An end-healing enzyme from *Clostridium thermocellum* with 5' kinase, 2',3' phosphatase, and adenylyltransferase activities

ALEXANDRA MARTINS and STEWART SHUMAN

Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10021, USA

ABSTRACT

We identify and characterize an end-healing enzyme, *CthPnkp*, from *Clostridium thermocellum* that catalyzes the phosphorylation of 5'-OH termini of DNA or RNA polynucleotides and the dephosphorylation of 2',3' cyclic phosphate, 2'-phosphate, and 3'-phosphate ribonucleotides. *CthPnkp* also catalyzes an autoadenylation reaction via a polynucleotide ligase-type mechanism. These characteristics are consistent with a role in end-healing during RNA or DNA repair. *CthPnkp* is a homodimer of an 870-amino-acid polypeptide composed of three catalytic domains: an N-terminal module that resembles the polynucleotide kinase domain of bacteriophage T4 Pnkp, a central metal-dependent phosphoesterase module, and a C-terminal module that resembles the nucleotidyl transferase domain of polynucleotide ligases. The distinctive feature of *CthPnkp* vis-à-vis known RNA repair enzymes is that its 3' end modification component belongs to the calcineurin-type phosphatase superfamily. It contains putative counterparts of the amino acids that form the dinuclear metal-binding site and the phosphate-binding site of bacteriophage λ phosphatase. As with λ phosphatase, the 2',3' cAMP phosphatase activity of *CthPnkp* is specifically dependent on nickel or manganese. We identify homologs of *CthPnkp* in other bacterial proteomes.

Keywords: RNA repair; DNA repair; end-healing; phosphoesterase; polynucleotide kinase

INTRODUCTION

When breakage of a 3'-5' phosphodiester in DNA or RNA results in the formation of 5'-PO₄ and 3'-OH termini at the break site, the ends can be rejoined to each other (or to new partner strands) by the action of DNA-specific or RNA-specific polynucleotide ligases. However, when breakage occurs with the opposite polarity, resulting in 5'-OH and 3'-PO₄ (or 2',3' cyclic PO₄) termini, the broken ends must be "healed" before they can be sealed. Healing entails two steps: (1) hydrolysis of the 3'-PO₄ (or 2',3' cyclic phosphate) to form a 3'-OH, and (2) phosphorylation of the 5'-OH to form a 5'-PO₄ end. The bacteriophage T4 proteins polynucleotide kinase/phosphatase (Pnkp), RNA ligase 1 (Rnl1), and RNA ligase 2 (Rnl2) are the prototypal RNA healing and sealing enzymes (Richardson 1965; Novogrodsky and Hurwitz 1966; Novogrodsky et al. 1966; Cameron and Uhlenbeck, 1977; Silber et al. 1972; Ho and Shuman 2002).

T4 Pnkp and Rnl1 function in vivo to repair a break in the anticodon loop of *Escherichia coli* tRNA^{Lys} triggered by phage-activation of a host-encoded anticodon nuclease PrrC (Amitsur et al. 1987). Depletion of tRNA^{Lys} by PrrC blocks phage protein synthesis and arrests the infection before it can spread. However, Pnkp and Rnl1 repair the broken tRNAs and thereby thwart the host defense mechanism.

Whereas bacteriophage tRNA restriction provided the initial insights to RNA repair biology, little attention has been paid to the issue of whether bacteria themselves (as opposed to phage) might specify enzymes that heal and seal broken RNAs. The recent discovery and characterization of an Rnl2-like RNA ligase encoded by *Deinococcus radiodurans* provided the first clue to the existence of an RNA repair capacity in bacteria (Martins and Shuman 2004a). To date, there has been no description of an end-healing enzyme in bacteria analogous to T4 Pnkp, even though Pnkp-like enzymes have been identified and characterized from a variety of bacteriophages, eukarya, and a eukaryotic virus (Blondal et al. 2005; Martins and Shuman 2004b; Zhu et al. 2004; Bernstein et al. 2005).

Here we identify a polynucleotide end-healing enzyme from the bacterium *Clostridium thermocellum*, an anaerobic thermophilic bacterium that has attracted biotechnology

Reprint requests to: Stewart Shuman, Molecular Biology Program, Sloan-Kettering Institute, New York, NY 10021, USA; e-mail: s-shuman@ski.mskcc.org; fax: (212) 717-3623.

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interest because of its capacity to convert cellulose to ethanol (Demain et al. 2005). The proteome of *C. thermocellum* strain ATCC 27405 (available at NCBI) includes an 870-amino-acid polypeptide containing an N-terminal module that resembles the kinase domain of T4 Pnkp, a central module that resembles a metal-dependent phosphoesterase, and a C-terminal module that resembles the nucleotidyl transferase domain of polynucleotide ligases (Fig. 1). We show here that the purified recombinant *C. thermocellum* protein has an intrinsic 5'-OH RNA/DNA kinase activity and a ribonucleotide 2',3' phosphatase activity. These results are consistent with a role in end-healing during RNA or DNA repair. Thus, we name the enzyme *CthPnkp*. We also show that *CthPnkp* reacts with ATP to form a covalent enzyme-adenylate adduct via a polynucleotide ligase-type mechanism. The presence of homologs of *CthPnkp* in other bacterial proteomes suggests that RNA repair might be relevant to bacterial physiology.

RESULTS

Identification of a *C. thermocellum* polynucleotide kinase with a novel domain structure

The amino acid sequence of the 870-amino-acid *C. thermocellum* polynucleotide kinase is shown in Figure 1, aligned to the sequences of homologous proteins of similar size encoded by *Kineococcus radiotolerans*, *Nostoc sp. PCC 7120*, and *Streptomyces coelicolor*. The N-terminal 150-amino-acid segment of the *C. thermocellum* polypeptide displays primary structure similarity to the polynucleotide kinase domains of coliphage, vibriophage, mycobacteriophage, and baculovirus Pnkp enzymes (Martins and Shuman, 2004b; Zhu et al. 2004). In particular, *CthPnkp* and its bacterial homologs contain the three peptide motifs that comprise the active site of T4 Pnkp (Wang and Shuman, 2001, 2002; Galburt et al. 2002; Wang et al. 2002), which are highlighted in Figure 1. The kinase active site is composed of (1) a classical P-loop motif (GxxGxGKS) that coordinates the β phosphate of the NTP donor; (2) an essential distal Arg side-chain in the RxxxR motif, which also coordinates the NTP β phosphate; (3) an essential Arg side-chain in the DxxR motif, which coordinates the 3' phosphate of the 5'-OH acceptor; plus (4) an essential Asp side-chain in the DxxR motif, which has been suggested to function as a general acid to activate the 5'-OH for direct nucleophilic attack on the NTP γ phosphate.

CthPnkp diverges from the viral Pnkp end-healing enzymes with respect to the domains flanking the kinase module. The phosphatase domains of viral Pnkp enzymes, which are situated downstream of the kinase domain, exemplify the DxDT superfamily of metal-dependent phosphotransferases that act through a covalent aspartyl phosphate intermediate (Collet et al. 1998). *CthPnkp* bears no similarity to the acyl phosphatases; rather, its

central segment contains the signature motifs of a different metal-dependent calineurin-like phosphoesterase superfamily (pfam00149 at NCBI) that includes phosphoprotein phosphatases and diadenosine tetraphosphatases (Goldberg et al. 1995; Voegtli et al. 2000). The phosphoesterase motifs

FIGURE 1. (Legend on next page)

GDIHG, GDLVDR, and GNHG are highlighted in Figure 1. A Blast search revealed extensive conservation of primary structure between *CthPnkp*-(180–400) and the 221-amino-acid phosphoprotein phosphatase of bacteriophage λ . *CthPnkp* contains the full constellation of conserved residues that bind a dinuclear metal cluster and coordinate the phosphate in the active site in the crystal structure of λ phosphatase (Voegtli et al. 2000).

Further sequence gazing revealed similarity of the C-terminal segment of *CthPnkp* to the nucleotidyl transferase domains of DNA ligases, RNA ligases, and mRNA capping enzymes, which comprise a superfamily of proteins that catalyze nucleotidyl transfer to polynucleotide 5' ends via covalent enzyme-(lysyl-N)-NMP intermediates (Shuman and Lima 2004). We identified within the C-terminal segment of *CthPnkp* putative counterparts of nucleotidyl transferase motifs I (KxxGxR), III (VCLDCELM), IV (EGMVV), and V (AVKxR), which are conserved in the three other bacterial proteins aligned in Figure 1. It is noteworthy that the candidate motif I sequence of the bacterial Pnkp proteins (KHMGSR) deviates from the KxDGxR motif I sequence characteristic of most ATP-dependent polynucleotide ligases and GTP-dependent mRNA capping enzymes. The motif I lysine is the nucleophile that attacks the NTP substrate to form the enzyme-NMP intermediate. Also, whereas polynucleotide ligases and capping enzymes contain additional domains flanking the core nucleotidyltransferase module that are essential for ligation/capping activity (typically an OB-fold domain located distal to motif V) (Subramanya et al. 1996; Odell et al. 2000; Shuman and Lima 2004), we detected no such flanking elements in *CthPnkp*.

Polynucleotide kinase activity of recombinant *CthPnkp*

To address whether the *C. thermocellum* protein possesses any of the enzymatic functions suggested by these sequence comparisons, we produced the 870-amino-acid *CthPnkp* protein in *E. coli* as a His₁₀-tagged fusion and purified it from a soluble bacterial extract by adsorption to Ni-agarose and elution with buffer containing imidazole. SDS-PAGE analysis showed that the protein preparation consisted

principally of a ~97-kDa polypeptide corresponding to His₁₀-*CthPnkp* (Fig. 2A). A ladder of minor polypeptides in the 45- to 70-kDa range likely corresponded to proteolytic fragments of *CthPnkp*. The polynucleotide kinase activity of *CthPnkp* was demonstrated by the transfer of ³²P_i from [γ -³²P]ATP to the 5'-OH terminus of a 36-mer oligodeoxyribonucleotide to form a 5' ³²P-labeled oligonucleotide product that was resolved from free ATP by polyacrylamide gel electrophoresis (Fig. 2C, WT).

FIGURE 2. Kinase and adenylyltransferase activities of recombinant *CthPnkp* proteins. (A) Aliquots (5 μ g) of the Ni-agarose preparations of recombinant full-length wild-type (WT) His₁₀-*CthPnkp*; full-length mutant proteins K531A, H532A, and R536A; and truncated proteins *CthPnkp*-(1–425), *CthPnkp*-(433–870), and *CthPnkp*-(462–870) were analyzed by SDS-PAGE. The Coomassie blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (B) Adenylyltransferase activity. Reaction mixtures (10 μ L) containing 50 mM Tris-acetate (pH 7.0), 5 mM MgCl₂, 5 mM DTT, 20 μ M [α -³²P]ATP, and 2 μ g of the indicated protein preparation were incubated for 15 min at 45°C. The products were analyzed by SDS-PAGE and visualized by autoradiography. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (C) Kinase activity. Reaction mixtures (10 μ L) containing 50 mM Tris-acetate (pH 7.0), 5 mM DTT, 10 mM MgCl₂, 100 pmol of a 36-mer 5'-OH oligodeoxyribonucleotide 5'-d(TGTAGTCACTATCG GAATAAGGGCGACACGGATATG), 100 μ M [γ -³²P]ATP, and 50 ng of the indicated protein preparation were incubated for 30 min at 45°C. The products were analyzed by PAGE and visualized by autoradiography.

FIGURE 1. A new family of bacterial Pnkp proteins. The amino acid sequence of *C. thermocellum* Pnkp (Cth; NCBI accession ZP_00312808) is aligned to homologous polypeptides encoded by *Kineococcus radiotolerans* (Kra; NCBI accession ZP_00354175), *Nostoc sp. PCC 7120* (Nostoc; NCBI accession BAB75430), and *Streptomyces coelicolor* (Sco; NCBI accession NP_630089). Gaps in the alignment are indicated by dashes. Positions of amino acid side-chain identity or similarity in all four proteins are indicated by dots above the alignment. The polynucleotide kinase, phosphoesterase, and nucleotidyltransferase motifs are highlighted in shaded boxes. The translation start sites and stop sites of truncated versions of *CthPnkp* are indicated by arrows.

FIGURE 3. Glycerol gradient sedimentation of *CthPnkp*. Sedimentation was performed as described in Materials and Methods. (A) Aliquots (10 μ L) of the even-numbered gradient fractions were analyzed by SDS-PAGE. The Coomassie blue-stained gel is shown. The positions of the recombinant Pnkp protein and the internal standards catalase, BSA, and cytochrome *c* are indicated. (B) Adenylyltransferase reaction mixtures (10 μ L) containing 50 mM Tris-acetate (pH 7.0), 10 mM $MgCl_2$, 5 mM DTT, 100 μ M [γ - ^{32}P]ATP, and 2 μ L of the indicated glycerol gradient fractions were incubated for 15 min at 45°C. The products were analyzed by SDS-PAGE and visualized by autoradiography. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (C) Kinase reaction mixtures (10 μ L) containing 50 mM Tris-acetate (pH 7.0), 5 mM DTT, 10 mM $MgCl_2$, 100 pmol of a 36-mer 5'-OH oligodeoxyribonucleotide, 100 μ M [γ - ^{32}P]ATP, and 2 μ L of a 1:20 dilution of the indicated glycerol gradient fractions were incubated for 30 min at 45°C. The products were analyzed by PAGE and visualized by autoradiography. (D) Phosphatase reaction mixtures (50 μ L) containing 50 mM Tris-acetate (pH 7.0), 5 mM $NiCl_2$, 5 mM 2',3' cAMP, and 4 μ L of the even-numbered gradient fractions were incubated for 30 min at 45°C. Release of P_i was measured with the malachite green reagent.

The native size of *CthPnkp* was gauged by sedimentation through a 15%–30% glycerol gradient. Marker proteins catalase (248 kDa), BSA (66 kDa), and cytochrome *c* (13 kDa) were included as internal standards. The majority of the applied *CthPnkp* sedimented as a discrete peak between BSA and catalase (Fig. 3A). A minor fraction was present in a more rapidly sedimenting shoulder. The peak of the kinase activity profile coincided with the *CthPnkp* protein peak in fractions 8–10 (Fig. 3C). A low level of kinase

activity was seen in more rapidly sedimenting fractions that contained the 97-kDa *CthPnkp* polypeptide, and a second minor peak of activity was detected in fraction 14 that did not coincide with a peak of the 97-kDa recombinant polypeptide. We suspect that this activity resides within an N-terminal proteolytic fragment of *CthPnkp* that was resolved from the native enzyme during sedimentation. A plot of the *S* values of the three standards versus fraction number yielded a straight line (data not shown). An *S* value of 7.5 was determined for the peak component of *CthPnkp* by interpolation to the internal standard curve. As the apparent *S* value of *CthPnkp* was larger than expected for a 100-kDa globular monomer, we surmise that *CthPnkp* is probably an asymmetrically shaped homodimer.

Characterization of the kinase activity

The kinase reaction of *CthPnkp* was characterized initially by using a 36-mer 5'-OH DNA substrate. The extent of label transfer from [γ - ^{32}P]ATP to the oligonucleotide acceptor was proportional to input *CthPnkp* (Fig. 4A). From the slope of the titration curve, we calculated a turnover number of $\sim 17 \text{ min}^{-1}$. Activity was optimal from pH 5.5–8.0, declined to half of the peak value at pH 5.0, and was abolished at pH ≤ 4.0 (Fig. 4B). The enzyme required a divalent cation cofactor. Various metals were tested at 5 mM concentration for their ability to support kinase activity. Magnesium was most effective, manganese was about half as active as magnesium, cobalt about one-fourth as active, and calcium and nickel were even less effective (Fig. 5A). No activity was observed with 5 mM cadmium, copper, or zinc (Fig. 5A). Kinase activity increased with ATP concentration in the range of 0.25 to 30 μ M and plateaued at $\geq 60 \mu$ M (Fig. 4D). From a double reciprocal plot, we calculated a K_m of 16 μ M ATP. *CthPnkp* catalyzed the transfer of $^{32}P_i$ from [γ - ^{32}P]ATP to the

FIGURE 4. Characterization of the 5' kinase reaction. (A) Enzyme titration. Reaction mixtures (10 μ L) containing 50 mM Tris-acetate (pH 7.0), 5 mM DTT, 10 mM $MgCl_2$, 100 μ M [γ - ^{32}P]ATP, 100 pmol 36-mer 5'-OH DNA, and *CthPnkp* as specified were incubated for 30 min at 45°C. (B) pH dependence. Reaction mixtures (10 μ L) containing 50 mM buffer (either Tris-formate at pH 3.5; Tris-acetate at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0; or Tris-HCl at pH 7.5, 8.0, 8.5, 9.0 or 9.5), 5 mM DTT, 10 mM $MgCl_2$, 100 μ M [γ - ^{32}P]ATP, 100 pmol 36-mer 5'-OH DNA, and 8 ng *CthPnkp* were incubated for 30 min at 45°C. (C) ATP dependence. Reaction mixtures (10 μ L) containing 50 mM Tris-acetate (pH 7.0), 5 mM DTT, 10 mM $MgCl_2$, 100 pmol 36-mer 5'-OH DNA, 8 ng *CthPnkp*, and [γ - ^{32}P]ATP as specified were incubated for 30 min at 45°C.

FIGURE 5. Metal specificity and RNA kinase activity. (A) Reaction mixtures (10 μL) containing 50 mM Tris-acetate (pH 7.0), 100 μM [$\gamma\text{-}^{32}\text{P}$]ATP, 100 pmol 36-mer 5'-OH DNA, 8 ng *CthPnkp*, and either no divalent cation (lane –) or 5 mM MgCl_2 , MnCl_2 , CaCl_2 , CdCl_2 , CoCl_2 , NiCl_2 , or ZnCl_2 as specified were incubated for 30 min at 45°C. The products were analyzed by PAGE and visualized by autoradiography. The relative kinase activities (normalized to the magnesium-containing reaction, defined as 100%) are indicated below the lanes. (B) RNA kinase activity. Reaction mixtures (10 μL) containing 50 mM Tris-acetate (pH 7.0), 5 mM DTT, 10 mM MgCl_2 , 100 μM [$\gamma\text{-}^{32}\text{P}$]ATP, 50 ng of *CthPnkp*, and 100 pmol of 5'-OH-terminated 18-mer, 15-mer, 12-mer, or 9-mer oligonucleotides as specified were incubated for 30 min at 45°C. The products were analyzed by PAGE and visualized by autoradiography.

5'-OH terminus of either a 18-mer DNA or 18-mer RNA oligonucleotide (Fig. 5B). RNAs with chain lengths of 18, 15, 12, or 9 nucleotides were phosphorylated to similar extents by *CthPnkp* (Fig. 5B). Shorter 5'-OH substrates were not tested.

2',3' Phosphatase activity of recombinant *CthPnkp*

The 3' end-healing phase of the phage tRNA restriction pathway entails the conversion of a 2',3' cyclic phosphate terminus to a 2'-OH, 3'-OH terminus that is suitable for ligation to a 5'- PO_4 strand (Amitsur et al. 1987). To test whether *CthPnkp* might catalyze an analogous 3' end-healing reaction, we assayed the recombinant protein for its ability to release phosphate from 2',3' cyclic adenosine monophosphate (2',3' cAMP). Phosphate release was measured colorimetrically by using the malachite green method (Lanzetta et al. 1979). *CthPnkp* hydrolyzed 2',3' cAMP to P_i in the presence of either nickel or manganese (Fig. 6A). Activity with either 5 mM nickel or manganese was optimal at pH 6.5 to 7.5 and

declined sharply as the pH was raised to ≥ 8.5 or lowered to ≤ 4.5 (Fig. 6A). The Ni-dependent phosphatase activity cosedimented with the *Cth1Pnkp* polypeptide during glycerol gradient sedimentation (Fig. 3D).

Phosphate release from 5 mM 2',3' cAMP at pH 7.0 was optimal at 5–20 mM NiCl_2 or MnCl_2 (data not shown). Neither magnesium, calcium, copper, cobalt, cadmium, nor zinc was able to support 2',3' cAMP phosphatase activity (data not shown). *CthPnkp* protein titration experiments showed that phosphatase specific activity in the presence of nickel was threefold higher than activity in the presence of manganese (data not shown). The fastidious requirement of *CthPnkp* for either nickel or manganese is a property shared with bacteriophage λ phosphatase, an enzyme that is structurally homologous to the phosphoesterase domain of *CthPnkp* (Zhuo et al. 1993).

P_i release by *CthPnkp* displayed a hyperbolic dependence on the concentration of the 2',3' cAMP substrate (Fig. 6B). From a double reciprocal plot, we calculated a K_m value of 4.4 mM and a k_{cat} of $\sim 60 \text{ min}^{-1}$. Whereas *Cth1Pnkp* readily released P_i from 2',3' cAMP; it was fourfold less effective in releasing P_i from 3',5' cAMP (Fig. 6C). The release of P_i from 2',3' cAMP likely entails two enzymatic activities: a 2',3' cyclic phosphodiesterase and a phosphomonoesterase. *CthPnkp* displayed vigorous phosphomonoesterase activity with either 3'-AMP or 2'-AMP, but not 5'-AMP (Fig. 6C). Control experiments verified that the 2',3' cAMP substrate was refractory to hydrolysis by alkaline phosphatase, whereas the 3'-AMP and 2'-AMP substrates were sensitive to alkaline phosphatase (data not shown).

Covalent adenylyltransferase activity of recombinant *CthPnkp*

An adenylyltransferase activity of recombinant *CthPnkp* was evinced by label transfer from [$\alpha\text{-}^{32}\text{P}$]ATP to the *CthPnkp*

FIGURE 6. Characterization of the 2',3' phosphatase activity. (A) pH dependence. Reaction mixtures (50 μL) containing 50 mM buffer (either Tris-acetate at pH 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0; or Tris-HCl at pH 7.5, 8.0, 8.5, or 9.0), 5 mM DTT, 5 mM 2',3' cAMP, and either 5 mM MnCl_2 and 2 μg *CthPnkp* or 5 mM NiCl_2 and 1 μg *CthPnkp* as specified were incubated for 30 min at 45°C. (B) cAMP dependence. Reaction mixtures (50 μL) containing 50 mM Tris-acetate (pH 7.0), 10 mM NiCl_2 , 1 μg *CthPnkp*, and 2',3' cAMP as specified were incubated for 30 min at 45°C. (C) Substrate specificity. Reaction mixtures (50 μL) containing 50 mM Tris-acetate (pH 7.0), 10 mM NiCl_2 , 1 μg *CthPnkp*, and 5 mM 2',3' cAMP, 3',5' cAMP, 2' AMP, 3' AMP, or 5' AMP as specified were incubated for 30 min at 45°C.

polypeptide to form a covalent enzyme-adenylate adduct (Fig. 2B). The adenylyltransferase activity profile during glycerol gradient sedimentation paralleled the sedimentation profile of the 97-kDa *CthPnkp* polypeptide (Fig. 3B). *CthPnkp* required a divalent cation cofactor to form the covalent adduct. MgCl_2 and MnCl_2 supported approximately equivalent activity at the optimal concentrations of 2.5 mM and 0.6 mM, respectively (Fig. 7). Cobalt and calcium were less effective in supporting enzyme adenylation at 5 mM concentration; little or no adenylation was supported by 5 mM nickel, cadmium, or zinc (Fig. 7). *CthPnkp* adenylation was optimal at pH 5.0–5.5 in Tris-acetate buffer (Fig. 8A). Activity fell precipitously as the pH was lowered to ≤ 4.5 , but declined gradually as the pH was incrementally raised to 9.5 in Tris-HCl buffer (Fig. 8A). The yield of *CthPnkp*-AMP complex reached saturation at $\geq 16 \mu\text{M}$ ATP (Fig. 8B). Half-saturation was achieved at $\sim 5 \mu\text{M}$ ATP. We calculated that $\sim 25\%$ of the *CthPnkp* protein preparation could be adenylylated in vitro with ^{32}P -AMP. *CthPnkp* was unreactive with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (data not shown).

Lys531 is required for adenylyltransferase activity

The $^{531}\text{KHMGSR}^{536}$ peptide of *CthPnkp* is a putative counterpart of the motif I of the polynucleotide ligase/capping enzyme superfamily, which is typically KxDGxR and contains the lysine nucleophile to which the NMP is covalently attached. The contribution of Lys531 to the activities of *CthPnkp* was surmised from the effects of a single alanine substitution K531A. The

FIGURE 7. Divalent cation specificity and dependence of the adenylyltransferase reaction. (A) Divalent cation specificity. Reaction mixtures (10 μL) containing 50 mM Tris-acetate (pH 7.0); 100 μM $[\alpha\text{-}^{32}\text{P}]\text{ATP}$; 2 μg *CthPnkp*; and either no divalent cation (lane —) or 10 mM MgCl_2 , MnCl_2 , CaCl_2 , CdCl_2 , CoCl_2 , NiCl_2 , or ZnCl_2 as specified were incubated for 15 min at 45°C . (B) Divalent cation dependence. Reaction mixtures (10 μL) containing 50 mM Tris-acetate (pH 7.0); 5 mM DTT; 100 μM $[\alpha\text{-}^{32}\text{P}]\text{ATP}$; 2 μg *CthPnkp*; and either 0, 0.63, 1.25, 2.5, 5, or 10 mM MgCl_2 (●) or MnCl_2 (○) were incubated for 15 min at 45°C . The yield of enzyme-AMP adduct (E-AMP) is plotted as a function of divalent cation concentration.

FIGURE 8. pH- and ATP-dependence of the adenylyltransferase reaction. (A) Reaction mixtures (10 μL) containing 50 mM buffer (either Tris-formate at pH 3.5; Tris-acetate at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0; or Tris-HCl at pH 7.5, 8.0, 8.5, 9.0, or 9.5), 5 mM DTT, 10 mM MgCl_2 , 100 μM $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, and 2 μg *CthPnkp* were incubated at 45°C for 15 min. (B) Reaction mixtures (10 μL) containing 50 mM Tris-acetate (pH 7.0), 5 mM DTT, 10 mM MgCl_2 , 2 μg *CthPnkp*, and $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ as specified were incubated at 45°C for 15 min.

K531A mutant protein was produced in bacteria and purified from soluble lysates by nickel-affinity chromatography (Fig. 2A). K531A was apparently inert in enzyme-adenylate formation (Fig. 2B) but retained activity as a polynucleotide kinase (Fig. 2C) and a 2',3' cAMP phosphatase (data not shown). A parallel titration experiment comparing the extent of adenylation of wild type versus K531A verified that its activity was undetectable at a level of sensitivity of $\leq 0.5\%$ of the wild type. These findings are consistent with Lys531 being the site of covalent adenylation.

Additional alanine substitutions were introduced in place of flanking motif I residues His532 and Arg536. The H532A protein retained adenylyltransferase (Fig. 2B) and polynucleotide kinase (Fig. 2B) activities. A titration experiment showed that H532A was 40% as active as was wild-type *CthPnkp* (data not shown). The R536A mutant was impaired for adenylyltransferase activity (Fig. 2B), although it retained polynucleotide kinase activity (Fig. 2C). A protein titration indicated that R536A was 5% as active in adenylation as wild-type *CthPnkp*. The corresponding motif I Arg side-chain of other members of the nucleotidyl transferase superfamily comprises part of the nucleotide binding pocket, where it typically makes contact with the ribose sugar of the NTP substrate (Subramanya et al. 1996; Odell et al. 2000; Shuman and Lima 2004).

Separable kinase and adenylyltransferase domains of *CthPnkp*

We produced recombinant His₁₀-tagged versions of two N-terminal deletion mutants, *CthPnkp*-(433–870) and *CthPnkp*-(462–870), that contained the nucleotidyl transferase motifs but lacked the signature motifs of the kinase and phosphoesterase enzyme families. The translation start sites of the truncated proteins are indicated by arrows in

Figure 1. The truncated proteins were purified from soluble *E. coli* extracts by Ni-agarose chromatography (Fig. 2A). Both proteins displayed autoadenylation activity (Fig. 2B) but were inert with respect to polynucleotide kinase (Fig. 2C) and 2',3' cAMP phosphatase (data not shown). We conclude that the C-terminal 409-amino-acid segment of *CthPnkp* comprises an autonomous adenylyltransferase domain.

An N-terminal His₁₀-tagged fragment, *CthPnkp*-(1–425) was produced and purified in parallel (Fig. 2A). *CthPnkp*-(1–425) had no detectable adenylyltransferase activity (Fig. 2B) but was active as a polynucleotide kinase (Fig. 2C). Thus, the N-terminal 425-amino-acid segment is an autonomous kinase domain. *CthPnkp*-(1–425) was 10% as active as was full-length *CthPnkp* in releasing P_i from 2',3' cAMP (data not shown).

DISCUSSION

Here we identified and characterized a new end-healing enzyme from *C. thermocellum* that catalyzes the phosphorylation of 5'-OH termini of DNA or RNA polynucleotides and the dephosphorylation of 2',3' cyclic phosphate, 2'-phosphate, and 3'-phosphate ribonucleotides. These activities suggest a role for *CthPnkp* in RNA or DNA repair. We show that *CthPnkp* also catalyzes an autoadenylation reaction via a polynucleotide ligase-type mechanism. However, we have been unable to demonstrate a strand-joining activity of *CthPnkp* by using single-stranded or duplex substrates that are readily sealed by prototypal DNA and RNA ligases studied in this laboratory (data not shown). The substrates tested included the following: a nicked DNA duplex (with 3'-OH and 5-PO₄ termini at the nick) composed of synthetic DNA oligonucleotides, a nicked RNA duplex composed of synthetic RNA oligonucleotides, a nicked RNA–DNA hybrid duplex formed by annealing 3'-OH and 5-PO₄ RNA strands to a DNA template strand, and an 18-mer single-stranded 5'-PO₄ RNA oligonucleotide.

Although the compaction of 5' kinase, 2',3' cyclic phosphodiesterase or 3' phosphatase, and covalent adenylyltransferase activities within a single polyfunctional protein has been described previously for yeast tRNA ligase (Apostol et al. 1991; Sawaya et al. 2003) and baculovirus RNA ligase (Martins and Shuman 2004b), the *CthPnkp* enzyme is novel with respect to its domain order and the structure and mechanism of its phosphoesterase component. Baculovirus RNA ligase consists of an N-terminal adenylyltransferase domain that resembles T4 RNA ligase 1 fused to a C-terminal end-healing domain that resembles T4 Pnkp. The baculovirus end-healing domain is composed of a proximal 5' kinase module (similar to that of *CthPnkp*) and a distal 3' phosphatase module that belongs to the acyl-phosphatase superfamily of phosphotransferases (Martins and Shuman 2004b). Yeast tRNA ligase consist of an N-terminal adenylyltransferase domain (again similar to T4 RNA ligase 1), a central T4-like kinase domain, and a C-terminal 2',3' cyclic phos-

phodiesterase domain that belongs to the 2H superfamily of phosphotransferases (Apostol et al. 1991; Mazumder et al. 2002; Sawaya et al. 2003). *CthPnkp* and its bacterial homologs are unique in that they have an adenylyltransferase domain appended at the C-terminus. Moreover, the adenylyltransferase domain of *CthPnkp* does not have the structural signatures upstream of motif I that are characteristic of the Rnl1-type subfamily of RNA ligases (Wang et al. 2003), which includes baculovirus RNA ligase and yeast tRNA ligase.

The most distinctive feature of *CthPnkp* vis-à-vis known RNA repair enzymes is that its 3' end modification component belongs to the calcineurin-type phosphoesterase superfamily. It contains putative counterparts of the amino acids that form the dinuclear metal-binding site and the phosphate-binding site seen in the crystal structure of λ phosphatase (Voegtli et al. 2000). As with λ phosphatase (Zhuo et al. 1993), the 2',3' cAMP phosphatase activity of *CthPnkp* is specifically dependent on either nickel or manganese, to the exclusion of magnesium and various other divalent cations. A functionally analogous Ni-dependent 2',3' cAMP phosphatase activity was described recently for the *E. coli* tRNA nucleotidyltransferase (the CCA-adding enzyme) (Yakunin et al. 2004). However, the C-terminal domain of CCA-adding enzyme responsible for the 2',3' phosphatase activity belongs to the HD superfamily of metal-dependent phosphotransferases (Aravind and Koonin 1998; Yakunin et al. 2004), which are structurally unrelated to the *CthPnkp*-type phosphoesterase module. A manganese-dependent 2',3' cAMP phosphatase activity associated with bacteriophage RM378 Pnkp also belongs to the HD superfamily (Blondal et al. 2005).

We propose that *CthPnkp* exemplifies a new class of end-remodeling enzyme. In addition to the putative trifunctional homologs of *CthPnkp* encoded by *K. radiotolerans*, *Nostoc*, and *S. coelicolor*, we have identified putative trifunctional kinase-phosphatase-adenylyltransferase homologs in several other bacterial species, including *Fusobacterium nucleatum*, *Bacillus licheniformis*, and *Helicobacter hepaticus* (Fig. 9). We have also identified bifunctional Pnkp homologs in *Deinococcus radiodurans* and *Thermobifida fusca* that are composed only of the kinase and the phosphoesterase domain equivalents of *CthPnkp*, with no equivalent of the adenylyltransferase domain (data not shown). It appears that Pnkp enzymes are present in many bacteria, which hints at an as yet unexplored role for RNA repair in bacterial physiology.

MATERIALS AND METHODS

Materials

Oligodeoxyribonucleotides were purchased from Biosource International. Oligoribonucleotides were purchased from Dharmacon and deprotected as instructed by the vendor. Concentrations of oligonucleotide stock solutions were determined by UV absorbance at 260 nm, using extinction coefficients cal-


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Fnu  LLLLRGAQASGKSTWVTENNLEPYTLSDAKIRLNINPIL-----NEDGSIE-
Bli  LVLLRGCGPTGKSTWIRDHQINQYTLSDQRLIHQSPVL-----NLEGKYD-
Hhe  MVLNRGTPASGKSTWIANMGLSEYTLSDSLRLMASSPVLLPKSKSNALSESADSTKSH

Fnu  -----ISQKNKLTWELLYKLEMRMENGDFTIIDATHSDIKLMNKYRDLANITYKY
Bli  -----ISQKNDGKVKLLPSLLEEMERGEFTIVDATHAKQSMISGYKALVLKYRY
Hhe  ALDSLAFTGQNDQKQVWKLFTLLEMRLLKQGDFTIIDATHSTIKALRAYETLANITYRY

Fnu  TIYYLEFD-TPLEELCKRNRERVGYKYVPEKVIERTWETIKRNEKLPS--VLKKINSI--
Bli  RAYVDFPNVPLETALLRNRAEHRKRVPSVVMHAMERIL-SEPAPSWTTVIKPEEF--
Hhe  RLIVIDFSHIPLEVALQNRACR-GYKSVFVDILESMTYKTLQDSLTLPSRYVLKSDETAP

Fnu  -----DEIINFYTAADVNEYKKVIIIGDIHSC-----AKPLKEVLKDFSEE
Bli  -----ADAMQYKPRRLDSYKIHVIGDVHGC-----FTVLDLSYKGRLED
Hhe  LQDKTHQSMLHPNFINLNTAQIHHIGDIHGCFTLLHYLIHTSLTHSLQDAIRILEQS

Fnu  NL-----YVFGDYFDGIAQAVETFKIILDLLEKPNVILTECHENNNSVKK
Bli  DE-----LYITGDMVDRIENAKVQVFLRIKDRKNVILLEGNHID-KYLKQ
Hhe  QFAPEICARFLNPKAYIIFLGDYIDRGVQNAQVVRLLGIMDLFNVCLLEGHHE----R

Fnu  FIYDEEKYTKSFDETTLQPLLEEFELDYIKAGLKKIYKRLRQCFTFEFRGKFLCTHGG
Bli  YGHDVEVTRSSVFNKKT-KPELDEAGFD--KRDIRELARFHQIAYFTYGDQYITTHGGI
Hhe  WLYKGKEDISTNEFSITFLKDLLENGGVGSKDTHRLYSKLRQCAYYTYDNKIVLCTHGGI

Fnu  PLVPLKAL-VSAREMIKGVGYETEIGEISYENYKGLCQDFIQVHGRG----INDGE
Bli  SALPDNLFTATSQLINGVGGYETDIDHVTKNMEG---RNIQIHGRHNMFLPVHAAA
Hhe  PTLNNLLVATKQLIYSGSGYE-DMQEC-AKSPALSTASNTYQVGHNRNREKHPMRVYE

Fnu  YSYCLEGRVEFGSELKVLITDNDGNLEKSGIKNDVYNRGLKLP--MSGVTEK-----
Bli  KSYNLEGGVHGGHRLRVVITDRSG-TQTHEIKNDVFKTSAP-PLT--SHHAHEE-LTVEH
Hhe  RNFALGGVEFGSALRVLLITKTSLPPSVIKTMTPTQSSLMQDSLMGRHTSKDNFTLIY

Fnu  -AKKFNTA--NELINEMIGHKFIN-----VKECDY-NLISLNFNRDAFNKKKWNDLTI
Bli  -----FLKHLDEHDYVS-----EQKLAGGISFNFTRKAFQERKWDQSVSM
Hhe  MQNKHNTSALNALHQSGSTFLFQKLARANALKEKKFGDISFNFTEKAPFSKQWNTLTC

Fnu  KARGLFVDRDSGEVKIRSYNKFNYGER-HVNRLYLHKYATYFIRVFKYNGFLGLASV-
Bli  KARGLFIRNNTNEIVRSYNKFNIEERLITKMHVLTNLRFPVTVYDKANGFLGTGVGY-
Hhe  KARGLFIDTNPHIYARSYDKFNYQEREETSDETLREKLTYPVNVFIKNGFLGTMSTH

Fnu  -----INGDVV-----LTSKSVTSKGYKDIF-----QSIWDKVEDSVKEL--LK
Bli  -----NSETDELV-----FTSKSYTSSGQKDGH-AKWVEELFYKTFDASQTE--AMK
Hhe  SALSDTKNANKNTYNNVLQNPFTITSKSDPTSPVAHIMRNLIAESLRKTAQDSIQEVSELY

Fnu  QTMIEENCTAVFEVVSPEYDPHIKYDKHELYLLDPIENKLDLDTHNIDLEFSENLMKKV
Bli  EYVKKRNVSLVFEVVLPEKDPHIIEYSDKLVLLDIVKRMKYE----KLLYEDVLRFAE
Hhe  EEKTRNLSLVFEVLEPQDDPHIAYEKPVILLDAIYNTPDY----AKLPFTLECALGN

Fnu  EFSSNLLTKKEELTRLANYDELYNPLHERTMSLEE-----FEGYVLCDSNGL
Bli  TFRVECKQKVTIFHQWTFYKWLVSNS-DPSIEE-----EGYVIEDSAGF
Hhe  KFG--PICKEKIT-LHCWKEVESEFLYCES-ALESNSLPAQGRKKNGTIEGYVIEDSAGF

Fnu  MFKPKLPYYMLWKGRRTWLER-YRTALL--KGKRIEIRDEKDNHRHFKFLLEL----
Bli  MTKLKLFPYQYKLPFYQYKWFIRGKIKH-RLGAAARSPOHEALPHSEHVFLAWAKTKD
Hhe  MFKYKGAYYRAWKALREIVEQCARTRALPKSGKGMQGDSEAYYELEGAFIQWLQYIEHN

Fnu  GKDKLQGLSLIIDVREMYEKS 730
Bli  -SEYFKNQSVIAIRNDPYNE 728
Hhe  GFESLESSSLIDREAFRLSL 845

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FIGURE 9. Bacterial Pnkp/adenylyltransferase homologs. The aligned amino acid sequences of putative trifunctional kinase-phosphatase-adenylyltransferases from *Fusobacterium nucleatum* (Fnu; NCBI accession EAA24876), *Bacillus licheniformis* (Bli; NCBI accession YP_079636), and *Helicobacter hepaticus* (Hhe; NCBI accession AAP78353) are shown. Gaps in the alignment are indicated by dashes. The polynucleotide kinase, calcineurin-type phosphoesterase, and nucleotidyltransferase motifs are highlighted in shaded boxes.

culated according to their base compositions. 2',3' cAMP, 2'AMP, 3'AMP, and 5'AMP were purchased from Sigma. Malachite green reagent was purchased from BIOMOL Research Laboratories. *C. thermocellum* genomic DNA was obtained from ATCC.

Recombinant CthPnkp

The *C. thermocellum* gene encoding an 870-amino-acid Pnkp homolog (NCBI accession ZP_00312808) was amplified by two-stage PCR from genomic DNA with *Pfu* DNA polymerase by using primers designed to eliminate internal NdeI and BamHI sites (by introducing translationally silent mutations) while introducing an NdeI restriction site at the start codon and a

BamHI site 3' of the stop codon. The PCR products were digested with NdeI and BamHI and inserted into pET16b to generate an expression plasmid encoding the *CthPnkp* polypeptide fused to an N-terminal His₁₀ tag. Alanine substitution mutations were introduced by PCR using the two-stage overlap extension method. N-terminal truncation alleles *CthPnkp*-(433–870) and *CthPnkp*-(462–870) were generated by PCR amplification using sense primers that introduced an NdeI site at the Met433 or Met462 codons. The C-terminal truncation alleles *CthPnkp*-(1–425) was constructed by PCR amplification using an antisense primer that introduced a stop codon in lieu of the coding for Pro426 and a BamHI site immediately 3' of the stop codon.

Wild-type and mutant pET-*CthPnkp* plasmids were transformed into *E. coli* BL21(DE3). Cultures (1 L) of *E. coli* BL21(DE3)/pET-*CthPnkp* were grown at 37°C in Luria-Bertani medium containing 0.1 mg/mL ampicillin until the A₆₀₀ reached ~0.6. The cultures were chilled on ice for 30 min, adjusted to 0.1 mM isopropyl-D-thiogalactopyranoside and 2% ethanol, and then incubated at 17°C for 16 h with continuous shaking. Cells were harvested by centrifugation, and the pellet was stored at –80°C. All subsequent procedures were performed at 4°C. Thawed bacteria were resuspended in 30 mL of buffer A (50 mM Tris-HCl at pH 7.5, 0.5 M NaCl, 10% sucrose). Lysozyme, PMSF, and Triton X-100 were added to final concentrations of 1 mg/mL, 1 mM, and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and insoluble material was removed by centrifugation. The soluble extracts were applied to 1 mL columns of Ni-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer A. The columns were washed with 8 mL of the same buffer and then eluted stepwise with 4 mL aliquots of 25, 50, and 200 mM imidazole in buffer B (50 mM Tris HCl at pH 8.0, 0.5 M NaCl, 10% glycerol). The polypeptide compositions of the column fractions were monitored by SDS-PAGE. The His₁₀-*CthPnkp* proteins adsorbed to the column and were recovered predominantly in the 200 mM imidazole eluates. Protein concentrations were determined by SDS-PAGE analysis of serial dilutions of the *CthPnkp* preparations in parallel with serial dilutions of a BSA standard. The gels were stained with Coomassie blue, and the staining intensities of the *CthPnkp* and BSA polypeptides were quantified using a Digital Imaging and Analysis System from Alpha Innotech Corporation. Approximately 8 mg of full-length wild-type *CthPnkp* was recovered from a 1-L bacterial culture.

Polynucleotide kinase assay

Reaction mixtures (10 μL) containing a 5'-OH oligodeoxynucleotide or oligoribonucleotide substrate, [γ-³²P]ATP, and other components as specified in the figure legends were incubated at 45°C. The kinase reactions were initiated by adding *CthPnkp* and terminated with formamide/EDTA. The products were analyzed by electrophoresis through a 15-cm 18% polyacrylamide gel containing 7 M urea in 45 mM Tris-borate, 1 mM EDTA at 10 W constant power. The radiolabeled oligonucleotide products were visualized by autoradiography of the gel and quantified by scanning the gel with a Fujix BAS2500 imaging apparatus.

Adenylyltransferase assay

Reaction mixtures (10 μ L) containing 50 mM Tris-acetate (pH 7.0), 5 mM DTT, 5 mM $MgCl_2$, [α - ^{32}P]ATP, and *CthPnkp* as specified were incubated for 15 min at 45°C. The reactions were quenched with SDS, and the products were analyzed by SDS-PAGE (12% acrylamide). The protein-[^{32}P]AMP adduct was visualized by autoradiography of the dried gel and quantified by scanning the gel.

Phosphatase assay

Reaction mixtures (50 μ L) contained 50 mM Tris-acetate (pH 7.0), 5 mM 2',3' cyclic AMP, 5 or 10 mM $NiCl_2$ or $MnCl_2$, and other components as specified in the figure legends. The reactions were quenched by adding 1 mL of malachite green reagent. Release of phosphate was determined by measuring A_{620} and interpolating the value to a phosphate standard curve.

Glycerol gradient sedimentation

An aliquot (200 μ g) of *CthPnkp* was mixed with catalase (100 μ g), BSA (100 μ g), and cytochrome *c* (100 μ g). The mixture was applied to a 4.8 mL 15%–30% glycerol gradient containing 50 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 2 mM EDTA, 2 mM DTT, and 0.1% Triton X-100. The gradient was centrifuged for 18 h at 4°C in a Beckman SW55Ti rotor at 50,000 rpm. Fractions (~0.2 mL) were collected from the bottom of the tube. Aliquots were analyzed by SDS-PAGE and assayed for kinase and phosphatase activity as specified in the figure legends.

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