Characterization of a 5′-Polynucleotide Kinase/3′-Phosphatase from Bacteriophage RM378*

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A polynucleotide kinase from the thermophilic bacteriophage RM378 that infects the thermophilic eubacterium Rhodothermus marinus was identified, expressed, and purified. This polynucleotide kinase was demonstrated to have a 5′-kinase domain as well as a 3′-phosphohydrolase domain. The RM378 polynucleotide kinase had limited sequence similarity to the 5′-kinase domain of the T4 bacteriophage polynucleotide kinase, but apparent homology was not evident within the 3′-phosphohydrolase domain. The domain order of RM378 polynucleotide kinase was reversed relative to that of the T4 polynucleotide kinase. The RM378 phosphohydrolase domain displayed some sequence similarity with the bacterial polA polymerase family, including an HD motif characteristic of the diverse superfamity of metal-dependent HD phosphohydrolases. The RM378 polynucleotide kinase was biochemically characterized and shown to possess 5′-kinase activity on RNA and single- and double-stranded DNA at elevated temperatures. It also showed phosphohydrolase activity on 2′:3′-cyclic adenosine monophosphate. This description of the RM378 polynucleotide kinase, along with the recently described RM378 RNA ligase, suggests that the RM378 bacteriophage has to counter a similar antiphage mechanism in R. marinus as the one that the T4 phage has to counter in Escherichia coli.

Recently, we described the identification and characterization of a thermostable RNA ligase from the thermophilic bacteriophage RM378 that is homologous to the T4 RNA ligase 1 and infects the thermophilic eubacterium Rhodothermus marinus (1–4). The biological role of T4 bacteriophage RNA ligase 1 is to counteract certain Escherichia coli host defenses that are based on the degradation of tRNA molecules (5–8). The best known of these systems is the suicidal anticondon nuclease system, which is activated upon inhibition of the E. coli DNA restriction-modification system by the T4 viral Stp polypeptide. The anticondon nuclease cleaves tRNA3′-5′ at its wobble position, yielding 2′:3′-cyclic phosphate and a 5′-hydroxyl group (5, 7, 8). Whereas the RNA ligase 1 is essential for ligation of the cleaved tRNA molecules, the polynucleotide kinase (PNK)1 has the important role of making the tRNA fragments appropriate substrates for the ligation step (6, 9–12). T4 PNK is a nucleic acid processing enzyme that has two major functions as follows: (i) to remove the 2′:3′-cyclic phosphate from the 5′ tRNA fragment; and (ii) to add a phosphate group to the 5′-hydroxyl group of the 3′-tRNA fragment using ATP as the phosphate donor (6, 10–12). The RNA ligase 1 and the PNK are thus part of the same system, acting in concert to repair dysfunctional translational machinery.

The T4 PNK is the founding member of a large 5′-kinase/3′-phosphohydrolase family that has been studied extensively for the last 35 years. The PNK family includes polynucleotide kinases from wide variety of organisms. The biological role of the eukaryotic 5′-kinase/3′-phosphohydrolase is to mend broken nucleic acids strands, making them appropriate substrates for repair by nucleic acid ligases and playing a notably important role in the repair of DNA nicks and gaps (13–15).

Biochemical and mutational analysis have shown that T4 PNK is a homotetramer with no kinetic cooperativity (11, 16–18). The 5′-kinase and 3′-phosphohydrolase activities have been shown to reside in distinct and separate domains, with an N-terminal 5′-kinase domain and a C-terminal 3′-phosphohydrolase domain. The 5′-kinase domains of PNK enzymes contain the nucleotide binding motif GXXXXGKS/T (a Walker A box or P-loop), which is a common motif in phosphotransferases as well as in other proteins containing nucleotide binding domains (16, 17, 19). Mutational data from T4 PNK has shown that in addition to residues in the P-loop motif (Lys-15 and Ser-16), residues Asp-35, Arg-38, Asp-85, and Arg-126 are all essential for the 5′-kinase activity (20). In addition, studies have suggested a role of residues Asp-85 and Asp-126 in the quaternary structure integrity, resulting in mixture of dimers and tetramers when these positions are changed by site-directed mutagenesis (16, 17, 20).

The sequence analysis and mutational data on the T4 PNK/3′-phosphohydrolase domain have shown that the metal-dependent phosphatase family motif DXXDXT is found in the PNK family and is essential for the phosphohydrolase activity of the domain (16, 17, 20). Sequence analysis of PNK shows that the 3′-phosphatase domain of T4 PNK is distantly related to other phosphatase families like the histidi nol phosphatase family and the acid phosphatase (HAD) superfamily (16, 20). The crystal structure of the T4 PNK was solved by Galburt et al. and confirmed that there were two functionally distinct structural domains (18). The N-terminal 5′-kinase domain was structurally similar to those of the adenylate kinase (Adk) osine 2′:3′-cyclic monophosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; PEG, polyethylene glycol; 3′-TMP, thymidine 3′-monophosphate.
family, and the 3'-phosphatase domain was a member of the HAD hydrolase superfamily (16, 18).

Here we describe a polynucleotide kinase from the thermophilic bacteriophage RM378 that infects *R. marinus*. As compared with T4 PNK, the RM378 PNK has analogous activity while utilizing a phosphohydrodrolase domain of different origin and having the kinase and phosphohydrodrolase domains in a reversed relative order as compared with those of T4 PNK.

**EXPERIMENTAL PROCEDURES**

**Cloning of the pnk Gene from RM378 Bacteriophage Genome**—All standard molecular biology protocols were done as described by Sambrook *et al.* (21). Chemicals and media were purchased from Sigma or Merek unless otherwise noted. Oligonucleotides were purchased from MWG Biotech Inc. and Eurogentec Inc. The full-length polynucleotide kinase gene was amplified by standard PCR from RM378 bacteriophage DNA using the KinR-Ase forward primer (5'-dccaggatttaatatgc-cgaaccttcattacaacatc-3') and the KinR-Bam reverse primer (5'-dgccgctgacctcgctctctcaacatc-3') with Dynazyme™ DNA polymerase (Finzymes Oy) as recommended by the manufacturer. The PCR product was cloned into pJOE 3075 vector with a connected His tail to the C-terminal. Five clones were sequenced for verification of the DNA sequence, and a vector-PNK clone named pJOE-PNK was selected for expression experiments. pJOE-PNK was transformed into CodonPlus® BL21 RIL *E. coli* cells (Stratagene Inc.), and the strain was cultivated at 37 °C in a 1-liter Bioflow 3000 fermentor, and the expression was induced with 1 mM isopropyl-1-thio-galactopyranoside. The harvested and frozen cells were incubated with lysozyme and sonication. The crude cell extract was centrifuged in a SA-600 rotor (Sorvall Inc.) at 10,400 × g for 1 h. The supernatant was collected and applied to an XK 26/10 50-ml column (Amersham Biosciences) packed with chelating Sepharose and charged with nickel ions. The column was applied to an XK 26/10 50-ml column (Amersham Biosciences) packed with nickel ions. The release of phosphate was measured at pH optimum, the standard phosphohydrolase assay conditions used were the determination of pH, the cation, and the apparent temperature optimum. The phosphohydrolase domain was eluted with 200 mM imidazole. Elution was performed in the same buffer with 500 mM imidazole in a stepwise manner (20 and 40%). The eluted protein was concentrated and run on 12% SDS-PAGE and stained with Coomassie Blue. SDS-PAGE analysis (Fig. 2). The recombinant PNK protein was collected and run on 12% SDS-PAGE and stained with Coomassie Blue. The recombinant PNK protein was collected and run on 12% SDS-PAGE and stained with Coomassie Blue.

**Characterization of the PNK/5'-Kinase Activity**—The standard PNK assay developed by Richardson (12) that measures the conversion of γ-32P-labeled ATP was used for characterization of the RM378 PNK with some minor modifications. Standard reaction conditions were 50 mM MOPS buffer, pH 8.5, 1 mM dithiothreitol, 10 mM MgCl2, 25 μg/ml bovine serum albumin, 5% PEG 6000, 100 μM ATP (mixture of normal and γ-32P-labeled ATP), and 0.5 mg/ml partial microccocal nuclease-digested calf thymus DNA or 10–100 μM DNA/DNA oligomers and 0.05 mg/ml PNK enzyme incubated at 70 °C for 15–30 min.

**Characterization of the PNK/3'-Kinase Activity**—After the determination of pH, the cation, and the apparent temperature optimum, the standard phosphohydrodrolase assay conditions used were potassium acetate buffer, pH 6.5, 5 mM MnCl2, 1 mM dithiothreitol, 10 mM KCl, 0.1–5 mM cyclic 2′,3′-cyclic adenosine monophosphate (cAMP), and 0.05 mg/ml PNK. Reaction time was 30–60 min at 65–70 °C. The reaction was quenched by adding 90 μl of Biolum Green reagent (Biolum Research Laboratory, Plymouth Meeting, PA) to the 10-μl reaction volume. The release of phosphate was measured at A250 nm in a Sunrise Absorbance Reader (Tecan Group Ltd, Maennedorf, Switzerland) and compared with a phosphate standard curve.

**Characterization of the phosphohydrodrolase activity was done using two substrates, thymidine 3'-monophosphate (3'-TMP) and cAMP. Determination of pH optimum was done with MOPS and potassium acetate buffers using 0.1 mM cAMP and 0.05 mg/ml PNK. Apparent temperature optimum was also done under the standard condition by assaying at different temperatures. Substrate concentration curves were done for cAMP and 3'-TMP under the standard conditions using different amount of substrates. All assays were done in triplicate, and the mean value was calculated.

**RESULTS**

**Sequence Analysis, Overexpression, and Purification of the RM378 PNK**—Although the initial screening of the RM378 bacteriophage genome (GenBank™ accession number NC_004735) using standard BLAST analysis (22) identified the *RnlA* gene, which encodes a homolog to the RNA ligase 1 in the T4 phage (2), we could not find an open reading frame with similarity to the T4 PNK (psdT) gene by using standard bioinformatic protocols. Our working model was that *R. marinus* contained a tRNA degradation system similar to that of *E. coli*, and we therefore hypothesized that a polynucleotide kinase would be present in the RM378 genome as part of a system to overide a tRNA degradation anti-pherage mechanism. However, we could not rule out the possibility that the tRNA degradation system in *R. marinus* was very different from the one in *E. coli*, given the distant evolutionary relationship between these two bacterial species (3). We therefore redefined our search by looking specifically for T4-like 5'-kinase domains and found a hit with open reading frame tag Rm378p110 for a putative PNK within the RM378 genome. This putative polynucleotide kinase gene designated *pnk* (GenBank™ accession number NP_835697), which has previously been described as having similarity to poly(A) polymerases from eubacteria, was 1086 bp in length and coded for a 361-amino acid polypeptide with a calculated mass of 42.1 kDa. The putative PNK had reversed domain orientation relative to T4 PNK, with the 5'-kinase domain on the C terminus of the putative PNK protein. Amino acid sequence similarity searches with the N terminus of the *pnk* showed sequence similarity to the superfamily of HD metal-dependent phosphohydrolase (23), which meant that RM378 PNK could also contain a 3'-phosphohydrolase activity analogous to the activity observed in T4 PNK (24).

The amino acid sequence of the putative RM378 PNK/5'-kinase domain was compared with those of the T4 and mycobacteriophage CJW1 5'-kinase domains (Fig. 1A) (19, 25). Alignments were done using the ClustalX program (26). Although the overall similarity was low, only 13 and 20% to T4 and mycobacteriophage CJW1, respectively, a characteristic nucleotide binding P-loop motif was conserved. The putative RM378 PNK/3'-phosphohydrolase domain was compared with those of *Clostridium acetobutylicum* poly(A) polymerase and *Desulfitobacterium hafniense* tRNA nucleotidyltransferase/poly(A) polymerase (Fig. 1B). Again, similarity was low, but an HD box, which is the main characteristic for the superfamily, was present (29). It was our hypothesis that this HD phosphohydrolase domain might have similar activity as that of the T4 PNK family-like phosphohydrolase even if they do not share any sequence similarity.

The putative *pnk* gene was amplified from the RM378 viral genome cloned into the pJOE-His expression vector with the *pnk* open reading frame in-frame with a His tag. The gene product was overexpressed in *E. coli* and purified to near homogeneity using nickel affinity chromatography. The yield of purified recombinant RM378 PNK was 2 mg/g cells (wet weight). Aliquots from the purification procedure were collected and run on 12% SDS-PAGE and stained with Coomassie blue R-250 (27). The purification was estimated as >90% by SDS-PAGE analysis (Fig. 2). The recombinant PNK protein was studied by analytical gel filtration to evaluate the oligomeric state of the protein. Conclusive results were not obtained, because peaks from the gel filtration column ranging in size from 34 to 200 kDa all displayed 5'-kinase activity (data not shown).
Characterization of the RM378 PNK/5'-Kinase Activity—We utilized the PNK assay of Richardson (12) to characterize the RM378 5'-kinase domain compared with those of the corresponding T4 and CJW1 domains was 13 and 20% overall, respectively.

B, the phosphohydrolase domains of RM378 PNK, C. acetobutylicum putative poly(A) polymerase (Cac PolA), and D. hafniense tRNA nucleotidyltransferase/poly(A) polymerase (Dha tRNAnt). The HD motif is boxed. The sequence identity of the RM378 phosphohydrolase domain compared with those of the C. acetobutylicum HD phosphohydrolase domain and the D. hafniense HD phosphohydrolase domain was 23 and 20% overall, respectively.

The results showed relatively good stability up to 60–65 °C, but at 70 °C enzymatic activity was only observed for 1 h (Fig. 3C). The effects of divalent cations were tested by doing the standard assay in presence of Mg\(^{2+}\) or Mn\(^{2+}\) at different concentrations. The results showed that a divalent cation was essential for the 5'-kinase reaction and that the maximum activity was reached in 10 and 100 \(\mu\)M for Mn\(^{2+}\) and Mg\(^{2+}\), respectively (data not shown). Activity was similar for Mn\(^{2+}\) and Mg\(^{2+}\), but Mn\(^{2+}\) concentrations of >100 \(\mu\)M severely inhibited the reaction (data not shown). The effects of varying NaCl and KCl concentrations on the PNK activity were also examined, and the results showed that both salts caused a steady decrease in activity at concentrations of >10 mM (data not shown). The effect of spermidine was limited, with a ~10%
increase in activity at a 1 mM concentration (data not shown). On the other hand, PEG 6000 significantly increased the 5'-kinase activity (3–4-fold) in a 5–15% concentration but inhibited the 5'-kinase reaction at higher concentrations (Fig. 3D).

The effect of ADP concentration on 5'-kinase activity, dephosphorylation, and phosphate exchange was studied in an assay with 10 μM ATP and an oligomer and by titrating the ADP concentration. As seen in Fig. 4A, the 5'-kinase activity decreased as the ADP concentration was increased to complete inhibition at 1 mM ADP. If only ADP and [32P]labeled oligomer were assayed with the PNK, the level of dephosphorylation increased as the ADP concentration was increased as seen in Fig. 4A. Additionally, when labeling an already phosphorylated oligomer, the exchange reaction was 2–4% overall when compared with kinase activity on a 5'-hydroxylated oligonucleotide independent of the ADP concentration from 0 to 0.1 mM ADP. At a 1 mM ADP concentration the reaction was inhibited.

Titration curves for ATP, r(A20), and single-stranded d(A20) oligomers were done to calculate the $K_m$ constants and find the maximum velocity of the 5'-kinase reaction. The results are shown for ATP, r(A20), and d(A20) in Fig. 4, B and C, respectively. The RM378 PNK had a $K_m$ constant of 20 μM for ATP. The RM378 PNK did not discriminate between RNA and DNA oligomers in any degree, but the PNK showed somewhat better activity on single-stranded DNA when compared with RNA. The $K_m$ constants were 1.3 and 1.5 μM for r(A20) and d(A20), respectively, and the $V_{max}$ values were 160 and 220 μmol·mg$^{-1}$·h$^{-1}$ for r(A20) and d(A20), respectively. Turnover numbers were estimated as 1.9/sec and 2.6/sec for the phosphorylation of d(A20) and r(A20), respectively. The 5'-kinase activity on blunt end double-stranded DNA (micococcal nuclease digested calf thymus DNA) was similar to that of single-stranded DNA (data not shown).

To investigate the completeness of the 5'-kinase reaction, 20 μM r(A20) and d(A20) oligomers were labeled with a 10 μM [32P]-labeled ATP mixture under optimal conditions. The results, shown in Fig. 4D, demonstrate that the ATP was depleted when labeling was done with a limited amount of ATP. Also, hydroxylated oligomers (1–25 μM) were fully phosphorylated (>95%) in the reaction using 100 μM ATP (data not shown). The RM378 PNK therefore appears to be an excellent enzyme for nucleic acid labeling at an elevated temperature.

Characterization of the RM378 PNK/3' Phosphohydrolase Activity—The characterization of the 3'-phosphohydrolase activity was done using both cAMP and 3'-TMP. We hypothesized that cAMP might work better than 3'-TMP as a substrate for HD phosphohydrolase because cAMP was the preferred substrate for the HD domain of E. coli tRNA nucleotidyl transferase as described by Yakunin et al. (24). Consistent with our hypothesis, the RM378 PNK had more phosphohydrolase activity on cAMP relative to 3'-TMP (Fig. 5, C and D). We studied the pH profile and found using MOPS that activity was only seen from pH 6–7, which is out of the MOPS stable pH range. We subsequently used potassium acetate buffer from pH 4–6 in the comparison and observed a pH optimum of 6, with >50% activity in the range pH 5.5–7.0 as seen in Fig. 5A. We only observed activity on cAMP during the initial experiments and, therefore, only used this substrate in the general characterization of the 3'-phosphohydrolase activity. Activity was relatively low on cAMP at pH 6 using Mg$^{2+}$. When Mg$^{2+}$ was replaced with Mn$^{2+}$ in the 3'-phosphohydrolase assay on the cAMP substrate, the activity increased, resulting in an ~10-fold increase in activity at 1 mM concentration as seen in Fig. 5D.

Temperature optimum of the 3'-phosphohydrolase activity was 75 °C, although the enzyme showed good activity (>50%) from 65–80 °C as seen in Fig. 5B. As before, the enzyme was stable up to 60–65 °C for 2 h (data not shown). We also titrated the cAMP and 3'-TMP substrates in the 3'-phosphohydrolase reaction and found $V_{max}$ to be 13.5 μmol·mg$^{-1}$·h$^{-1}$ and 1.5 μmol·mg$^{-1}$·h$^{-1}$ for cAMP and 3'-TMP, respectively. The $K_m$ constants for cAMP and 3'-TMP were 0.7 and 0.06 mM, respectively. The substrate titrations curves for the PNK phosphohydrolase activity are shown in Fig. 5C. Turnover numbers were estimated at 0.16/sec and 0.017/sec for cAMP and 3'-TMP, respectively. The RM378 was able to dephosphorylate 80% of the 2'-3'-cAMP substrate under optimal conditions using 1 mM cAMP (data not shown).

A comparison was done between RM378 and T4 PNK using the cAMP, 3'-TMP, and d(A15)-3'PO$_4$ oligomer as substrates, all at a 0.2 mM concentration using 20 units (by the PNK unit definition by Richardson (12)) in 20-μl reaction volumes with potassium acetate buffer, pH 6, with both Mg$^{2+}$ and Mn$^{2+}$ as the divalent cation. The reactions were carried out for 2 h at 37 and 70 °C for T4 and RM378 PNK, respectively. As seen in Fig. 5D, the two enzymes exhibited different substrate preferences. Although T4 PNK showed good phosphohydrolase activity on 3'-TMP and the oligomer 3'-phosphate, the RM378 PNK revealed much better activity on the cAMP versus 3'-TMP and no detectable activity on the d(A15)-3'PO$_4$ oligomer under the experimental conditions.

DISCUSSION

The RM378 polynucleotide kinase is a bifunctional enzyme that carries out the same or very similar processes as those of the T4 polynucleotide kinase. The T4 PNK and RNA ligase will mend and ligate broken tRNA molecules after cleavage with the anticodon nuclease in pre-E. coli strains. The anticodon nuclease suicidal mechanism has the purpose of limiting the T4 phage infection and is an interesting example of altruistic behavior among bacteria (7, 8). Our studies suggest that the phage RM378 needs to counter similar RNA degradation mechanisms in R. marinus. This observation is based on the fact that the bacteriophage seems to be armed with both RNA ligase (2) and polynucleotide kinase.

The PNK and the RNA ligase are two components with a common purpose and presumably act in conjunction with each other. It is therefore not surprising to find the corresponding genes located close to each other in the genome of bacteriophage T4, separated by ~1400 bp and running in the same direction. It may seem logical to expect similar organization in the genome of bacteriophage RM378, although the overall general organization of the two phage genomes is not similar as judged from identified homologous genes (4). Intensified efforts to locate the PNK gene after our discovery of the RNA ligase gene did not identify a likely candidate open reading frame in the
immediate vicinity of the RNA ligase gene, but the PNK gene was finally located still relatively nearby, 6400 bp downstream but running in the opposite direction.

The RM378 PNK only shared its 5′-kinase domain with the PNK family and revealed no apparent homology to the 3′-phosphatase domain in that family. Similarity with the T4 5′-kinase is still low, but they share the P-loop GXXXXGK(S/T) motif, which is characteristic of many phospho-transferase families. The 5′-kinase domain is located on the C-terminal end of the RM378 PNK in contrast to the T4 PNK, suggesting that some kind of domain rearrangement had taken place, because known polynucleotide kinases from viral origin have the same domain arrangement as that of the T4 PNK. T4 PNK and homologs found previously in other bacteriophages, including coliphage RB69, phage Aeh1, and, very recently in the mycobacteriophages Omega and Cjw1 and the vibriophage KVP40, all have the kinase-phosphatase order of domains in contrast to the phosphatase-kinase order in mammalian PNKs (25). Although distinctly different from both of these groups because of its unique phosphatase domain, RM378 PNK resembles the mammalian PNKs rather than other phage PNKs in terms of domain arrangement (25). This is a very interesting observation, because eukaryotic PNKs function as repair enzymes on double-stranded DNA. The eukaryotic PNKs show relatively good homology to the viral 5′-kinase domains but less to the 3′-phosphohydrolase domains and contain relatively analogous activity, although they only phosphorylate DNA.

BLAST results revealed that the 3′-phosphohydrolase domain of RM378 PNK was related to the HD superfamily of phosphohydrolases, defined by the characteristic HD motif (23). The HD phosphohydrolase superfamily is distantly related to the phosphodiesterase family. The natural substrates of members of the phosphodiesterase family are 3′:5′-cyclic AMP and GMP, but they are inhibited by other forms of NMP.
such as AMP and 2'-3'-cyclic AMP. We did not discover any relationship between the RM378 PNK and the phosphodiesterase family. Sequence analysis and BLAST searches revealed that the RM378 PNK N-terminal domain shares similarity with the HD domains of poly(A) polymerases of bacterial origin. Poly(A) polymerases are responsible for mRNA adenylation, which is a control mechanism for RNA degradation in bacteria. Poly(A) polymerases belong to the nucleotidyl transferase superfamly, which includes CCA and the nucleotidyl transferases poly(A) polymerase and DNA polymerase β (28–30). Recently, Yakunin et al. characterized a template-independent nucleotidyltransferase, (ATP(CTP):tRNA nucleotidyltransferase that comprises two domains, an N-terminal nucleotidyltransferase domain and a C-terminal HD domain containing 2'-3'-cyclic phosphodiesterase and phosphatase activities (24). The RM378 PNK phosphohydrolase domain shows similar activity at elevated temperatures on 2'-3'-cyclic AMP, confirming the phosphohydrolase activity of the domain. The HD phosphohydrolase domains are found together with a variety of other types of domains displaying various domain architectures. Many of the proteins thus formed seem to have a function in nucleotide metabolism through a fusion of a HD domain to a nucleotidyl transferase, a helicase, or an RNA binding domain (23). The RM378 PNK is thus another variation on this theme.

A number of hypothetical genes, previously annotated as being similar to poly(A) polymerases such as those in E. coli (AAN81685), C. acetobutylicum (GenBank™ NP_347389), and, more interestingly, in Deinococcus radiodurans DRB0098 (GenBank™ NP_051631), showed limited similarity to the whole RM378 PNK protein and shared similar size and same domain orientation. These putative genes share both the 5'-kinase P-loop and the 3'-phosphohydrolase HD motif, which suggests that they are in fact polynucleotide kinases like the RM378 PNK. We therefore propose that these gene products constitute a new subfamily of polynucleotide kinases. Apart from the data presented here, evidence supporting existence of this HD-Adk PNK subfamily is already accumulating (31, 32).

First, D. radiodurans contains a RNA ligase of family 2 involved in tRNA repair, revealing a potential need for a PNK involved in the mending of RNA ends (33). Second, Liu et al. showed that the D. radiodurans DRB0098 hypothetical gene, which is in an operon with two genes, including an ATP-dependent DNA ligase-like gene, showed a recA-like expression pattern. That is, the gene was induced in the recovering phase after the irradiation of D. radiodurans cells. This may suggest that the DRB0098 gene product plays a part in nucleic acid repair in D. radiodurans (31, 32).

Although the fold of the phosphatase domains in T4 and RM378 PNKs may be quite different and the order of the kinase and phosphatase domains reversed, it is not excluded that the relative spatial location of these domains in the two enzymes may be quite similar within the monomer or within a tetramer. Interestingly, the structural organization of the T4 PNK tetramer suggests that different ends at the site of the cut in cleaved tRNA may access both kinase and phosphatase active sites simultaneously (16). However, it is still possible that the domains could be acting independently in the sense that a certain spatial relationship between the domains is not strictly required and may thus be different in the two phage proteins.

Characterization of the RM378 PNK protein showed that it was a moderately thermostable protein with an apparent temperature optimum of 70 °C and was stable up to 60–65 °C. This temperature optimum is consistent with the R. marinus natural environment as well as with R. marinus optimum growth conditions at 65 °C (1). The PNK exhibits high activity on both RNA and DNA and has similar activity on double-stranded blunt end DNA as compared with single-stranded DNA oligomers. The 5'-kinase activity was dependent upon a divalent cation, and both Mg²⁺ and Mn²⁺ worked equally well. The main component that increased the activity of the protein was PEG 6000, where a 5–15% addition resulted in increasing the activity 3–4-fold. Similar effects of addition have been reported from nucleotidyl transferase enzymes like T4 PNK and from T4...
DNA and RNA ligase (34–37). This work did not reveal whether this increase in activity was due to stabilization effects on the protein or to the steric exclusion caused by PEG and many other bio-polymers (38).

The RM378 PKN/5′-kinase activity was inhibited in the presence of ADP. When only ADP and 5′-phosphorylated oligomer were present, dephosphorylation of the oligomer was observed. These results demonstrate that the catalyzed reaction is reversible, as is the case with T4 PNK (39–41). When ATP, ADP, and a 5′-hydroxylated oligomer were incubated together in a mixture, an increasing concentration of ADP inhibited the 5′-kinase reaction. These observations suggest that the two components (ADP and ATP) are competing for the nucleotide-binding site. Interestingly, the exchange reaction was not significantly affected by the ADP concentration apart from inhibition of the kinase reaction at a high concentration. The overall exchange phosphorylation sequence data and data from the structural organization of the PNK domains and may suggest that the protein domains

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