A Single Catalytically Active Subunit in the Multimeric Sulfolobus shibatae CCA-adding Enzyme Can Carry Out All Three Steps of CCA Addition*

Received for publication, May 18, 2004, and in revised form, July 16, 2004
Published, JBC Papers in Press, July 19, 2004, DOI 10.1074/jbc.M405518200

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The CCA-adding enzyme is a member of the nucleotidyltransferase superfamily because the enzyme synthesizes a precise sequence without using a nucleic acid template (16, 22). Five different models have been proposed to explain CCA addition in the absence of a nucleic acid template (5, 16, 20, 23–26). First, the two-site model postulates at least two nucleotide binding sites, one for CTP and the other for ATP, and supports a role for the tail domain of the enzyme in tRNA binding.

The 3′-terminal CCA sequence of mature tRNA is essential for efficient aminocacylation (1, 2) as well as for translation because it base pairs with the large rRNA near the peptidyltransferase center (3, 4). The CCA-adding enzyme ATP(CTP):tRNA nucleotidyltransferase builds and repairs the 3′-terminal CCA sequence of tRNA. Although this unusual RNA polymerase has no nucleic acid template, it can construct the CCA sequence one nucleotide at a time using CTP and ATP as substrates. We found previously that tRNA does not translocate along the enzyme during CCA addition (Yue, D., Weiner, A. M., and Maizels, N. (1998) J. Biol. Chem. 273, 29693–29700) and that a single nucleotidyltransferase motif adds all three nucleotides (Shi, P.-Y., Maizels, N., and Weiner, A. M. (1998) EMBO J. 17, 3197–3206). Intriguingly, the CCA-adding enzyme from the archaeon Sulfolobus shibatae is a homodimer that forms a tetramer upon binding two tRNAs. We therefore asked whether the active form of the S. shibatae enzyme might have two quasi-equivalent active sites, one adding CTP and the other ATP. Using an intersubunit complementation approach, we demonstrate that the dimer is active and that a single catalytically active subunit can carry out all three steps of CCA addition. We also locate one UV light-induced tRNA cross-link on the enzyme structure and provide evidence suggesting the location of another. Our data rule out shuttling models in which the 3′-end of the tRNA shuttles from one quasi-equivalent active site to another, demonstrate that tRNA-induced tetramerization is not required for CCA addition, and support a role for the tail domain of the enzyme in tRNA binding.

This paper is available on line at http://www.jbc.org
model proposes that the growing 3'-end of the tRNA is progressively sequestered within a pocket near the active site; progressive packing of this pocket would create a binding site for the incoming nucleotide, and CCA synthesis would cease when the pocket was full (16, 28). Fourth, the "scrunching-shuttling" model proposes that two identical subunits of the tRNA-induced tetramer enzyme would function nonequivalently (24). One subunit would add C74 and then "scrunch" or bulge C74 to allow the addition of C75; the CC terminus would then be long enough to "shuttle" to a nonequivalent subunit, which would add A76. Fifth, the "protein-assisted scrunching" model (20) proposes that the scrunching of the 3'-terminal CC (but not C74 alone) would cause a conformational change that switches the specificity of the nucleotide binding pocket from CTP to ATP.

The scrunching-shuttling model of CCA addition attempts to explain the otherwise puzzling observation that the dimeric Sulfolobus shibatae CCA-adding enzyme is a tRNA-inducible tetramer that binds two tRNA molecules (24). To test whether the growing 3'-end of tRNA can shuttle within the tetramer from one quasi-equivalent active site that adds CTP to a second quasi-equivalent active site that adds ATP (24), we performed intersubunit complementation experiments. Using the wild type S. shibatae CCA-adding enzyme as well as three different mutants that block the addition of C74 (Y95V), the addition of A76 (H93V), or the addition of both CTP and ATP (K153A), we generated heterodimers by the reassortment of tagged and untagged subunits under partially denaturing conditions. We found that a single wild type subunit in an immobilized heterodimer can still add CCA; moreover, by cross-linking the tRNA substrate to the enzyme before or after subunit reassortment, we were able to show that the inactive subunit in an active heterodimer contacts the tRNA substrate. Thus, our data rule out shuttling models in which the 3'-end of the tRNA shuttles from one quasi-equivalent active site to another (24) but are consistent with a protein-assisted scrunching model for a CCA addition (20) in which the scrunching of CC (but not C74 alone) switches the specificity of nucleotide binding site from CTP to ATP.

MATERIALS AND METHODS

Preparation of tRNA Substrates—tRNA substrates lacking A, CA, or CCA (tRNA-DC, tRNA-DC, or tRNA-D, where D is the discriminator base) were prepared as described previously by in vitro transcription of FokI- or BbsI-digested pmEsBDeCCA encoding Bacillus subtilis tRNA(20, 17). Following purification by electrophoresis in a 12% polyacrylamide gel containing 8% urea, the tRNA band was visualized by UV shadowing, excised, eluted, and concentrated by ethanol precipitation.

Unagged and Histidine-tagged CCA-adding Enzymes—Unagged and histidine-tagged S. shibatae CCA-adding enzymes were expressed and purified as described previously (6, 16, 17). Individual mutant H93V, Y95V, and K153A are described in detail elsewhere, and untagged mutants (H93V, Y95V, and K153A) were constructed by the site-directed mutagenesis method (Qiagen). Briefly, 290 μl of CCA-adding enzyme in phosphate-buffered saline (50 mM potassium phosphate, pH 7.2, 150 mM NaCl) at a concentration of 1 mg/ml was added to each well of an Ni-NTA HisSorb plate and incubated for 1 h at room temperature. After incubation, the wells were washed four times with phosphate-buffered saline containing 0.5% Tween detergent to remove nonspecifically bound proteins. The bound proteins were eluted with 100 μl of 1 M NaCl in 0.5 M Tris-HCl, pH 8.0, 0.1% SDS, and 0.5 M, respectively; and tRNA was increased to 2 μg in solution (to recover CCA-adding enzyme in Fig. 5, in solution (to recover CCA-adding enzyme on the HisSorb plate), which would be equivalent to 100 nM enzyme if it were free to diffuse throughout the 100-μl reaction volume. After incubation at 55 °C for 3 min the reactions were withdrawn from the Ni-NTA HisSorb plates, and the tRNA was purified by phenol extraction and concentrated by ethanol precipitation using glycerol as a carrier. Reaction products were resolved by denaturing 12% PAGE and quantified by phosphorimaging (Amersham Biosciences).

CCA Addition to UV Light-cross-linked tRNA-Enzyme Complexes—UV light-cross-linking of tRNA to enzyme was carried out essentially as described previously (16); S. shibatae CCA-adding enzyme was denatured with 8 M urea for 1 h, diluted into 4 μl, and incubated for 10 min at room temperature with 5 μg of unlabelled tRNA-D in standard reaction buffer lacking CTP and ATP. After irradiation at 254 nm with a hand-held UV light (Spectronics Corp.) for 10 min on ice, uncross-linked tRNA was removed by Amicon YM-30 filtration (Millipore). To generate heterodimers bearing cross-linked tRNA, 5 μg of cross-linked tRNA-enzyme complex was mixed with 20 μg of histidine-tagged wild type or mutant enzymes, and the reactions were denatured and annealed; heterodimers were recovered on a Ni-NTA affinity column, eluted with imidazole in buffer A, dialyzed into buffer B, and concentrated by Amicon YM-30 filtration. The CTP addition to UV-cross-linked tRNA-enzyme complexes was assayed using 0.5 μM [α-32P]CTP and 50 μM ATP; ATP addition was assayed by using 0.5 μM [α-32P]ATP and 50 μM CTP. All reactions were incubated at 70 °C for 10 min, analyzed by 10% SDS-PAGE, and quantified by phosphorimaging (Amersham Biosciences).

tDNA Minihelix for UV Cross-linking—DNA minihelices with a 13-base pair stem were generated by annealing equimolar amounts of two oligonucleotides, namely 5'-CCG/TGCTCACCCG-3' and 5'-GGTGATGGCGGG-3' (DNAgency). The oligonucleotides were brought to a concentration of 15 μM in 5 mM HEPES (pH 7.9), 50 mM NaCl, and 10 mM MgCl2, denatured by heating to 80 °C for 3 min, and quick-cooled on ice for 10 min (29). A 200-μl reaction containing 6 μM (1200 pmol) tDNA minihelix and 6 μl of tDNA minihelix (1200 pmol) S. shibatae enzyme was generated as described (30–32). When required, the tDNA minihelix was 5'-labeled with T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol; Amersham Biosciences) as described by the supplier (New England Biolabs).

Tryptic Digestion and Purification of the Cross-linked Peptide—After

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Methods

A protocol for generating heterodimers by dissociation and reassociation of tagged and untagged homodimers. tRNA is depicted as a boomerang-shaped object; H indicates a hexahistidine tag; WT is wild type. B, heterodimers can be formed by dissociation of homodimers in 6 M urea followed by reassociation upon dilution. Untagged homodimers are removed by purification over a Ni-NTA affinity column. Left section, tagged and untagged enzymes stain equally well with Coomassie Brilliant Blue. Middle section, complete conversion of tagged homodimers to tagged heterodimers can be achieved by denaturation in 6 M urea; 6 M guanidine HCl (Gu) is no more effective a denaturant than 6 M urea. Right section, formation of heterodimers and/or heterotetramers is unaffected by the addition of equimolar tRNA before denaturation. All denaturation and renaturation steps are performed in Buffer B, which contains 100 mM KCl and 10 mM MgCl2 to facilitate tRNA renaturation (see “Materials and Methods”).

Results and Discussion

Generating Heterodimers That Contain a Single Active Subunit—We established previously that the S. shibatae CCA-adding enzyme has only a single active site (28) and that the enzyme can faithfully add CTP and ATP to tRNA substrates that have been cross-linked to the enzyme by UV light irradiation (16). These data ruled out conventional translocation of the tRNA minihelix along the enzyme as CCA is added and suggested instead that the 3′-end of the tRNA substrate refolds (16) or “scrunches” (20) to reposition the growing 3′-end in a shuttle model, the tRNA minihelix would remain fixed on the enzyme; however, subsequent discovery that the dimeric S. shibatae enzyme is a tRNA-inducible tetramer suggested a very different model in which the growing 3′-end could shuttle within the tetramer from one quasi-equivalent active site that adds CTP to a second quasi-equivalent site that adds ATP (24). The quasi-equivalent sites would have different nucleotide specificity either because the tetramer is intrinsically asymmetric or because tRNA binding induces an asymmetric conformational change. In the simplest version of this shuttling model, the tRNA minihelix would remain fixed on the enzyme; however, the 3′-end of the shorter tRNA-N and tRNA-NC substrates could only reach the CTP addition site, whereas the 3′-end of the longer tRNA-NCC substrate could reach the ATP addition site. We set out to test this alternative model by asking whether an active subunit can complement inactive subunits within a heterodimeric enzyme.

To generate heterodimers of mutant and/or wild type enzymes, hexahistidine-tagged and untagged homodimers were combined pairwise, denatured in urea, and renatured by stepwise dilution, and dimers bearing the hexahistidine tag were recovered on Ni-NTA resin (Fig. 1A). In addition to wild type, three different S. shibatae mutant enzymes were used as follows: Y95V blocks the addition of C74 but not the addition of C75; H93V blocks the addition of A76; and K153A blocks both CTP addition and ATP addition.1

As shown in Fig. 1B, 6 M urea is sufficient to cause a complete reassortment of subunits. A 1:4 mixture of tagged and untagged subunits was denatured in 0, 2, 4, 6, or 8 M urea and renatured by dilution, and tagged dimers were recovered on a Ni-NTA column. Above 6 M urea, the ratio of tagged to untagged subunits is constant and roughly 5:4, as expected for complete subunit reassortment. Confirming the selectivity of the purification, only tagged subunits are retained on Ni-NTA resin when the urea denaturation step is omitted (Fig. 1B). Although complete subunit reassortment requires 6 M urea, significant reassortment is seen at 4 M urea, a concentration that is only mildly denaturing for most proteins; thus, it seems likely that urea preferentially weakens the dimer interface rather than completely denaturing the subunits.

Heterodimers were generated as in Fig. 1, eluted from the Ni-NTA resin with imidazole, concentrated by Amicon YM-30 filtration, and assayed for CCA-adding activity (Fig. 2). Intriguingly, heterodimers of a wild type subunit with the C74-defective Y95V mutant, the A76-defective H93V mutant, and the catalytically inactive K153A mutant were all able to add CCA. We cannot conclude from this experiment alone that CCA is added by a single catalytically active subunit, because the addition of the tRNA substrate induces heterodimers to form tetramers (24). These tetramers could, in principle, juxtapose two wild type subunits as postulated by the “shuttling” model, where one quasi-equivalent active site would add CTP and the
CCA Addition by a Single Catalytically Active Subunit

Our dissociation/reassociation protocol allows specific (Fig. 2). wild type subunit, nor is activity of the heterodimer mutant—shown; are well behaved; heterodimerization does not inactivate the other ATP. We can conclude, however, that the heterodimers have been evident. The average of three independent experiments is upon formation of heterotetramers (a dimer of heterodimers) would linear range for 3 min with excess tRNA; a 2-fold inhibition of activity mutants under these conditions (Fig. 3), and wild type subunits did not reassort with tagged subunit reassortment step was included before immobilization, all three heterodimers displayed wild type activity (Fig. 3). Heterodimers cross-linked to tRNA were eluted with A single wild type subunit conferred full CCA-adding enzyme on the HisSorb plate results in roughly half-maximal enzyme activity, not quarter-maximal as would be expected for a bimolecular reaction (Fig. 3C, right panel). Second, equivalent picomoles of subunits have comparable activity whether immobilized as heterotetramers (Fig. 3C, left panel) or heterodimers (Fig. 3C, right panel).

\( tRNA \) Cross-linked to One Subunit of the Heterodimer Serves as Substrate for the Other Subunit—To ask whether the inactive subunit in an active heterodimer (Fig. 3) plays a direct role in CCA addition, **tRNA** was UV light-cross-linked to heterodimers before subunit reassortment. This analysis was greatly simplified by the observation that UV light cross-linking of **tRNA** to the enzyme either in the presence of 4 M urea or following exposure to 4 M urea during the renaturation protocol generates only one of the two cross-linked complexes seen when cross-linking was performed in the absence of urea (Fig. 4; compare Ref. 16). Although we do not understand why exposure to 4 M urea prevents the formation of complex 1 or why it slightly reduces the yield of complex 2 (Fig. 4), both complex 1 and complex 2 are sensitive to proteinase K and RNase T1 digestion as expected for covalent **tRNA**/protein complexes (data not shown). Complexes 1 and 2 may be cross-linked to different sites on the **tRNA**, different sites on the enzyme subunit, or different subunits within the enzyme dimer (see Fig. 6). In any event, \( tRNA \) cross-linked to the enzyme was nearly as good a substrate as free **tRNA** under standard assay conditions (16), adding C75 and A76 with >80% efficiency (data not shown).

To prepare heterodimers in which **tRNA** was cross-linked to the inactive subunit, we cross-linked **tRNA** to inactive subunits before subunit reassortment but in the presence of 4 M urea to generate complex 2 (Fig. 5A). Uncross-linked \( tRNA \) was removed by Amicon YM-30 filtration (Millipore), the cross-linked inactive subunits were reassorted with tagged wild type subunits by stepwise dilution from 8 M urea, and heterodimers were recovered by affinity chromatography on Ni-NTA resin (Fig. 5A). Heterodimers cross-linked to \( tRNA \) were eluted with imidazole and assayed under standard conditions with labeled CTP or ATP, and the products were resolved by 10% SDS-PAGE (Fig. 5B). A single wild type subunit conferred full CCA-adding activity on a heterodimer containing \( tRNA \) cross-linked to either a C74- or A76-defective subunit (Fig. 5B, first lanes of all four panels), whereas a fully defective subunit did not confer activity on the equivalent heterodimer (Fig. 5B, second lanes of all four panels). This experiment argues against the possibility that association with a wild type subunit can allosterically re reactivate the K153A mutant; allosteric reactivation of the Y95V and H93V mutants is also unlikely because these \( S. \ shibatae \) mutations, when mapped onto the crystal structure of the highly homologous \( A. \ fulgidus \) CCA-adding enzyme (18, 19), lie on the surface of the nucleotide binding pocket. Because a single active subunit in a heterodimer can add CCA (Fig. 3), we conclude that an active subunit can add **tRNA** to \( tRNA \) that is cross-linked to the inactive subunit within a heterodimer.

**Identifying the Binding Site for the tRNA TyC Stem-Loop Region**—The CCA-adding enzyme recognizes primarily the top half of **tRNA** (acceptor and TyC stem-loop) (16), and the **tDNA** minihelices corresponding to the top half of \( E. \ coli \) **tRNA** are good substrates for the \( E. \ coli \) CCA-adding enzyme as long as the 3′-terminal residue is a ribonucleotide (29). To identify residues within the CCA-adding enzyme that are involved in binding the top half of **tRNA**, we synthesized a **tDNA** minihelix that spans the acceptor stem and the TyC stem, carries a 3′-terminal ribonucleotide corresponding to C74, and is site-specifically labeled with a photoactivatable bromocyt-
dine residue at position 61 near the \( T_{\Psi}C \) loop (Fig. \( 6A \)). UV cross-linking of the 5'-labeled tDNA minihelix to the \( S. \) \( shibatae \) CCA-adding enzyme in the absence of urea generated a single cross-linked complex (Fig. \( 6B \), lane 2); no cross-linked complex was formed when the same tDNA minihelix lacked bromocytidine (Fig. \( 6B \), lane 4). After trypsin digestion, cross-linked peptides were purified by ion exchange over DEAE-Sepharose (Amersham Biosciences) and subjected to aminoterminal microsequencing (Yale Howard Hughes Medical Institute/Reck Biotechnology Resource Laboratory), which identified the tryptic peptide NIGQYYLNIGPQYYSETIDDFIQK. As discussed below, this peptide may be near the \( T_{\Psi}C \) loop when the tDNA is docked to the structure of the highly
homologous *A. fulgidus* apoenzyme (18) and could participate in tRNA binding (Fig. 6C).

The *S. shibatae* CCA-adding enzyme is highly homologous to the *A. fulgidus* CCA-adding enzyme (28), which is known to dimerize through its body and tail domains (18, 19). The dimensions of the resulting cleft in the *A. fulgidus* dimer and the distribution of electrostatic surface potential suggest that both the body and tail domains may be involved in tRNA binding (18). Indeed, when tRNA is docked onto the *A. fulgidus* structure (18) using tRNA protection and interference data as a strong constraint (16), the acceptor and T\(\beta\)C stem of tRNA are located within the cleft, whereas the tail domain of the catalytically active subunit may interact with the T\(\beta\)C loop (Fig. 6C). This docking also places the T\(\beta\)C loop close to a region of the catalytically inactive subunit in the *A. fulgidus* dimer (18, 19).

We mapped the cross-linked *S. shibatae* peptide onto the structure of the *A. fulgidus* apoenzyme docked to tRNA (Fig. 6C; adapted from Ref. 18), because the class I *A. fulgidus* and *S. shibatae* CCA-adding enzymes are highly homologous in sequence (28) and function (17) and, thus, presumably in structure. The cross-linked peptide spans region \(\beta\)14-\(\alpha\)N of the catalytically active subunit and thus may correspond to complex 1 obtained with intact tRNA (Fig. 4). The T\(\beta\)C loop could also potentially interact with helix \(\alpha\)L or region \(\alpha\)H-\(\beta\)8 of the inactive subunit (18, 19), perhaps corresponding to complex 2 ob-
tetramerization of the CCA-adding enzymes are tRNA-inducible tetramers (but see tion. It will be interesting to see whether other class I archaeal (24). Just as importantly, the data demonstrate that tRNA-

subunit adds CTP and a quasi-equivalent subunit adds ATP (24). These data rule out shuttling models in which one B

can carry out all three steps of CCA addition in a heterodimer

steps of CCA addition (Fig. 3); and 2) a single active subunit

subunit in an immobilized heterodimer can carry out all three

adding enzyme can add CCA. 1) A single catalytically active

region of the inactive subunit or both may interact

CCA-adding enzyme does not provide any hints regarding the nature of the dimer/dimer interface in the tRNA-induced tetramer (18).

Implications for the Mechanism of CCA Addition—We have presented the following two kinds of evidence that a single catalytically active subunit within a dimeric S. shibatae CCA-adding enzyme can add CCA. 1) A single catalytically active subunit in an immobilized heterodimer can carry out all three steps of CCA addition in a heterodimer containing tRNA that is cross-linked to the inactive subunit (Fig. 5B). These data rule out shuttling models in which one subunit adds CTP and a quasi-equivalent subunit adds ATP (24). Just as importantly, the data demonstrate that tRNA-induced tetramer formation (24) is not required for CCA addition. It will be interesting to see whether other class I archaeal CCA-adding enzymes are tRNA-inducible tetramers (but see Ref.19), as this may help to explain whether tRNA-inducible tetramerization of the S. shibatae enzyme is fortuitous or essential for some activity other than CCA addition (24).

We have also presented evidence based on UV cross-linking experiments with intact tRNA (Fig. 5) and a site-specifically labeled tDNA minihelix (Fig. 6) that identify one UV light-induced cross-link and suggest the location of a second. The cross-link between tRNA and the catalytically inactive subunit (Fig. 5) is likely to be between the T6C loop and helix αL or region αH–β8 (18, 19). The cross-link between 5-bromocytidine at position 61 of the tDNA minihelix and the enzyme involves region β14–αN of the active subunit (18, 19). Although the 5-bromocytidine is remote from β14-αN when mapped onto intact tRNA (Fig. 6C), it could approach β14-αN more closely if the end of the tDNA minihelix T-stem were to fray or if the tDNA minihelix itself were to slide along the binding cleft, enabling the minihelix to function as a substrate for a CCA addition in one extreme register (29) and as a substrate for UV cross-linking in the other.

The UV cross-links support a role for the tail domain in tRNA binding, consistent with the observation that deletion of the tail of the homologous A. fulgidus CCA-adding enzyme generates a monomer with lower affinity for tRNA substrate (19). Indeed, effective tRNA binding may require not only the presence of the tail domain but stabilization of this domain by interactions across the dimer interface between the juxtaposed body and tail domains (18, 19). It is surprising that the tDNA minihelix can serve as an efficient substrate for CCA addition (29) and also cross-link to the β14-αN region, because a 13-base pair stem is not long enough to reach between the active site and the cross-linking site. The simplest interpretation is that the tDNA minihelix can slide along the tRNA binding cleft, implying that the tail domain of catalytically active subunit or the helix αL region of the inactive subunit or both may interact with the T6C loop so as to block tRNA translocation after each nucleotide addition (Fig. 6C). This possibility could potentially explain why class I archaeal CCA-adding enzymes are dimers but class I eukaryotic poly(A) polymerases function as monomers (12, 33).

Acknowledgments—We thank Arnold Bailey for helpful discussions and especially for suggesting the use of HisSorb plates to prevent tRNA-induced tetramerization. We are also grateful to Yong Xiong for creating Fig. 6C.

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*J. Biol. Chem.* 2004, 279:40130-40136.
doi: 10.1074/jbc.M405518200 originally published online July 19, 2004

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