The CCA-adding Enzyme Has a Single Active Site*

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The CCA-adding enzyme (tRNA nucleotidytransferase) adds the essential 3′-terminal CCA sequence to tRNA. The eubacterial, eukaryotic, and archaeal CCA-adding enzymes all share a single active-site signature motif, which identifies these enzymes as belonging to the nucleotidytransferase superfamily. Here we show that mutations at Asp-53 or Asp-55 of the Sulfolobus shibatae signature sequence abolish addition of both C and A, demonstrating that a single active site is responsible for addition of both nucleotides. Mutations at Asp-106 (and to a lesser extent, at Glu-173 and Asp-215) selectively impaired addition of A, but not C. We have previously demonstrated that the tRNA acceptor stem remains fixed on the surface of the CCA-adding enzyme during C and A addition (Shi, P.-Y., Maizels, N., and Weiner, A. M. (1998) EMBO J. 17, 3197–3206). Taken together with this new evidence that there is a single active site for catalysis, our data suggest that specificity of nucleotide addition is determined by a process of collaborative templating: as the single active site catalyzes addition of each nucleotide, the growing 3′-end of the tRNA would progressively refold to create a binding pocket for addition of the next nucleotide.

The CCA-adding enzyme (ATP (CTP): tRNA nucleotidytransferase) adds the essential 3′-terminal CCA sequence to tRNA (1, 2). This enzyme is present in all three living kingdoms (3). In organisms that do not encode the 3′-terminal CCA sequence, including eukaryotes, many eubacteria, and some archaea, the CCA-adding enzyme carries out an essential step in tRNA biosynthesis. In organisms like Escherichia coli where all tRNA genes encode CCA, the CCA-adding enzyme repairs 3′ termini depleted by exonucleolytic attack, but is not absolutely essential for survival (4). The CCA-adding enzyme can use tRNA-CC, tRNA-C, or tRNA-N (containing only the discriminating base) as substrate. It completes the terminal CCA sequence by adding one nucleotide at a time. Despite the fact that the CCA-adding enzyme has no nucleic acid template, incorporation is highly specific and apparently error-free under physiological conditions: neither UTP nor GTP is incorporated into tRNA, and the enzyme does not add oligo(C) to tRNA-C when deprived of ATP or oligo(A) to tRNA-CC when deprived of CTP (3, 5).

We recently cloned the CCA-adding enzyme from the thermophilic archaeon Sulfolobus shibatae (3). The archaeal CCA-adding enzyme contained a clear match with the active-site signature of the nucleotidytransferase superfamily (6), but it exhibited no strong homology to the eubacterial and eukaryotic CCA-adding enzymes (which are very similar to one another). This enabled us to divide enzymes of the nucleotidytransferase superfamily into two distinct classes (3). Class I enzymes include the archaeal CCA-adding enzymes (Fig. 1), eukaryotic poly(A) polymerases, DNA polymerase β, and kanamycin nucleotidytransferase. Class II enzymes include eubacterial and eukaryotic CCA-adding enzymes and eubacterial poly(A) polymerases. Class II enzymes share 25 kDa of strong N-terminal homology, but diverge at the C termini (3, 7). In contrast, Class I enzymes exhibit little obvious similarity to each other or to class II enzymes outside of the active-site signature.

The nucleotidytransferase superfamily active-site signature (6) contains two carboxylates (DXX(E)) that are thought to occupy the same positions in space as seen in the crystal structures of DNA polymerase β (8, 9) and kanamycin nucleotidytransferase (10). These two carboxylates, together with a third contributed by a more distant region of the protein, coordinate two divalent metal cations that stabilize the trigonal bipyramidal transition state generated by S_n2 attack of a hydroxyl group on a phosphoester bond (11). Three carboxylates are necessary for DNA polymerase β activity: Asp-190 and Asp-192 within the conserved signature (12) and the distant Glu-256 (13). Similarly, three carboxylates are necessary for bovine poly(A) polymerase activity: Asp-113 and Asp-115 within the signature and the distant Asp-167 (7). These data suggest that class I and II enzymes of the nucleotidytransferase superfamily use the same reaction mechanism as many other polymerases (14, 15). The alignment of Martin and Keller (7) predicted that the third carboxylate should fall within an RRD motif in class II enzymes and within an RRD motif in class I enzymes as previously shown for DNA polymerase β and poly(A) polymerase. However, no RRD motif could be identified by inspecting the sequences of the four known archaeal CCA-adding enzymes. We therefore set out to mutate systematically each of the conserved carboxylates in the S. shibatae CCA-adding enzyme, keeping in mind that the archaeal enzyme might have dispensed with the need for a third carboxylate, as have a number of DNA and RNA polymerases (see “Discussion”).

Sequence alignments predicted that the aspartate residues at positions 53 and 55 within the active-site signature of the S. shibatae CCA-adding enzyme would be critical to catalysis. Here we report that mutation of either Asp-53 or Asp-55 abolishes addition of both CTP and ATP by this enzyme. No third carboxylate seems to be absolutely required, although mutation of Asp-106 (and to a lesser extent, Glu-173 and Asp-215) diminishes ATP but not CTP addition. These data demonstrate that one catalytic center is responsible for addition of both CTP and ATP. In principle, a single catalytic center could translo-
cated along the tRNA molecule to add all three nucleotides of the terminal CCA sequence. However, we have recently found that the tRNA acceptor stem does not translocate or rotate as each nucleotide is added to the 3'-end of tRNA-C, but remains fixed upon the surface of the enzyme (16). Taken together, the observations that there is a single active site and that RNA polymerization occurs without translocation argue that the tRNA would progressively refold to create a binding pocket for addition of the next nucleotide, until addition ceases when the CCA-binding pocket is full.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Deletion**—To facilitate protein purification and site-directed mutagenesis, the _S. shibatae_ cca gene (3) was cloned into the pET22x(b) vector (Novagen), which supplies a hexahistidine tag through a six-residue tether (LAAAL). The resulting construct was used to ensure that no mutations had been introduced by polymerase chain reaction.

Site-directed mutagenesis was carried out according to Kunkel et al. (17). _E. coli_ strain C2356 ( _dut_ , _ung_ ) was transformed with pET22ca-His and infected with helper phage M13K07, and single-stranded phage DNA (corresponding to the CCA-coding strand) was prepared by multiple phenol and chloroform extractions. Single-stranded DNA was annealed with mismatched oligonucleotides (synthesized at the W. M. Keck Biotechnology Center, Yale School of Medicine) (Table I) by heating to 90 °C, followed by slow cooling to 25 °C in 10 min in 0.1 M Tris-HCl, 0.5 M NaCl, and 1 mM MgCl₂, 0.4 mM each dNTP, and 0.75 mM ATP. Reactions were stopped by addition of EDTA, and plasmids were transformed into _E. coli_ strain BL21(DE3) (Table I; by heat-
galactopyranoside for 3 h at 37 °C to induce gene overexpression. N. The purpose of each oligonucleotide is indicated on the right. Oligonucleotides for site-specific mutagenesis are complementary to the coding strand. Restriction sites are underlined; degenerate positions are indicated by a slash; and mutant codons are in lowercase.

### Table I

| Oligonucleotide | Use or mutation generated |
|-----------------|--------------------------|
| 1. AGCAGAAGCTTTAACCAAGTCGGGT | 3′-PCR primer for WT |
| 2. CTGAGATGCTTTAGAATTTCCTCTT | 3′-PCR primer for ΔC30 |
| 3. TCGAGATGCTTTAGAATTTCCTCTT | 5′-PCR primer for WT and ΔC30 |
| 4. GATAGAAGCTTTAACCAAGTCGGGT | D53A, D55A, D55E, D63A/D65A |
| 5. CAATGCTGGGACTATATAGCTATGCTTTC | D106A |
| 6. GCCTATGCTGCTTTCGCATCTCCTACAC | E114A |
| 7. CATGCTGCTGCTTTCGCATCTCCTACAC | E104A |
| 8. ATGGTGGTTCTAGCTACTGCAGT | D124A |
| 9. TAGGATCTCCACTGGGGT | E139A |
| 10. GCCTAACCCTAGCTTTCCTCT | D143A |
| 11. GTAGGCTTAAACCAGCAGCTTTCC | E144A |
| 12. CTGCTACTCTCAAGAGGCCCCTG | E161A |
| 13. ATAGCTAGCTTAATTGGGTCGTTGCT | E173A |
| 14. GGATTAACCTTGGTACGCCAGAAAAGC | E209A |
| 15. TAGGATCTCCACTGGGGT | D215A |
| 16. CATGCTGCTTTCGCATCTCCTACAC | D218A |
| 17. CAGCCCGCTAAAGCAGCTTTCCT | G156D |

* PCR, polymerase chain reaction; WT, wild-type enzyme.

trimming the 3′-ends of the unfractonated tRNA (100 mg total in 25 ml) with snake venom phosphodiesterase I (1.2 units/25 ml; Sigma) for 1 h at 20 °C in 100 mM Tris-HCl (pH 9.0), followed by phenol/chloroform extraction. Under these conditions, partial digestion by the 3′-exonuclease leaves a tRNA mixture with N, NC, and NCC ends, where N is the discriminator base; addition of both CTP and ATP increases by ~10-fold after this treatment. To remove any rRNA fragments and degradation products, 3′-end-trimmed tRNA was bound to Q-Sepharose (2 mg of tRNA/ml column; Amersham Pharmacia Biotech) in 20 mM Tris-HCl (pH 7.5), washed with the same buffer containing 0.4 M NaCl, and eluted with a 0.4–1.0 M NaCl gradient, and the pooled tRNA fractions were precipitated with ethanol. All mutant CCA-adding enzymes were assayed using a single batch of 3′-end-trimmed tRNA substrate. Bacillus subtilis tRNAaas lacking the 3′-CA sequence was transcribed in vitro by T7 RNA polymerase using FolI-digest ed pmBsDCCA as template (a kind gift of N. Pace, University of California, Berkeley, CA). The runoff transcript was gel-purified as described (16). tRNA was quantified assuming an A260 nm of 24 at 1.0 mg/ml.

### RESULTS

**Signature Sequence Carboxylate Mutants**—All CCA-adding enzymes belong to the nucleotidyltransferase superfamily and share a similar active site signature, which contains a DXD motif. Based on crystal structures for two enzymes in this family, DNA polymerase β and kanamycin nucleotidyltransferase, the carboxylates in the signature motif are thought to chelate the two metal ions involved in phosphoester bond transfer (11). Martin and Keller (7) demonstrated by mutational analysis that the two carboxylates in the active-site signature motif are indeed essential for catalysis by another enzyme in this family, bovine poly(A) polymerase. The S. shibatae CCA-adding enzyme contains a clear DXD motif in the predicted active site (3). To determine if the aspartate residues in this motif are critical for catalysis, we generated four mu-
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Initial reaction rates are shown and were measured as described under “Experimental Procedures” using standard conditions with 5–120 ng of His-tagged purified enzyme/60-μl reaction. All rates were normalized to the wild-type enzyme concentration.

| Enzyme                          | ATP addition | CTP addition |
|---------------------------------|--------------|--------------|
| C-terminal deletions            | %            | %            |
| WT                              | 100          | 100          |
| ΔC30                            | 0.2          | 0.6          |
| ΔC135                           | <0.05        | <0.05        |
| Signature sequence mutation     | %            | %            |
| WT                              | 100          | 100          |
| D53A                            | <0.05        | 0.6          |
| D55A                            | <0.05        | <0.05        |
| D53A/D55A                       | <0.05        | <0.05        |
| D55E                            | 0.3          | 1.0          |

*WT, wild-type enzyme.

We first sought a tRNA substrate concentration that gave half-maximal CTP-adding activity at saturating ATP and CTP. CTP-adding activity was assayed using 0.125–4 μM tRNA at high (200 μM ATP and 50 μM CTP) and low (50 μM ATP and 12.5 μM CTP) nucleotide concentrations. As shown in Fig. 3, 1 μM tRNA was nearly saturating at both NTP concentrations, and we therefore used 0.25 μM tRNA for subsequent assays.

Next we sought ATP and CTP concentrations that gave half-maximal ATP- and CTP-adding activities at fixed 0.25 μM tRNA. We assayed CTP-adding activity using 3–50 μM ATP at fixed 25 μM CTP (Fig. 4A) and using 1.5–25 μM CTP at fixed 50 μM ATP (Fig. 4B). This experimental protocol was designed to test whether the ATP- and CTP-binding sites of the archaeal enzyme interact with each other, as appeared to be the case for the rabbit liver CCA-adding enzyme (24, 25). We found that CTP-adding activity was unaffected by up to 50 μM ATP (Fig. 4A) and was half-maximal at 3 μM CTP (Fig. 4B). Subsequent CTP addition assays were therefore performed at 3 μM CTP + 50 μM ATP.

Similarly, we assayed ATP-adding activity using 6.2–100 μM ATP at fixed 50 μM CTP and found that 100 μM ATP was not saturating (Fig. 5A). ATP was therefore fixed at 100 μM, and ATP-adding activity was assayed by varying CTP from 3 to 50 μM (Fig. 5B). ATP addition was reduced at 50 μM CTP, the lowest CTP concentration tested. This was expected, as the tRNA substrates prepared by partial digestion of mature tRNA with venom phosphodiesterase lack the 3′-terminal CA and require addition of C before A. Subsequent ATP addition assays were therefore performed at 100 μM ATP + 3 μM CTP.

All mutant CCA-adding enzymes, including point mutations and both deletions, were then retested for ATP- and CTP-adding activities under challenge conditions (Fig. 6, left and right panels, respectively). Some level of CTP- and ATP-adding activities (5–90%) was evident in all mutants, except those in the signature sequence (D53A, D55A, D55E, and D53A/D55A) and the two C-terminal deletions (ΔC30 and ΔC135). A number of the other mutants retained considerable activity under these conditions. However, in three mutants, activity was reduced to 20% or less of wild-type levels. D215A was defective in both CTP- and ATP-adding activities (16 and 13% of wild-type levels, respectively), and E173A was also defective in both CTP- and ATP-adding activities (19 and 7% of wild-type levels, respectively). Most intriguingly, D106A retained 90% of normal CTP-adding activity, but was only 14% as active as the wild-type enzyme when assayed for ATP-adding activity.

A Shared GXGXXG Motif?—The E. coli CCA-adding enzyme
contains a potential ATP-binding motif (GXGXXG) just downstream of the active-site signature sequence, and a mutation in the second G in the motif (G70D) had previously been shown to diminish ATP- but not CTP-adding activity by 7–10-fold in crude extract (26). However, this GXGXXG motif is not conserved in any other of the highly homologous class II nucleotidyltransferases such as the CCA-adding enzymes of a fungus (Saccharomyces cerevisiae), a higher plant (Lupinus albus), a metazoan (Caenorhabditis elegans), and a variety of other eu-bacterial CCA-adding enzymes and poly(A) polymerases (Acidaminococcus fermentans, B. subtilis, Hemophilus influenzae, Mycobacterium leprae, and Thermus aquaticus). Curiously, the S. shibatae CCA-adding enzyme also contains a GXGXXG sequence downstream of the signature sequence, and this motif is partially conserved in other highly homologous class I CCA-adding enzymes (M. jannaschii, M. thermoautotrophicum, and A. fulgidus) (Fig. 1). Although similarity between class I and II nucleotidyltransferases is apparently confined to the active-site signature sequence, we nonetheless asked whether the GXGXXG motif of S. shibatae (a class I CCA-adding enzyme) might be functionally equivalent to that of E. coli (a class II CCA-adding enzyme). As shown in Fig. 6, neither CTP- nor ATP-adding activity was significantly affected by a G156D mutation in the second G of the S. shibatae sequence. Thus, the GXGXXG motif is not conserved, either structurally or functionally, in class I or II CCA-adding enzymes. Most likely, the G70D mutation (26) affects ATP addition indirectly, perhaps by altering overall protein structure rather than the ATP-binding site.

D106A Preferentially Affects ATP Addition—To confirm the
selective deficiency in A addition by mutant D106A, mutant enzymes that retained significant activity using venom-treated E. coli tRNA as substrate (Fig. 6) were reassayed under the same challenge conditions for ATP-adding activity (left panels) and CTP-adding activity (right panels) using in vitro transcribed B. subtilis tRNA<sup>CC</sup>-t as substrate. Upper and lower panels represent two different gels, and asterisks indicate labeled nucleotide. WT, wild-type enzyme.

**DISCUSSION**

We have identified two active-site carboxylates in the CCA-adding enzyme from the thermophilic archaeon S. shibatae. Asp-53 and Asp-55 both fall within the conserved nucleotidyltransferase signature sequence, and four different mutations at these residues (D53A, D55A, D55E, and D53A/D55A) abolish addition of both CTP and ATP. Thus, the CTP- and ATP-adding reactions appear to use the same catalytic center, although strictly speaking, we cannot rule out distinct but overlapping catalytic sites.

To explain how the CCA-adding enzyme can accurately polymerize CCA without recourse to a nucleic acid template, Masiakowski and Deutscher (24, 25) proposed that the active site contains multiple "subsites" recognizing the body of the tRNA, the 3′ terminus of the tRNA primer, and each of the donor nucleotides. This model was supported by extensive kinetic data using partially purified rabbit liver enzyme and state-of-the-art, oligonucleotide and tRNA-derived substrates. Using powerful new tools for protein overexpression and purification, site-directed mutagenesis, oligonucleotide synthesis, and in vitro transcription, we have now been able to revisit these earlier conclusions. Our results make it very unlikely that the CCA-adding enzymes contain multiple subunits.

The active sites of many polymerases (including the three best studied members of the nucleotidyltransferase superfamily: DNA polymerase β, poly(A) polymerase, and kanamycin nucleotidyltransferase) contain three key carboxylates that chelate two divalent metal ions (7–10, 15, 27, 28). Typically, two of these carboxylates are adjacent to one another or separated by a single residue in the primary sequence and can usually be identified by inspection of the primary sequence. The third carboxylate is more distant in primary sequence, although close in three-dimensional space, and it is often less obvious from sequence analysis. In the two other class I enzymes examined so far, DNA polymerase β and bovine poly(A) polymerase, a third carboxylate is clearly essential for activity (7, 13). An alignment of rat DNA polymerase β, bovine poly(A) polymerase, and kanamycin nucleotidyltransferase (7) suggested that the third carboxylate in the S. shibatae CCA-adding enzyme should fall within an RXD motif. As no conserved RXD motif was present in the four known archaeal CCA-adding enzymes (S. shibatae, M. jannaschii, M. thermoautotrophicum, and A. fulgidus), we performed alanine scanning mutagenesis of all 12 conserved or partially conserved carboxylates in the archaeal enzymes. None of the mutations in these conserved carboxylates abolished activity under standard assay conditions (saturating tRNA and CTP and nearly saturating ATP). A third carboxylate therefore appears not to be essential for CCA-adding activity.

Three mutants exhibited significant defects (<20% activity) under challenge conditions, in which concentrations of tRNA, CTP, and ATP were limiting. E173A and D215A were defective in both CTP- and ATP-adding activities. This could indicate a global effect on enzyme structure, or because our data suggest that there is only a single catalytic site, one or both mutations could influence the structure of the active site. The most interesting mutant was D106A, which retained normal CTP-adding activity, but did reduce dNTP binding (31). In contrast, the structurally equivalent Asp-705 of Klenow fragment and Asp-
110 of reverse transcriptase play critical roles in both dNTP binding and catalysis (32–34). T7 RNA and DNA polymerases provide two other exceptions to the rule. Only two active-site carboxylates have been identified in T7 RNA polymerase (27, 35). These two residues (Asp-537 and Asp-812) are far apart in the protein sequence, but both are important for metal binding and catalysis (36), and there is no candidate for a third carboxylate nearby in the crystal structure (37). The high resolution structure of T7 DNA polymerase complexed with a primer-template and a deoxynucleoside triphosphate revealed two metals interacting with the strictly conserved Asp-475 and Asp-654, but not with Glu-655 (38). Thus, two carboxylates suffice for binding two active-site metals that stabilize the trigonal bipyramidal transition state required for phosphoester bond transfer.

If two carboxylates suffice for other polymerases, one must then ask what is the function of the third carboxylate in other enzymes of the nucleotidyltransferase superfamily? This residue could participate in catalysis, nucleotide triphosphate binding, or both as discussed below. We speculate that the third carboxylate in these other class I enzymes may be a component of a dNTP- or ATP-binding pocket. If so, mutations in the third carboxylate might have a greater effect on poly(A) polymerase (a template-independent enzyme) than on DNA polymerase β, where the template strand can help to select the incoming nucleotide.

Our phylogenetic analysis suggests that the CCA-adding enzyme and poly(A) polymerase may have interconverted at least once over the course of evolution (3): the eubacterial poly(A) polymerase and CCA-adding enzymes are highly homologous class I enzymes, whereas the eukaryotic poly(A) polymerase and CCA-adding enzymes belong to different classes of the nucleotidyltransferase superfamily and share no obvious similarity outside the active-site signature sequence (3). Thus, the eukaryotic CCA-adding enzyme could have “degenerated” into a poly(A) polymerase, or poly(A) polymerase might have acquired the ability to add CCA. The intraconversion of these two enzyme activities might also explain why some RNA and DNA polymerases preferentially add untemplated adenosines during runoff transcription (39–41) or polymerase chain reaction amplification (42, 43).

Finally, we propose that the CTP- and ATP-binding pockets of the CCA-adding enzyme represent a collaboration between the enzyme and the tRNA primer. Our reasoning is straightforward. 1) We have shown here that the S. shibatae CCA-adding enzyme has only a single active site because CTP and ATP additions are both disabled by mutations in either carboxylate (Asp-53 or Asp-55) of the nucleotidyltransferase active-site signature sequence. 2) We have recently shown that the tRNA acceptor stem remains fixed upon the surface of the S. shibatae and E. coli CCA-adding enzymes as each new nucleotide is added to the growing 3′-end; the tRNA does not rotate or translocate relative to the enzyme (16). 3) Judging by the compact crystal structures of two other class I enzymes, DNA polymerase β (8, 9) and kanamycin nucleotidyltransferase (10), the active site is unlikely to move relative to the tRNA-binding site.

If neither the tRNA nor the active site moves as each nucleotide is added to the growing 3′-end of the tRNA, the 3′-end itself must move. We therefore propose the following model for RNA polymerization without translocation. As the active site catalyzes addition of each new nucleotide, the growing 3′-end of the tRNA would progressively refold to create a binding pocket for addition of the next nucleotide. Specifically, binding of tRNA-NCC to the enzyme would create an ATP-binding pocket. This would be consistent with earlier kinetic studies showing that tRNA-N and tRNA-NC are poor substrates for ATP addition, tRNA-NCC is a poor substrate for CTP addition, and mutations in the 3′-terminal Cys-75 in tRNA-NCC prevent ATP addition (10, 24, 25). The enzyme and the growing 3′-end would collaboratively template CCA addition, and addition would cease when the CCA-binding pocket was full. We wish to emphasize that this model is likely to apply to both class I (archaeal) and class II (eubacterial and eukaryotic) CCA-adding enzymes because the active sites of the two classes are highly homologous (3), and the tRNA does not rotate or translocate relative to either class of enzyme during CCA polymerization (16).

Collaborative templating is not radical or unprecedented. Selection of the correct nucleotide by template-directed polymerases also represents an obvious, although seldom acknowledged, collaboration between enzyme and substrate. The incoming nucleotide fits into a flat, narrow pocket; the incoming nucleotide forms hydrogen bonds with the edge of the template base on the floor of the pocket, and it stacks between the two flat hydrophobic walls of the pocket defined by the previous base pair (n–1) and a critical tyrosine that behaves like the next base pair (n+1). Functionally equivalent tyrosines are found in Tag (44), T7 (38), and E. coli (45) DNA polymerases and human DNA polymerase α (31). Thus, although the primer-template is a major determinant specifying the incoming nucleotide, template-dependent polymerases recognize the incoming sugar (ribose or deoxyribose) and provide one of the two hydrophobic walls that sandwich the incoming nucleotide, conferring greater stability and specificity on this key hydrophobic bonding interaction. The possibility that the specificity of CCA addition is determined by collaborative templating provides a mechanistically plausible answer to the long-standing question of how the CCA-adding enzyme specifically adds the CCA sequence without an external nucleotide template.

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