A comparative analysis of two conserved motifs in bacterial poly(A) polymerase and CCA-adding enzyme

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ABSTRACT

Showing a high sequence similarity, the evolutionary closely related bacterial poly(A) polymerases (PAP) and CCA-adding enzymes catalyze quite different reactions—PAP adds poly(A) tails to RNA 3'-ends, while CCA-adding enzymes synthesize the sequence CCA at the 3'-terminus of tRNAs. Here, two highly conserved structural elements of the corresponding Escherichia coli enzymes were characterized. The first element is a set of amino acids that was identified in CCA-adding enzymes as a template region determining the enzymes' specificity for CTP and ATP. The same element is also present in PAP, where it confers ATP specificity. The second investigated region corresponds to a flexible loop in CCA-adding enzymes and is involved in the incorporation of the terminal A-residue. Although, PAP seems to carry a similar flexible region, the functional relevance of this element in PAP is not known. The presented results show that the template region has an essential function in both enzymes, while the second element is surprisingly dispensable in PAP. The data support the idea that the bacterial PAP descends from CCA-adding enzymes and is now fixed in a conformation specific for A-addition.

INTRODUCTION

According to their active site signature, CCA-adding enzymes and poly(A) polymerases (PAP) are members of the nucleotidyltransferase superfamily where they are grouped in two different classes, according to their structural properties (1–4). As members of class II, bacterial CCA-adding enzymes and PAP share a high similarity in their amino acid sequence and show a conserved set of individual motifs involved in catalysis [Figure 1A; (3,5,6)]. Yet, both enzymes have different activities and functions within the cell. While the PAP adds homopolymeric stretches of A-residues to RNAs and tags these transcripts for degradation, the CCA-adding enzyme synthesizes and maintains the invariant sequence C-C-A at the 3'-end of tRNAs, which is an essential prerequisite for aminoacylation (7–11). Interestingly, the CCA-adding enzyme polymerizes the CCA-terminus without employing a nucleic acid as a template. Instead, a set of amino acids (EDxxR, located in motif D) is found in the single nucleotide-binding pocket of the enzyme that forms Watson–Crick-like hydrogen bonds with the base moieties of the bound nucleotides and thereby specifies the nature of the incorporated nucleotides (12).

Another important functional element in the CCA-adding enzyme is a region of 10–12 amino acids located between motifs A and B in the N-terminal part of the enzyme (Figure 1A and B). Being not resolved in crystal structures, this protein region seems to be highly flexible and not involved in structurally defined domains (12,13). Although this element is not conserved at the sequence level and varies dramatically between individual CCA-adding enzymes, it is required for the addition of the terminal A-residue of the CCA triplet, since a corresponding deletion leads to enzymes with a restricted (CC-adding) activity. Accordingly, the absence of this region is a common feature of naturally occurring CC-adding enzymes found in some bacteria (14).

In contrast to CCA-adding enzymes (12,13,15) and class I PAP (16,17), crystal structures of class II PAP are not yet available. Consequently, little is known about the polymerization and nucleotide-recognition mechanism of bacterial PAP. Interestingly, these enzymes carry both the amino acid template and the flexible region identified in class II CCA-adding enzymes, representing a further indication of the close evolutionary connection (4,18,19). Nevertheless, it is surprising that an enzyme that is specific for the incorporation of A-residues has

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a nucleotide-binding site that can in principle also recognize CTP. The existence of the second investigated element in the PAP, the flexible region, was inferred from sequence alignments with a CCA-adding enzyme of known structure as well as structure prediction programs. Being an essential element required for A-addition in CCA-adding enzymes, it is conceivable that the predicted flexible region in the PAP also participates in the polyadenylation reaction.

Here, these two elements of the *Escherichia coli* PAP were analyzed by individual amino acid replacements, deletions as well as reciprocal exchanges and compared with the corresponding mutations in the CCA-adding enzymes (*E. coli*, P06961; *Bst*, *B. stearothermophilus*, Q7SIB1). The indicated secondary structure elements of the *B. stearothermophilus* enzyme are taken from the corresponding crystal structure analysis (12) and allow a precise alignment of the regions carrying the putative flexible loop. Highly conserved positions are indicated in red. The flexible element is shown in green (CCA-adding enzymes) or blue (PAP). While motifs A to E show considerable sequence conservation, the flexible loops differ dramatically. Obviously, their main function is to introduce a defined flexibility into the proteins.

**Figure 1.** Conserved elements in CCA-adding enzyme and PAP. (A) Elements under investigation. A short flexible loop region is a newly identified element located between motifs A and B in the N-terminal part of CCA-adding enzymes, where it is an essential element involved in A incorporation. A second region in motif D represents the amino acid template identified in CCA-adding enzymes. Flexible loop as well as amino acid template are also present in the bacterial PAP. (B) Sequence alignment of *E. coli* PAP (Ec, *E. coli*, SwissProt accession number P0ABF1) and CCA-adding enzymes (Ec, *E. coli*, P06961; Bst, *B. stearothermophilus*, Q7SIB1). The indicated secondary structure elements of the *B. stearothermophilus* enzyme are taken from the corresponding crystal structure analysis (12) and allow a precise alignment of the regions carrying the putative flexible loop. Highly conserved positions are indicated in red. The flexible element is shown in green (CCA-adding enzymes) or blue (PAP). While motifs A to E show considerable sequence conservation, the flexible loops differ dramatically. Obviously, their main function is to introduce a defined flexibility into the proteins.

**MATERIALS AND METHODS**

**Construction of recombinant clones**

The genes of CCA-adding enzyme and PAP from *E. coli* were cloned as described (18). Plasmids carrying the corresponding wt cDNA sequences were used to construct protein variants carrying specific amino acid replacements or deletions according to a modified QuickChange site-directed mutagenesis protocol (Stratagene, Edinburgh, UK). Single amino acid exchanges were introduced in the corresponding plasmids via QuickChange site-directed mutagenesis PCR (Stratagene). Loop replacements were done using PCR products containing the information for the flexible loop regions [amplified with appropriate primers in a standard PCR reaction using *Pfu* DNA polymerase (Roche, Mannheim, Germany)]. The PCR products were used as megaprimers in a second site-directed mutagenesis-PCR. The same method was

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applied to exchange the flexible loop by a spacer of 10 glycine residues.

### Protein expression and purification

All PAP variants were expressed in *E. coli* BL21(DE3) or *E. coli* BL21(DE3)pLys (Novagen, Darmstadt, Germany). Freshly transformed cells were grown at 30–37°C in 500 ml LB medium containing 30 μg/ml kanamycin and [for *E. coli* BL21(DE3)pLys] 33 μg/ml chloramphenicol. Expression was induced at an OD$_{600}$ of 1.5–1.7 by addition of IPTG to a final concentration of 200 μM–1 mM. After 1–1.5 h of incubation at 30–37°C, cells were harvested by centrifugation. The variant PAP Δ136–145 was induced at an OD$_{600}$ of 0.7 (IPTG at 133 μM) and further growth for 20 min at 30°C. Cells were harvested by centrifugation, lysed by lysozyme treatment and sonication in ice-cold buffer A (20 mM Tris/HCl, pH 7.6, 0.5 M NaCl, 10 mM MgCl$_2$, 5 mM imidazole, 1 mM DTT and 100 μg/ml lysozyme). After centrifugation for 30 min at 24000g at 4°C, the protein in the supernatant was purified by FPLC on a 1 ml Hitrap Chelating Sepharose column (GE Healthcare, Munich, Germany) and eluted with 500 mM imidazole. Fractions containing the enzymes were identified by SDS–PAGE, pooled and dialyzed against buffer B (20 mM Tris/HCl, pH 7.6, 0.5 M NaCl, 5 mM MgCl$_2$, 1 mM DTT and 10% glycerol). Wild-type (wt) as well as variants of the *E. coli* CCA-adding enzyme were purified as previously described (18). All proteins were stored in the presence of 40% (v/v) glycerol at −20°C.

### Preparation of RNA substrates

tRNA substrates (human mt tRNA$^\text{Tyr}$ and tRNA$^\text{Tyr} + \text{CC}$) were prepared as previously described (20). The rpsO mRNA used as a PAP substrate was prepared as a T7 RNA polymerase run off transcript in the presence of α$^32$P-UTP according to the supplier’s instructions (New England Biolabs, Ipswich, UK). Radiolabeled RNA molecules were purified by denaturing PAAG gel electrophoresis. Bands were cut out with a sterile blade, and the RNA was eluted by incubation in water at 4°C overnight. Transcripts were ethanol-precipitated in the presence of glycogen.

### Enzyme activity assays

A total of 4 pmol $^{32}$P-labeled tRNA substrate (human tRNA$^\text{Tyr}$ and tRNA$^\text{Tyr} + \text{CC}$) were incubated with 50–200 ng of recombinant enzyme in the presence of all four NTPs (10 mM each) or individual nucleotides (10 mM) in polyadenylation buffer [10 mM Tris/HCl, pH 7.6, 4 mM MgCl$_2$, 100 mM NaCl, 0.2 mM DTT, 0.2 mM EDTA, 1% glycerol, 0.1% Triton X-100 (21)] or CCA-addition buffer (30 mM HEPES/KOH, pH 7.6, 6 mM MgCl$_2$, 30 mM KCl, 2 mM DTT) in a total volume of 20 μl for 30 min to 2 h at 37°C. To increase the sensitivity of the analysis of PAP E211A and PAP D212A, 3 μCi $^{32}$P-ATP or $^{32}$P-CTP (3000 Ci/mmol) were added to the reaction. After ethanol precipitation, products were separated by electrophoresis on a 10% polyacrylamide gel containing 8 M urea and visualized by autoradiography.

### Kinetic analysis

For steady-state kinetic assays with eight data points, enzyme variants were tested at 30°C in a 10 μl reaction volume with CCA-addition buffer, 3 μM yeast tRNA$^\text{Phe}$ and 0.1 μCi α$^{32}$P-ATP (3000 Ci/mmol) included as a label. ATP was titrated between 0.005 and 0.6 mM. The reactions were stopped after 10 min by adding 20 μl EDTA (50 mM) and spotted to DE81 filter papers (Whatman, Maidstone, UK). Filters were washed with 35 ml buffer containing 0.3 M NH$_4$-formate and 10 mM pyrophosphate on a 10-place filter manifold (Amersham, Little Chalfont, Buckinghamshire, UK) and measured in a scintillation counter. Kinetic parameters of three independent experiments were analyzed using GraphPadPrism (curve fitting by nonlinear regression). The obtained $k_{cat}$ values are apparent values because the tRNA was not used at saturating amounts when NTPs were titrated. However, $K_M$ values are unaffected since the enzymes display a random sequential mechanism (22).

### Sequence analysis of reaction products

RNA 3′-ends were ligated to a DNA oligonucleotide carrying one single RNA nucleotide (UMP) at the phosphorylated 5′-end (5′-U-TACCTCATGGTCAAGCT GTT-3′). Ligation was performed in the presence of 50 mM Tris/HCl, pH 8.0, 10 mM MgCl$_2$, 1 mM hexamine cobalt chloride, 12.5% PEG 6000, 0.2 mg/ml BSA and 10 U T4 RNA ligase (New England Biolabs) over night at 16°C. Reverse transcription was carried out using MMLV-Reverse Transcriptase (New England Biolabs) with a primer complementary to the ligated oligonucleotide (5′-AACAGCTATGACCCTGAG-3′) according to the supplier’s protocol. Amplification of the cDNA was performed in a standard PCR by using the RT primer and a primer representing the 5′-part of tRNA$^\text{Tyr}$ (5′-GGTAAAATGGCTGAGTGAAG-3′). PCR products were cloned into pCR2.1-Topo vector according to the manufacturer’s instructions (Invitrogen, Karlsruhe, Germany) and sequenced.

### Computational analysis

Protein alignments were performed by ClustalW (23), protein secondary structure analysis was analysed using Psipred and PredictProtein (24–26). Sequences of *E. coli* CCA-adding enzyme and PAP were further adjusted by hand due to the available crystal structures of human and *Bacillus stearothermophilus* CCA-adding enzyme (12,13).

### RESULTS

Amino acids with templating function: PAP and CCA-adding enzymes use the same motif (EDxxR) for nucleotide selection

Crystal structure analysis as well as biochemical studies revealed a nucleotide selection mechanism mediated by a highly conserved base-pairing amino acid template (EDxxR) in the single nucleotide-binding pocket of class II CCA-adding enzymes (12,19). Furthermore, these enzymes carry a backup system that allows correct CCA synthesis...
even if the nucleotide-interacting arginine side chain of this amino acid template is replaced by mutations (27).

Interestingly, sequence alignments led to the identification of an identical EDxxR motif at the corresponding position in class II PAP [Figure 1B; (18)]. A similar involvement of this motif in nucleotide selection was demonstrated by introducing amino acid replacements that changed the nucleotide specificity of the E. coli PAP to GTP (19). However, it was unclear whether PAP carry a similar backup mechanism that allows correct nucleotide incorporation as it was recently described for CCA-adding enzymes (27). To address this question, the individual positions of the EDxXR motif were replaced by alanine in the E. coli PAP (Figure 2). The resulting enzyme variants were recombinantly expressed, purified by affinity chromatography and tested for activity with a tRNA substrate in the presence of all four nucleotides [tRNA is a readily accepted substrate for polyadenylation by the E. coli PAP; (18)]. To identify the nature of the incorporated nucleotides, the 3'-ends of the reaction products were ligated to an RNA/DNA oligonucleotide, amplified by RT/PCR and cloned. For variants PAP E211A and PAP D212A, 21 individual clones were analyzed by sequencing. Both enzyme forms specifically incorporated A-residues with a fidelity comparable to that of the wt enzyme (for the wt enzyme form, 11 clones of reaction products were analyzed). AMP was incorporated in >90%, followed by CMP (4.5–7.9%), while GTP was not accepted at all. Furthermore, PAP D212A showed a very rare incorporation of UMP (1.4%) (Figure 2).

PAP R215A, however, led to a dramatic loss in nucleotide specificity and the formation of poly(N) tails. From 56 sequences analyzed, all nucleotides except GMP were incorporated randomly into the tRNA substrate (CMP, 39.9%; UMP, 33.7%, AMP, 24.4% and GMP, 2%) (Figure 2). Similar results were obtained using rpsO mRNA as a substrate (data not shown). Hence, an artificial bias in nucleotide selection due to the substrate form (tRNA or mRNA) can be ruled out. These results demonstrate that this conserved set of amino acids is important for nucleotide selection not only in the CCA-adding enzyme but also in PAP. However, the PAP does not exhibit a backup mechanism that allows proper nucleotide selection in the R215A variant, as it was found in the CCA-adding enzymes.

**Analysis of a flexible loop region in PAP and CCA-adding enzyme**

Although crystal structures are not available for class II PAP, sequence alignments indicate an amazingly high similarity to class II CCA-adding enzymes, making it difficult to distinguish these proteins at the sequence level (3,5,6,18). In their catalytic core, both types of enzymes carry the same set of five short sequence motifs, including the above mentioned amino acid template. In the tRNA nucleotideyltransferases, an additional region important for nucleotide transfer was identified as a small domain of 10–12 amino acids that seems to represent a highly flexible loop as it is not resolved in the available crystal structures of the corresponding enzymes of *Homo sapiens*.

**Figure 2. Mutagenic analysis of the amino acid template in poly(A) polymerase.** While the wt enzyme shows *in vitro* only a low misincorporation of CTP, the R215A variant has a greatly reduced nucleotide specificity and incorporates randomly ATP, CTP and UTP. GTP is incorporated at a very low level. The other amino acid replacements (E211A and D212A) show almost no increase in misincorporation compared to the wt enzyme. These values indicate that the arginine residue in the amino acid template of the poly(A) polymerase determines the specificity of the nucleotide-binding pocket, while the other highly conserved residues play only a minor role in nucleotide selection.

The flexible loop: essential for CCA-addition, dispensable for polyadenylation

According to the alignment in Figure 1B, location as well as size of the predicted flexible loop in PAP are similar to that of the corresponding element found in the crystal structure of the CCA-adding enzyme of *B. stearothermophilus*. The same holds true for the putative loop region R65 to F74 in the CCA-adding enzyme of *E. coli*.

Initially, point mutations were introduced at positions 140 and 141 of the PAP, individually replacing the original amino acids D and R by alanine (Figure 3A). Correspondingly, amino acid replacements G70A and [position D99 to E110; (13)], *B. stearothermophilus* [positions G87 to E96; (12)] and the A-adding enzyme of *Aquifex aeolicus* [positions Y83 to P90; (28)]. Interestingly, this element does not seem to be conserved at the amino acid level, since loop regions from different CCA-adding enzymes show a high sequence variation. Yet, substitutions and deletions within this flexible loop interfere with A incorporation, indicating its functional importance (14,28).

Since the PAP catalyzes a very similar reaction (polyadenylation, corresponding to multiple rounds of A-addition), it is conceivable that this type of nucleotidytransferase might also carry such a flexible element required for A incorporation. As no crystal structures of bacterial PAP are available, structure prediction programs Psipred and PredictProtein (24,25) as well as a sequence alignment based on the structure of the *Bacillus* CCA-adding enzyme (12) were applied, leading to the identification of a similar putatively unstructured region at the corresponding position (G136 to Q145) in the *E. coli* enzyme (Figure 1B).

To investigate the functional importance of this flexible loop in the PAP, a detailed mutational analysis was performed on the corresponding *E. coli* enzyme and the resulting enzymatic activities were compared to those of analogous mutations in the CCA-adding enzyme of the same organism.
Y71A were introduced in the CCA-adding enzyme (Figure 3B). The mutated proteins were recombinantly expressed and tested for activity using a radioactively labeled tRNA as a substrate. Surprisingly, none of the point mutations interfered with the polyadenylation reaction of the enzyme, as both protein variants showed a poly(A) synthesis comparable to the wt enzyme, leading to polyadenylated RNA molecules with reduced electrophoretic mobility depending on the number of incorporated A-residues (Figure 3A, left panel). In contrast to these PAP variants, the loop mutants of the CCA-adding enzyme showed a reaction that differs dramatically from the wt CCA-addition. The wt enzyme incorporated a complete CCA triplet at the tRNA 3'-end, shifting the migration position of the reaction product compared to the mock incubation (Figure 3B, upper panel). Furthermore, an additional side reaction product of this enzyme, carrying a CCCA terminus, was also observed. The underlying in vitro activity of the E. coli CCA-adding enzyme for this reaction has already been described, where it was shown that this enzyme can incorporate three instead of two C-residues into certain tRNA substrates (18,29). The point mutations G70A and Y71A, however, incorporated one nucleotide less than the wt enzyme, leading to a different mobility of the corresponding tRNA band in the gel. Furthermore, when a tRNA substrate ending with CC was offered, the mutant CCA-adding enzymes were severely impaired in adding the terminal A-residue (Figure 3B, lower panel). Hence, these variants were fully active in CTP addition and catalyzed also the side reaction (CCC-addition), while the incorporation of A was severely affected. These results were corroborated in a kinetic analysis, where the mutants showed a 10-fold reduction in $k_{cat}$, whereas $K_M$ was only slightly affected (data not shown).

In a second approach, massive mutations were introduced into both enzymes. As the amino acid glycine is known to contribute to the flexibility of protein structures (30), positions G136 to Q145 in the PAP and R65 to F74 in the CCA-adding enzyme were replaced by a poly-glycine stretch (Gly10; Figure 3). In addition, the loop elements were completely removed, leading to deletion forms of PAP (Δ136–145) and CCA-adding enzyme (Δ65–74). The resulting variants were tested using the same substrates as for the point mutations. Consistent with the results described above, the PAP mutant forms readily incorporated stretches of A-residues into the tRNA substrate (Figure 3A, right panel), while the CCA-adding enzyme variants were again severely affected in A-addition and incorporated only two C-residues, an activity comparable to that of the point mutant forms G70A and Y71A (Figure 3B, right panel). In the Gly10 variant, $k_{cat}$ was 400-fold reduced compared to the wt CCA-adding enzyme, while the deletion variant Δ65–74 showed a 1200-fold...
reduction in $k_{\text{cat}}$ (data not shown), in agreement to recent findings (14). However, none of these mutations affected the apparent $K_M$, indicating that the flexible loop is not involved in ATP binding and has therefore, no impact on nucleotide affinity of the CCA-adding enzyme. For PAP, on the other hand, the experiments show that even dramatic mutations in the flexible loop do not affect the polyadenylation reaction, indicating that this element is not required for efficient and multiple A-additions.

As both enzymes share a considerable sequence homology (18), it was investigated whether these loop regions are interchangeable between CCA-adding enzyme and PAP and represent functional elements in the context of a different but closely related protein.

**Reciprocal exchanges of the flexible loop elements between PAP and the CCA-adding enzyme**

To test the functionality of the predicted flexible loop regions in the context of a closely related nucleotidyltransferase that is highly similar but catalyzes a different reaction, the region G136 to Q145 of the PAP was replaced by the sequence R65 to F74 of the CCA-adding enzyme, resulting in loop chimera 1 (PCP; chimeric PAP; Figure 4, left). Additionally, the reciprocal loop exchange was performed, leading to a chimeric CCA-adding enzyme carrying the flexible loop of the PAP (loop chimera 2; CPC; Figure 4, right). Again, both enzyme chimeras were recombinantly expressed and tested for activity under the described *in vitro* conditions in the presence of all four nucleotides. The loop chimera 2 (CPC) showed an activity identical to that of all other previously tested CCA-adding enzyme loop variants. The protein catalyzed the incorporation of two C-residues into a tRNA molecule, but was dramatically impaired in adding the terminal A of the CCA sequence (Figure 4, right panel). The kinetic analysis confirmed these results. While $K_M$ was only moderately affected (4-fold), the $k_{\text{cat}}$ value was 230-fold reduced compared to the wt enzyme (not shown). In the reciprocal loop chimera 1 (PCP), however, the loop replacement did not lead to a visible effect on polyadenylation. In agreement with the activities of the other PAP variants tested, the enzyme readily synthesized long stretches of poly(A) on the RNA substrate, indistinguishable from that of the wt protein. The composition of the poly(A) tails was determined by sequence analysis of 11 individual clones of the reaction products, showing A incorporation in 92.1%, while other NTPs were incorporated at very low rates (CMP: 6.3%, GMP: 0.8%, UMP: 0.8%), comparable to the reaction catalyzed by the wt enzyme.

Taken together, the loop manipulations led to consistent and uniform effects in the PAP as well as in the CCA-adding enzyme. Whereas, all tested PAP variants readily catalyzed a polyadenylation reaction indistinguishable from the wt enzyme, the loop variants of the CCA-adding enzyme were severely impaired in the addition of the terminal A-residue, while CC-addition was not affected. Although both proteins are evolutionary closely related, the incorporation of A-residues obviously depends on different structural requirements in these nucleotidyltransferases.

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**Figure 4.** Reciprocal loop exchange between CCA-adding enzyme and PAP. In chimera 1, the flexible loop of the CCA-adding enzyme was inserted into the PAP. The resulting enzyme (PCP) readily synthesizes long stretches of poly(A), indistinguishable from the wt enzyme reaction product. This activity indicates that the loop element of the CCA-adding enzyme is tolerated in the PAP context. In chimera 2, however, the flexible loop of PAP in the CCA-adding enzyme (CPC) reduces the enzymatic activity to the addition of two C-residues, which is indicated by a higher electrophoretic mobility compared to tRNAs with complete CCA ends (synthesized by the wt CCA-adding enzyme).
DISCUSSION

Representing members of class II nucleotidyltransferases, bacterial PAP and CCA-adding enzymes share a high homology in sequence and, consequently, are evolutionary closely related (3,4,18,31). While crystal structures are available for the CCA-adding enzymes, the structural organization of the corresponding bacterial PAP is not solved yet. Hence, these types of enzymes can currently only be compared by sequence alignments and comparative biochemical studies (18).

The PAP has no backup mechanism for polyadenylation

In the single nucleotide-binding pocket of class II nucleotidyltransferase enzymes, a set of highly conserved amino acids (EDxxR) forms a protein-based template region and recognizes the correct nucleotide to be incorporated into the RNA primer. In the CCA-adding enzyme, this motif recognizes either ATP or CTP, depending on the relative orientation of the amino acid side chains (12). Although having a strong influence in nucleotide selectivity, the conserved arginine in the binding pocket of CCA-adding enzymes is not the only determinant for correct CCA synthesis. Enzymes where this amino acid was replaced by alanine still show a high efficiency and accuracy in CCA incorporation, indicating that other, additional parameters determine the nucleotide specificity as well (27). Obviously, the interaction of tRNA primer and enzyme dictates the sequence specificity even in the absence of a functional templating region, leading to a backup mechanism for CCA-addition (27,32,33).

It was a surprising finding that bacterial PAP also carry an identical amino acid template at the corresponding position (18). While mutational analysis of the E. coli PAP enzyme revealed that the arginine residue of this motif also contributes dramatically to the selectivity of the binding pocket (19), the presented data indicate that PAP does not have a backup system like CCA-adding enzymes, where the RNA primer contributes to nucleotide selectivity. Hence, the R215A replacement completely abolishes polyadenylation, but allows random incorporation of A-, C- and U-residues [for G-addition, the individual positions of the EDxxR motif have to be replaced by amino acids forming hydrogen bonds with GTP (19)].

Obviously, the CCA-adding enzyme is optimized for proper CCA synthesis (even in the absence of a correct amino acid template), as any mistake would inevitably lead to a nonfunctional tRNA molecule (10). The existence of a backup mechanism emphasizes the vital importance of this activity. The PAP, on the other hand, is rather nonspecific. Even the wt form carrying an intact amino acid template does not discriminate efficiently against UTP or CTP (19,34,35). The resulting poly(A)-tail carrying these misincorporations is apparently still accepted by the cell as being functional. Hence, a backup mechanism for poly(A) synthesis is not required. Accordingly, the polyadenylation reaction seems to be exclusively protein-based and does not rely on the collaboration of RNA primer and protein, as it was demonstrated for CCA-adding enzymes (27,33). However, a chimeric enzyme carrying the N-terminal catalytic core of PAP and a C-terminal part of the CCA-adding enzyme has a bona-fide CCA-adding activity (18). This indicates that the catalytic core of PAP has an intrinsic ability to communicate with the tRNA primer during CCA synthesis, representing a further evidence for the close evolutionary relation between these types of enzymes (4,36). As PAP might be descendants of CCA-adding enzymes (3,18), this hidden collaboration between RNA and protein could be a relict of the CCA-adding activity of the ancestral protein.

The flexible loop is required for CCA-addition, but not for polyadenylation

Besides the amino acid template and the conserved motifs in the catalytic core (4,12,31), a second element in CCA-adding enzymes was recently identified to be essential for complete CCA synthesis. Crystal structures of CCA-adding enzymes show a short region between motifs A and B that is highly disordered and therefore not resolved in the structural analysis (12,13,28). This flexible element consisting of 10–12 amino acids seems to be involved in the incorporation of the terminal A-residue, since point mutations within this region interfere with A incorporation, while addition of C-residues is not affected (14,28,37). Interestingly, this loop region plays an important role in the catalytic activity of two subtypes of tRNA nucleotidyltransferases with restricted enzymatic activity. In some bacteria, CCA-addition is accomplished by the collaboration of enzymes with partial activities (14,38–40). A CC-adding enzyme incorporates the first two C-residues, while an A-adding enzyme adds the terminal AMP and completes thereby the CCA terminus. Whereas the A-adding enzyme carries a corresponding flexible loop required for A incorporation, the CC-adding enzymes lack this element due to a deletion. When a similar loop from a CCA-adding enzyme was transplanted into a CC-adding enzyme, the resulting chimera exhibited a complete CCA-adding activity and catalyzed also the incorporation of the terminal A-residue (14). These results support the observation that the flexible loop is an essential element for A-addition, while it is dispensable for addition of C-residues.

Therefore, one could expect that such an element is not only essential for A-addition mediated by CCA-adding enzymes, but is also involved in the similar polyadenylation reaction (consisting of multiple rounds of A incorporation) catalyzed by the closely related bacterial PAP. This idea was supported by several structure prediction algorithms that indicate a corresponding unstructured region in the E. coli PAP. However, none of the performed manipulations (point mutations, deletions and glycine replacements) within this region of the PAP enzyme affected the polymerization reaction. This demonstrates that, in contrast to the CCA-adding enzyme, the flexible loop in PAP is obviously not a functional element for A-addition. The reciprocal replacements of the loop containing the region between CCA-adding enzyme and PAP corroborate these conclusions. The flexible element of the CCA-adding enzyme does not interfere with the function and is therefore tolerated in the PAP context,
while the insertion of the corresponding PAP region into the CCA-adding enzyme abolishes A incorporation activity.

A possible function of this loop in the CCA-adding enzyme as an ATP-binding element has been discussed previously (37), based on its similarity to nucleotide-binding P loop structures (41,42). However, such an ATP-binding function is highly unlikely, as the mutant enzyme variants show only a slight increase in $K_M$, while $k_{cat}$ is dramatically reduced. This is supported by the analysis of a G70D loop mutation in the E. coli enzyme, where $K_M$ also remained unaffected (43). If the loop region would be required for ATP binding, the $K_M$ values should be strongly increased. Additionally, loop sequences of individual CCA-adding enzymes show a high sequence variation that makes a conserved ATP-binding function very unlikely. However, it is discussed that the flexibility of this loop is required for the structural rearrangement of the enzyme during CCA-addition (14). Tomita and co-workers could show that a crystal consisting of the A-adding enzyme from A. aeolicus bound to a tRNA primer ending with CC readily dissolved upon soaking in an ATP containing solution (28). Obviously, the binding and incorporation of ATP induces a conformational change of the protein, leading to the break-up of the crystal. This enzyme movement seems to reorganize the growing tRNA 3'-end and forms a ‘stacking arc’ including several amino acids of the enzyme and the last C-residue of the tRNA in order to allow proper ATP recognition in the binding pocket and to adjust the 3'OH of the tRNA-end initiating the next nucleotide transfer reaction (32,33,44). However, our data show that the loop element seems to be more than an unspecific mediator of protein flexibility. Since the replacement by a highly flexible glycine spacer in the CCA-adding enzyme does not allow A incorporation, flexibility alone is not sufficient for this reaction. Obviously, the spatial organization of this element has certain constrictions, allowing a defined and ordered mobility in the dynamic ribonucleoprotein complex required for A-addition.

**Evolutionary and mechanistic aspects**

In the light of the close evolutionary relation between CCA-adding enzyme and PAP, it is surprising that the enzymatic activity of the PAP is independent of the presence and/or structural organization of the flexible loop. It seems that polyadenylation does not require a defined conformational change of individual protein domains as it apparently happens during CCA-addition. The nucleotide-binding pocket of the PAP seems to be preformed for ATP binding and does not require any structural reorganization for the multiple incorporation of A-residues. Hence, the PAP might be considered as an enzyme locked in a conformation required for ATP interaction. Therefore, it is surprising that structure prediction programs indicate the existence of a flexible loop in this enzyme. However, being closely related to class II CCA-adding enzymes, it seems that these proteins might have interconverted into each other during evolution (4). The functional data presented here support such an interpretation based on sequence similarity. The PAP carries two essential elements required for CCA-addition. The amino acid residues in the template region can principally recognize both ATP and CTP, but the binding pocket is preformed for ATP interaction. The second element, the flexible loop region, is required for switching from CTP to ATP incorporation during CCA-addition, but has no function in polyadenylation. Thus, it seems that the bacterial PAP evolved out of a class II CCA-adding enzyme and represents now a former CCA-adding enzyme specialized in polyadenylation. This is further corroborated by the fact that the N-terminal catalytic core of PAP is able to synthesize a CCA-terminus if fused to the C-terminal region of a CCA-adding enzyme, demonstrating that it still has an intrinsic CCA-adding activity (18).

Taken together, the evolutionary scenario where a CCA-adding enzyme evolved into a PAP is strongly supported by the fact that structural elements important for proper CCA-addition are still present in bacterial PAP. It seems that these enzymes are now fixed in a conformation that interferes with C-addition and restricts the activity to the (multiple) addition of A-residues, leading to poly(A) tails. Replacing the C-terminal part of the PAP by the corresponding region of the CCA-adding enzyme obviously releases these conformational constraints and allows the catalytic core of PAP to adopt a conformation that is compatible with CTP incorporation and a subsequent switch to A-addition. The fact that some of the conserved elements of the catalytic core are still present in PAP but have no (flexible loop) or only a limited function (amino acid template) in polyadenylation indicates that the evolutionary separation of class II CCA-adding enzymes and PAP might have been a rather recent event in evolution.

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