stored at 22 μM in 0.1 M glycine (pH 9.0), 10 mM DTT with 40% glycerol at −20 °C. The wild-type enzyme purified from a separate plasmid without a His-

tag was provided by John Perona and Timothy Bullock (University of California, Santa Barbara).

Assays for *E. coli* CCA-adding enzyme

Minihelices (20 μM) were annealed in 10 mM Tris-HCl and 20 mM $MgCl_2$ by heating at 80 °C for 3 min, quick chill at 4 °C for 10 min, and incubated at 37 °C for 20 min. The annealed substrates were incubated at 37 °C with the *E. coli* enzyme (0.2 μM for the 34- and 35-mers, 2.0 μM for the 32- and 33-mers) in a 10-μL reaction. This consisted of 100 mM glycine, pH 9.0, 10 mM $MgCl_2$, 1 mM DTT, 50 μM ATP (or CTP) and 0.05 μM α-^{32}P-ATP (3,000 Ci/mmol) or 0.05 μM α-^{32}P-CTP (3,000 Ci/mmol) (Shi et al., 1998b). At defined time points, 3-μL aliquots of the reaction were removed and mixed with 3 μL of a denaturing dye containing 89 mM Tris-HCl, pH 8.1, 89 mM boric acid, 2 mM EDTA, 7 M urea, and bromophenol blue (0.1%) and xylene cyanol (0.1%). The reaction in each aliquot was separated by a denaturing 15% PAGE and the products were analyzed by a phosphorimager (Molecular Dynamics). When the substrates were labeled as in Figure 6B, 3,000 cpm of the labeled substrate were mixed with 30 μM of unlabeled substrate and incubated with 250 μM ATP and 250 μM CTP under the same reaction condition described above.

Preparation of size markers

Size markers were prepared by the CCA-enzyme with various valine minihelices and α-^{32}P-CTP under standard assay conditions for 1 h. The 33- and 34-mers were generated by incubating the enzyme (6 μM) with Val-32 and Val-33C (20 μM each), respectively. The 35-, 36-, and 37-mers were generated by incubating the enzyme (1.0 μM) with Val-34C, Val-35C, and Val-36C (20 μM each), respectively. Equal aliquots of each labeled marker were mixed to give a ladder.

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