Conversion of a DNA ligase into an RNA capping enzyme

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Received May 26, 1999; Revised and Accepted July 6, 1999

ABSTRACT

In eukaryotes, newly synthesised mRNA is ‘capped’ by the addition of GMP to the 5’ end by RNA capping enzymes. Recent structural studies have shown that RNA capping enzymes and DNA ligases have similar protein folds, suggesting a conserved catalytic mechanism. To explore these similarities we have produced a chimeric enzyme comprising the N-terminal domain 1 of a DNA ligase fused to the C-terminal domain 2 of a mRNA capping enzyme. This report shows that this hybrid enzyme retains adenylation activity, characteristic of DNA ligases but, remarkably, the chimera has ATP-dependent mRNA capping activity. This is the first observation of ATP-dependent RNA capping. These results suggest that nucleotidytransferases may have evolved from a common ancestral gene.

INTRODUCTION

The mRNA capping reaction is catalysed by RNA capping enzymes (guanylyltransferases) (1) and proceeds through a covalent GMP-enzyme intermediate in which the GMP is attached to the capping enzyme via a lysine residue (2,3). This lysine residue is part of a conserved motif (4,5), one of six co-linear sequence motifs, also found in DNA ligases, RNA ligases and tRNA ligases, with a similar spacing between them (Fig. 1A) (6). These similarities have led to the suggestion that nucleotidyl transfer by all of these enzymes must share a common mechanism and the enzymes are likely to have a similar structure (4,5). These proposals were supported by biochemical studies of mutant enzymes (6–13) and more recently by the structures of Chlorella RNA capping enzyme (4,5). These proposals were supported by biochemical studies of mutant enzymes (6–13) and more recently by the structures

MATERIALS AND METHODS

Expression and purification of recombinant ACE protein

The ACE chimera gene was amplified from plasmid templates containing the T7 ligase (16) and PBCV-1 Chlorella guanylyltransferase (17) genes using standard recursive PCR methods. The PCR products were cloned into the NcoI and XhoI restriction sites in the T7-based expression plasmid, pET21d (Novagen) and fused to a sequence encoding a C-terminal six histidine tag. The resulting plasmid, pET-ACE, confirmed by dideoxy DNA sequencing, was transformed into Escherichia coli B834 (DE3)pLysS. A 1 l culture of E. coli B834 (DE3)pLysS/pET-ACE was grown at 37°C in Luria–Bertani medium containing 0.1 mg/ml of ampicillin until the A600 reached 0.6. The culture was adjusted to 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG), and incubation was continued at 37°C for 4 h. The bacteria were resuspended in buffer A (50 mM Tris–HCl, pH 7.5) containing 0.15 M NaCl, and sonicated. The ACE protein was insoluble and was refolded as described previously for a proteolytic fragment of T7 ligase (16). Refolded ACE was bound to Nickel–Sepharose resin (Qiagen) pre-equilibrated in buffer A, containing 50 mM imidazole. The ACE protein was

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eluted with buffer A, containing 200 mM imidazole. The protein was dialysed overnight in buffer A to remove the imidazole.

**Enzyme–AMP (EpA) complex formation**

Reaction mixtures containing 50 mM Tris–HCl (pH 7.5), 5 mM dithiothreitol (DTT), 5 mM MgCl₂, 1 μM [α-³²P]ATP and ACE were incubated for 60 min at 37°C, and then the reaction was stopped by the addition of sodium dodecyl sulfate (SDS) (1% final concentration). Samples were electrophoresed on a 12% polyacrylamide gel containing 0.1% SDS. Label transfer to the ACE protein was visualised by autoradiographic exposure.
Gel filtration of the ACE–[α-^32^P]AMP complex

Reaction mixtures (50 μl) containing 50 mM Tris–HCl (pH 7.5), 2 mM DTT, 5 mM MgCl₂, 1 mM [α-^32^P]ATP and either 1 μg of ACE or Chi Capping enzyme were incubated for 30 min at 37°C. Native EpA complex was resolved from free ATP by gel filtration through a 1-ml column of Sephadex G-50 that had been pre-equilibrated with buffer B containing 50 mM NaCl; gel filtration was performed at 4°C. The elution profile was determined by Cerenkov counting of each fraction.

Preparation of RNA substrates

γ-^32^P-labelled triphosphate-terminated poly(A) was synthesised as described previously (2) and then converted to diphosphate-terminated poly(A) by treatment with Vaccinia virus capping enzyme. The RNA triphosphatase reaction mixture containing 50 mM Tris–HCl (pH 8.0), 5 mM DTT, 5 mM MgCl₂, 300 pmol of γ-^32^P-labelled triphosphate terminated poly(A) and 50 pmol of purified recombinant Vaccinia virus capping enzyme, was incubated for 1 h at 37°C. The quantitative release of ^32^Pi from poly(A) was verified by polyethyleneimine-cellulose thin-layer chromatography. The RNA product was then recovered by two rounds of precipitation with 10% trichloroacetic acid. The poly(A) was resuspended in 0.1 M Tris–HCl, pH 8.0, and then extracted with phenol–chloroform, ethanol precipitated and resuspended in 100 ml of 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA. Cap-labeled poly(A) [ApppA(pA)n] was prepared by the transfer of [^32^P]AMP from [α-^32^P]ATP to diphosphate-terminated poly(A) by using purified ACE. Reaction mixtures contained 50 mM Tris–HCl (pH 8.0), 5 mM MgCl₂, 5 mM DTT, 100 pmol of triphosphate-terminated poly(A), 5 mM [α-^32^P]-ATP, and 1 μg of purified recombinant Vaccinia virus capping enzyme. After incubation for 30 min at 37°C, unincorporated ATP was removed by multiple rounds of trichloroacetic acid precipitation. The RNA was recovered with phenol–chloroform and recovered by ethanol precipitation.

RESULTS AND DISCUSSION

The ACE protein, which consisted of residues 1–235 of T7 ligase and 232–330 of Chlorella RNA capping enzyme, was over-expressed in E.coli. A 39 kDa polypeptide corresponding to ACE was detectable by SDS–PAGE in whole-cell extracts of IPTG-induced bacteria. This apparently single ACE protein band was resolved into a doublet when less protein was electrophoresed on SDS–polyacrylamide gels. This protein doublet was also present after histidine tag purification of the chimera, suggesting it was not the result of proteolytic degradation of ACE. It was deduced that the recombinant ACE enzyme was probably forming a covalent protein–NMP complex

second step the NMP is transferred from the active site lysine of the enzyme to the 5′-phosphate end of the DNA (18) or 5′-diphosphate end of RNA (6) (Fig. 2).

To determine which NTP is utilised by ACE in this nucleotidytransferase reaction, purified ACE was incubated with various [α-^32^P]NTPs and a divalent cation (Fig. 3A). The ACE polypeptide was selectively labeled in the presence of [α-^32^P]ATP (Fig. 3B). The amount of EpA complex formed in the presence of [α-^32^P]ATP was proportional to the amount of added ACE protein (data not shown). [α-^32^P]CTP, GTP, UTP and other ribonucleoside triphosphates were inert in nucleotidytransferase reactions (Fig. 3B). Similarly, there was no label transferred to the 39 kDa polypeptide in the presence of γ-^32^P]ATP (data not shown). [ε-^32^P]dATP was, however, an extremely poor donor for enzyme–adenylate formation by ACE compared with [α-^32^P]ATP (data not shown). This indicated that the chimeric enzyme discriminates, in common with T7 DNA ligase (14,16), between ribose and deoxyribose sugars. ATP is 500-fold more effective than dATP in EpA formation by ACE.

The faster-migrating protein species of ACE (E) was converted quantitatively into the slower-migrating protein species ACE–AMP (EpA) by incubation of the enzyme preparation in the presence of ATP and magnesium (Fig. 4), as this drives the reaction equilibrium forward toward EpA intermediate formation (Fig. 2). All of the slower-migrating polypeptide, ACE–AMP, was shifted to the more rapidly migrating apo-protein after the enzyme was incubated with PPI and a divalent metal (Fig. 4). It is well established in other DNA ligase (18) and RNA capping enzyme (6,19,20) systems that incubation of EpN intermediate in the presence of PPI liberates NTP by reversing the nucleotidytransfer reaction (Fig. 2). EpA complex formation by ACE is dependent on a divalent cation co-factor, in common with other ligase and capping enzymes (6,18). This metal requirement was met by either magnesium or manganese (data not shown).
These experiments reveal that the recombinant ACE protein is an active transadenylase. It was reported previously that both intact T7 DNA ligase and the N-terminal domain I of T7 ligase also possess intrinsic transadenylase activity (21). This adenylation activity of recombinant T7 ligase domain I is greatly stimulated by incubation with recombinantly expressed OB domain 2 of T7 ligase (21).

We also reported (21) that recombinant T7 Domain 1 can ligate nicked and cohesive-ended DNA, but with a significantly reduced activity compared to the full-length T7 enzyme. The ACE protein retains the adenylation characteristics of ATP-dependent DNA ligases, but can this enzyme function as a DNA ligase in vitro? To investigate this possibility, purified recombinant ACE was incubated with known substrates of ATP-dependent DNA ligases, as described previously (16,22). No ligation intermediates, such as adenylated DNA or ligated DNA products were detected (data not shown), even when high concentrations of ACE were incubated with the radiolabeled nicked DNA substrates. How could the fusion of the OB domain 2 from an RNA capping enzyme with the N-terminal domain of DNA ligase have eliminated the intrinsic ligase activity of T7 ligase domain I?

The structures of T7 DNA ligase (14) and Chlorella capping enzyme (15) consist of two domains with a distinct groove running between them (Fig. 1B and C). The active site for both classes of enzyme is located at the base of this cleft, in the larger domain 1. Calculations of the charge distribution on the surface of the enzyme revealed that there is a large positive potential around the nucleotide-binding pocket that continues into the base of the cleft (14,15). The NTP-binding pocket is at the base of the cleft (Fig. 1B and C). It is known that NMP is transferred from the active site lysine residue to the bound polynucleotide in the second step of the reaction, therefore NTP and the DNA/RNA binding site must be in close proximity. These observations suggested that the polynucleotide must bind in the cleft between the two domains of these proteins (14,15). DNA ligase has a much larger cleft than the RNA capping enzyme and this may reflect the difference in polynucleotide substrate specificity. ACE is predicted to have a cleft intermediate of a size between that of the ligase and capping enzymes. This narrower cleft would exclude dsDNA from binding and this would explain why the chimeric enzyme does not retain DNA ligase activity.

The ACE fusion protein is clearly not a DNA ligase, but does it possess any other enzyme activities, such as RNA capping? To test if ACE has RNA adenytransferase (capping) activity, two RNA capping assays were employed. The first assay is designed to determine if ACE can transfer AMP (cap) to a standard mRNA substrate, poly(A) containing 5'-diphosphate ends (Fig. 2). Purified ACE was incubated with [α-32P]ATP and magnesium, and the enzyme–[α-32P]AMP complex was isolated by gel filtration. The ACE–[α-32P]AMP complex was then incubated with diphosphate-terminated poly(A) in the presence of magnesium. The RNA samples were digested with nuclease P1 and the products separated by thin-layer chromatography. Nuclease P1 digestion of the ACE reaction products liberated a single radioactive species corresponding to cap dinucleotide, AppPA (Fig. 5). The mobility of this species was similar to that of the cap dinucleotide, GpppA, synthesised by Chlorella virus capping enzyme (positive control reaction) and was distinct from free ATP (Fig. 5). This result demonstrates emphatically that ACE has ATP-dependent RNA capping activity, in contrast to known GTP-dependent RNA capping enzymes. ACE was unable to transfer AMP to triphosphateterminated poly(A) (data not shown), confirming that ACE transfers AMP, specifically, to the 5' end of diphosphate-terminated poly(A).

To confirm that the ACE protein–AMP complex is an intermediate in cap synthesis a second assay system measured whether AMP could be transferred from the RNA cap to the ACE protein via a reversal of the capping reaction (Fig. 2). [α-32P]AMP-labeled capped poly(A) was synthesised with ACE,
[α-32P]ATP and diphosphate-terminated poly(A). Unlabeled ACE protein was incubated with cap-labeled poly(A)-[AppA(pA)n] in the presence of magnesium, and the reaction products were analysed by SDS–PAGE. The cap-labeled poly(A) migrated near the bottom of the gel (Fig. 6). Inclusion of unlabeled ACE protein in the reaction resulted in the reversal of the capping reaction (Fig. 6); i.e. the label (AMP) was transferred to the ACE polypeptide (E-AMP) (Fig. 6). This reverse reaction was dependent on the inclusion of magnesium. This assay shows that the second step of the ACE catalysed ATP-dependent capping reaction is readily reversible and confirms that the ACE enzyme binds to the AMP capped RNA product. ACE is therefore a true mRNA capping enzyme.

The findings that an ATP-dependent DNA ligase can be converted into an ATP-dependent RNA capping enzyme, by fusing domain 1 of a DNA ligase with the OB domain of an RNA capping enzyme, are truly remarkable. This report establishes that nucleotidyltransferases work via a conserved catalytic mechanism. Some clues to the novel catalytic activity and which make specific contacts with the NTP molecule (14,15). Two crystal structures of Chlorella mRNA capping enzyme (15) have also provided direct evidence for a substantial conformational change during guanylation in the capping enzymes; this involves a 13 Å movement of the C-terminal OB domain 2 towards domain 1. By analogy, it was proposed that a similar conformational change might be required for catalysis by DNA ligases and other nucleotidyltransferases (15). During this conformational change by Chlorella capping enzyme, conserved residues in motifs V and VI in the OB domain are positioned in the active site and form specific interactions with the nucleotide (15).

The OB domain of ACE clearly confers a novel binding specificity for the 5' end of mRNA rather than for nicked DNA and promotes the capping of diphosphate-terminated mRNA. The OB fold has recently been found in the structures of many proteins that bind to single-stranded (ss) and double-stranded (ds) DNA and RNA (reviewed in 23). A number of co-crystal structures of these domains bound to DNA and RNA have established that the OB fold mediates polynucleotide recognition (23). All the current data confirms that the OB domains of nucleotidyltransferases play a dual role. First, the OB domain acts as a DNA/RNA recognition module, conferring the specificity of DNA ligases and RNA capping enzymes for their polynucleotide substrates. Second, the domain couples this recognition to nucleotidyl transfer by participating directly in the catalytic reaction (13,15,21). This mechanism is also supported by biochemical studies of domains 1 and 2 of T7 ligase. The larger domain 1 binds to both dsDNA and ssDNA in an

![Figure 5](image1.png) Covalent transfer of AMP to RNA is catalysed by the ACE protein. An autoradiogram of a thin-layer chromatography plate showing RNA capping activity. Aliquots of ACE–[^32P]AMP or Chlorella CE–[^32P]GMP complexes, prepared as described in the Materials and Methods, were incubated for 60 min at 37°C in reaction mixtures containing 50 mM Tris–HCl (pH 8.0), 5 mM MgCl₂, 5 mM DTT and 50 pmol of diphosphate-terminated poly(A). The mixtures were then extracted with phenol and chloroform-isooamyl alcohol (24:1). RNA was recovered from the aqueous phase by ethanol precipitation and resuspended in 10 mM Tris–HCl (pH 8.0). Aliquots were digested in 50 mM sodium acetate (pH 5.2) with 5 µg of nuclease P1 for 60 min at 37°C. The digests were analysed by thin-layer chromatography on polyethyleneimine-cellulose plates.

![Figure 6](image2.png) Reversibility of the ACE capping reaction. An autoradiograph of a 12% SDS–polyacrylamide gel showing the covalent exchange of α-[^32P]AMP from 5' labeled poly(A) to the ACE protein. The positions of the EpA complex and the cap-labeled poly(A) substrate are indicated. The reaction mixtures contained 50 mM Tris–HCl (pH 8.0), 5 mM DTT, 5 mM MgCl₂, 50 pmol of cap-labeled poly(A) [AppA(pA)n] and either 0, 10, 20, 80 pmol of ACE, respectively. After incubation for 60 min at 37°C, the reactions were terminated by adding SDS to a final concentration of 1%, and the samples were analysed by SDS–PAGE.
apparently non-specific fashion, however the OB domain 2, in contrast, binds only dsDNA. The OB domain of T7 ligase also dramatically enhances the adenylation activity of domain 1 (21).

The ACE protein has all the biochemical properties of an mRNA capping enzyme. It hydrolyses NTP, in the presence of magnesium, to form a covalent phosphoramide intermediate (EpN). The enzyme can specifically transfer this NMP moiety to the 5’ end of diphosphate-terminated mRNA and all of these nucleotidyl transfer reactions are readily reversible. However, unlike a conventional RNA capping enzyme, which transfers GMP to mRNA, ACE catalyses AMP-dependent capping. This report strongly suggests that DNA ligases and RNA capping enzymes have evolved from a common ancestral nucleotidyl-transferase, which has been adapted to perform a particular catalytic role in vivo. This evolution may reflect the switch from an RNA world to a more DNA centric one.

ACKNOWLEDGEMENTS

I would like to thank Professor Robin Carrell for his continued support and Drs Louise Serpell and Tim Dafforn for critically reading this manuscript. A.J.D. is a Royal Society University Research Fellow.

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