Published in final edited form as:

Biochemistry. 2013 July 9; 52(27): . doi:10.1021/bi400412x.

Structural and biochemical analysis of the phosphate donor specificity of the polynucleotide kinase component of the bacterial Pnkp•Hen1 RNA repair system

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Abstract

Clostridium thermocellum Pnkp is the end-healing and end-sealing subunit of a bacterial RNA repair system. *Cth*Pnkp is composed of three catalytic modules: an N-terminal 5'-OH polynucleotide kinase; a central 2',3' phosphatase; and a C-terminal ligase. The crystal structure of the kinase domain bound to ATP•Mg²⁺ revealed a rich network of ionic and hydrogen-bonding contacts to the α , β , and γ phosphates. By contrast, there are no enzymic contacts to the ribose and none with the adenine base other than a π -cation interaction with Arg116. Here we report that the enzyme uses ATP, GTP, CTP, UTP or dATP as a phosphate donor for the 5'-OH kinase reaction. The enzyme also catalyzes the reverse reaction, in which a polynucleotide 5'-PO₄ group is transferred to ADP, GDP, CDP, UDP or dADP to form the corresponding NTP. We report new crystal structures of the kinase in complexes with GTP, CTP, UTP and dATP in which the respective nucleobases are stacked on Arg116 but make no other enzymic contacts. Mutating Arg116 to alanine elicits a 10-fold increase in K_m for ATP, but has little effect on k_{cat} . These findings illuminate the basis for non-specific donor nucleotide utilization by a P-loop phosphotransferase.

RNA repair is a mechanism to rectify RNA breaks incurred during RNA processing and cellular stress. RNA repair enzymes that seal 2',3'-cyclic phosphate and 5'-OH ends are present in diverse taxa in all phylogenetic domains of life.^{1,2} The Pnkp•Hen1 RNA repair system is encoded in an operon present in diverse bacteria from many phyla.³⁻⁵ Pnkp is the end-healing and end-sealing component and comprises three catalytic domains: N-terminal kinase, central phosphatase, and C-terminal ligase.³ The kinase catalyzes phosphoryl transfer to the 5'-OH RNA end. The phosphatase domain releases P_i from 2'-PO₄, 3'-PO₄ or 2',3'-cyclic-PO₄ ribonucleotides.⁶⁻⁹ The ligase domain has covalent adenylyltransferase activity and, in a 1:1 complex with the N-terminal half of the Hen1 protein, is an RNA ligase.^{3,4,10,11} The C-terminal half of Hen1 is an autonomous Mn²⁺-dependent 3'-terminal ribose 2'-O-methyltransferase that places a 2'-OCH₃ mark at the RNA repair junction prior to ligation and thereby protects the junction from recurrent damage.^{4,5,12,13}

Crystal structures have been determined for each of the catalytic domains of *Clostridium thermocellum* Pnkp and Hen1.^{10-12,14,15} The kinase, phosphatase, ligase, and 2'-O-methyltransferase modules are members of the P-loop phosphotransferase, binuclear metallophosphoesterase, covalent nucleotidyltransferase, and AdoMet-dependent methyltransferase enzyme superfamilies, respectively. In each case, the core superfamily

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Accession numbers. The coordinates for the refined models of kinase•GTP•Mg²⁺, kinase•CTP•Mg²⁺, kinase•UTP•Mg²⁺, and kinase•dATP•Mg²⁺ have been deposited in the RCSB protein structure database (PDB ID codes 4JSY, 4JT2, 4JST, and 4JT4).

fold is embellished by distinctive structural elements that either mediate protein-protein interactions or dictate substrate/cofactor specificity.

The N-terminal 170-amino acid segment of *Clostridium thermocellum* Pnkp is an autonomous polynucleotide kinase module. It phosphorylates 5'-OH single-stranded RNA or DNA substrates using ATP as the phosphate donor. We recently reported crystal structures of the kinase domain bound to ATP•Mg²⁺ and ADP•Mg²⁺, reflective of substrate and product complexes respectively.¹⁴ The ATP donor is bound within a crescent-shaped groove formed by the P-loop (¹⁵GSSGSGKST²³) and an overlying lid composed of helices α6 and α7 and the connecting ¹²⁰RTDRQVE¹²⁶ peptide (Fig. 1A). The ATP α and β phosphates are engaged by a network of hydrogen bonds from Thr23 and the P-loop main-chain amides; the γ phosphate is anchored by the lid residues Arg120 and Arg123 and the P-loop Ser17. The P-loop lysine (Lys21) and the catalytic Mg²⁺ bridge the ATP β and γ phosphates. The P-loop serine (Ser22) is the sole enzymic constituent of the octahedral metal coordination complex, which also includes nonbridging β and γ phosphate oxygens of ATP and three waters (Fig. 1A). A secondary shell of atomic contacts to the metal-bound waters in the metal complex includes the Asp38 and Asp78 side chains. Structure-guided mutational analysis underscored the essential contributions of Lys21 and Ser22 in the ATP donor site and Asp38 and Arg41 in the putative phosphoacceptor site. Our studies suggested a catalytic mechanism whereby Asp38 (as general base) activates the polynucleotide 5'-OH for its nucleophilic attack on the γ phosphorus and Lys21 and Mg²⁺ stabilize the transition-state.¹⁴

It is notable that there are no protein contacts to the ATP ribose sugar or to the nitrogen atoms of the adenine nucleobase. The sole protein interaction with adenine is via a π-cation stack on the Arg116 side chain of the lid (Fig. 1A). This situation contrasts with many other P-loop enzymes, especially helicases, that make hydrogen bonds to the adenine N6 and N7 atoms via a conserved glutamine side chain that confers specificity for ATP or dATP as the phosphohydrolase substrate.^{16,17} Prompted by the *Cth*Pnkp kinase•ATP structure, we investigate the donor specificity of this exemplary bacterial RNA repair enzyme and probe the contributions of the Arg116 side chain. We report that the kinase is nonspecific with respect to the nucleotide substrate, being adept at phosphoryl transfer, in the forward and reverse direction, to guanosine, cytidine, uridine, and deoxyadenosine nucleotides. We also determine new atomic structures of the kinase in complexes with Mg²⁺ and GTP, CTP, UTP and dATP that reveal, in every case, an *anti* nucleoside conformation and an Arg116•nucleobase π-cation stack. We compare and contrast the nucleotide binding modes of polynucleotide kinases from diverse taxa.

Experimental Procedures

Kinase purification and mutagenesis

The pET28b-Smt3*Cth*Pnkp-(1-170) expression plasmid encoding the kinase domain was described previously.¹⁴ Missense mutations R116A, R120A, R123A, and R120A-R123A were introduced into the expression vector by the two-stage PCR-based overlap extension method. The Pnkp inserts were sequenced to confirm the presence of the desired mutation and the absence of unwanted coding changes. The plasmids were transformed into *E. coli* BL21(DE3). Recombinant protein production was induced by adjusting exponentially growing cultures (1 liter) to 0.3 mM IPTG and incubating them at 17°C for 15 h with continuous shaking. Cells were harvested by centrifugation and stored at -80°C. All subsequent procedures were performed at 4°C. Thawed cell pellets were resuspended in 50 ml of lysis buffer (50 mM Tris HCl, pH 7.5, 1.2 M NaCl, 25 mM imidazole, 10% glycerol). Lysozyme and Triton X-100 were added to final concentrations of 1 mg/ml and 0.2% respectively; one protease inhibitor tablet (Roche) was then added. The lysates were sonicated to reduce viscosity and insoluble material was removed by centrifugation for 1 h

at 14,400 rpm in a Sorvall SLA-1500 rotor. The soluble extracts were applied to 4 ml columns of Ni-nitriloacetic acid agarose (Qiagen) that had been equilibrated in buffer A (50 mM Tris HCl, pH 7.5, 200 mM NaCl, 10% glycerol). The columns were washed with 12 ml buffer A, and 12 ml of 100 mM imidazole in buffer A. The His-tagged *CthPnkp* proteins were recovered by serial step-wise elution with 200 mM imidazole and 300 mM imidazole in buffer A. The elution profiles were monitored by SDS-PAGE. Peak *CthPnkp*-containing fractions were pooled and supplemented with Smt3-specific protease Ulp1 (protein:Ulp1 in a molar ratio 50:1) and the mixture was dialyzed overnight against 4 liters of buffer B (50 mM Tris HCl, pH 7.5, 100 mM NaCl, 10% glycerol). The tag-free *CthPnkp* protein was separated from the cleaved His₁₀Smt3 tag by a second round of Ni-agarose chromatography, during which *CthPnkp* was recovered in the flow-through fraction. The Ni-agarose *CthPnkp*-(1-170) preparations were used for the assays of kinase specific activity reported in Fig. 6. For further purification of wild-type *CthPnkp*-(1-170) and the R116A mutant, the Ni-agarose preparations were adjusted to 10 mM EDTA, concentrated by centrifugal ultrafiltration, and gel-filtered through a column of Superdex-200 equilibrated in 50 mM Tris HCl pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM DTT, 1mM EDTA. The enzyme preparations were stored at -80°C. Protein concentrations were determined by using the BioRad dye reagent with bovine serum albumin as the standard.

Assay of the NTP donor specificity of the kinase reaction

Reaction mixtures (10 μ l) containing 50 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, 5 mM DTT, 100 μ M NTPs as specified, 0.05 μ M of a 10-mer H_0RNAp phosphoacceptor (5'- $\text{H}_0\text{AUCACGCUUpCp}$) labeled with ³²P at the penultimate phosphate (prepared as described²⁰), and 0.25 μ M kinase were incubated at 45°C for 30 min. The reactions were initiated by adding enzyme to reaction mixtures pre-warmed to 45°C and quenched by adding an equal volume of 90% formamide, 50 mM EDTA, 0.01% bromophenol blue/xylene cyanol. The mixtures were analyzed by electrophoresis (at 55 W constant power) through a 40-cm 20% polyacrylamide gel containing 8 M urea in 45 mM Tris borate, 1.2 mM EDTA. The ³²P-labeled RNAs were visualized by autoradiography, which showed that the H_0RNAp substrate was separated from the faster migrating pRNAp product (Fig. 1B). The extent of RNA phosphorylation was determined by scanning the gel with a FujiFilm BAS-2500 imager and quantifying pRNAp/(pRNAp + H_0RNAp).

Assay of the NDP acceptor specificity of the reverse kinase reaction

Reaction mixtures (10 μ l) containing 50 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, 5 mM DTT, 0.1 μ M of a 5'-³²P labeled 15-mer DNA oligonucleotide (pCCGACCAACGAAGGT), 100 μ M ADP, UDP, GDP, CDP or dADP as specified, and 0.25 μ M kinase were incubated at 45°C for 30 min. The reactions were quenched by adding 2 μ l of 5 M formic acid. An aliquot (5 μ l) of each reaction mixture was applied to a polyethyleneimine-cellulose TLC plate and then overlaid with 10 nmol of the corresponding cold NTP standards. Ascending TLC was performed with 0.45 M (NH₄)₂SO₄. The radiolabeled products were visualized by autoradiography (see Fig. 2A). Reverse kinase activity was evinced by label transfer from the ³²P-DNA oligonucleotide (pDNA, located at and trailing from the chromatographic origin) to the added NDP acceptor to form the respective ³²P-labeled NTPs that co-migrated with the cold NTP standards that were located by UV auto fluorescence. The extents of label transfer to the NDPs were determined by scanning the plate with a FujiFilm BAS-2500 imager and quantifying NTP/(NTP + pDNA).

Kinase assay by label transfer from ATP to a 5'-OH oligonucleotide acceptor

Reaction mixtures (10 μ l) containing 50 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, 5 mM DTT, 100 μ M [γ -³²P]ATP, 60 pmol of a 15-mer 5'-OH DNA oligonucleotide phosphoacceptor

($\text{H}_0\text{CCGACCAACGAAGGT}$), and wild-type or mutant kinase as specified were incubated for 30 min at 45°C. The reactions were quenched by adding an equal volume of 90% formamide, 50 mM EDTA, 0.01% bromophenol blue/xylene cyanol, and the mixtures were analyzed by electrophoresis (at 7 W constant power) through a 15-cm 20% polyacrylamide gel containing 8 M urea in 45 mM Tris borate, 1.2 mM EDTA. The ^{32}P -labeled DNAs were visualized and quantified by scanning the gel with a Fuji Film BAS-2500 imager.

Crystallization, diffraction data collection, and structure determination

The SeMet-kinase preparation of *CthPnkp*-(1-170)-V44M-L137M described previously¹⁴ was concentrated to 7.8 mg/ml and adjusted to 2 mM GTP, CTP, UTP or dATP plus 10 mM MgCl_2 and incubated for 10 min before aliquots of the protein solution (2 μl) were mixed on a cover slip with an equal volume of precipitant solution containing 100 mM sodium citrate, pH 5.6, 100 mM MgCl_2 , 16-18% (v/v) PEG-3350. Crystals were grown at 22°C by hanging drop vapor diffusion against a reservoir of the same precipitant solution. Single crystals were transferred to a solution containing 100 mM sodium citrate, pH 5.6, 2 mM NTP, 100 mM MgCl_2 , 16-18% PEG-3350, and 15% glycerol prior to freezing the crystals in liquid nitrogen. Diffraction data for each *CthPnkp*•NTP• Mg^{2+} complex were collected at NSLS beamline X25 equipped with a Pilatus 6M detector in 370 continuous increments of 0.5° each. The data were integrated and reduced with MOSFLM and SCALA. Diffraction statistics are compiled in Table 1. The kinase•NTP• Mg^{2+} crystals were in space group $\text{P}2_12_12_1$, had two protomers per asymmetric unit, and were isomorphous with the SeMet kinase•ATP• Mg^{2+} crystals reported previously.¹⁴ Structures of kinase•GTP• Mg^{2+} , kinase•UTP• Mg^{2+} , and kinase•dATP• Mg^{2+} complexes at 2.02 to 2.14 Å resolution and kinase•CTP• Mg^{2+} at 2.5 Å resolution were determined by refinement of the diffraction data against the SeMet kinase•ATP• Mg^{2+} structure (PDB ID 4GP7) from which ATP• Mg^{2+} and waters had been removed. The NTPs and Mg^{2+} were then modeled into the $\text{F}_o - \text{F}_c$ maps of the respective active sites. The models were iteratively rebuilt by hand in COOT¹⁸ and refined in PHENIX.¹⁹ NCS restraints and TLS B-factor refinements in PHENIX were carried out only for the kinase•CTP complex. The final models had excellent geometry, no Ramachandran outliers, and no large $\text{F}_o - \text{F}_c$ difference Fourier peaks (Table 1).

Results

NTP donor specificity of *CthPnkp* kinase

The kinase activity of the Pnkp protein was assayed by the transfer of phosphate from cold NTPs to a 10-mer 5'-OH RNA acceptor with a 3'- PO_4 terminus and a single ^{32}P -radiolabel between the 3'-terminal and penultimate nucleosides (Fig. 1B). In the presence of kinase and ATP, 98% of the input H_0RNAp strand was converted to a pRNAp product. When ATP was omitted, only 2.7% of the RNA substrate was phosphorylated, reflecting the presence of a trace level of NTP-bound kinase in the enzyme preparation. From the extent of ATP-independent product formation, we calculated that 0.26% of the enzyme preparation is preloaded with an NTP donor. The salient findings were that replacing ATP with either GTP, CTP, UTP or dATP resulted in 98% phosphorylation of the acceptor RNA strand (Fig. 1B). We conclude that the *CthPnkp* kinase does not require an adenine nucleobase or ribose sugar in the phosphate donor substrate. To query whether a nucleoside moiety was even necessary, we tested kinase activity in a reaction mixture containing 100 μM inorganic triphosphate in lieu of an NTP. We found that triphosphate did not support the kinase reaction (not shown). A mixing experiment showed that 100 μM triphosphate did not inhibit kinase activity in the presence of 100 μM ATP (not shown). These results suggest that a nucleoside moiety is needed for occupancy of the donor site on the kinase.

We gauged the kinetics of the RNA phosphorylation reaction under conditions of enzyme excess, whereby reactions containing 50 nM HORNAP and 100 μM ATP were initiated by adding 0.25, 0.5, 1.0 or 2.0 μM kinase. The kinetic profiles are shown in Fig. 1C, with each datum being the average of three independent experiments $\pm$ SEM. The data were fit by nonlinear regression to a one-phase association in Prism; the observed rate constants increased with enzyme concentration (k_{obs} values of $0.23 \pm 0.018 \text{ min}^{-1}$, $0.53 \pm 0.006 \text{ min}^{-1}$, $1.17 \pm 0.021 \text{ min}^{-1}$, and $2.17 \pm 0.082 \text{ min}^{-1}$ at 0.25, 0.5, 1.0 and 2.0 μM kinase, respectively) signifying that kinase• HORNAP association, rather than reaction chemistry, was rate-limiting under these conditions (i.e, in which the concentration of the 5'-OH acceptor strand was low).

NDP acceptor specificity of the reverse kinase reaction

The polynucleotide kinase enzymes of bacteriophages T4, KVP40, Omega and Cjw1 and of the archaeon *Pyrococcus horikoshii* can transfer a polynucleotide 5'- PO_4 to ADP to form ATP and a 5'-OH polynucleotide.²¹⁻²³ To assess if *CthPnk* can also catalyze reversal of the kinase reaction, we incubated the enzyme with 5' ^{32}P -labeled 15-mer DNA and Mg^{2+} in the presence of 100 μM ADP. This resulted in the formation of a ^{32}P -ATP product that was resolved from the oligonucleotide by PEI-cellulose TLC (Fig. 2A). 88% of the input polynucleotide 5'- PO_4 was converted to ATP; omission of the ADP acceptor resulted in no release of label from the ^{32}P -DNA. The finding that 100 μM GDP, CDP, UDP and dADP also served as acceptors for the reverse kinase reaction, resulting in the formation of ^{32}P -labeled GTP, CTP, UTP and dATP, respectively (Fig. 2A), fortified the conclusions from the experiments in Fig. 1B that the bacterial polynucleotide kinase is neither nucleobase nor sugar specific with respect to its nucleotide substrate. No label was released in the presence of 100 μM inorganic pyrophosphate (not shown).

Phosphoryl transfer from ^{32}P -DNA to NDPs to form ^{32}P -NTPs displayed a hyperbolic dependence on NDP concentration, with apparent K_m values of $18 \pm 1.5 \mu\text{M}$ ADP, $18 \pm 2.2 \mu\text{M}$ GDP, $32 \pm 5.0 \mu\text{M}$ CDP, $44 \pm 6.8 \mu\text{M}$ dATP, and $64 \pm 6.2 \mu\text{M}$ UDP (Fig. 2B). The kinetics of the reverse kinase reaction were analyzed under conditions of enzyme excess (0.1 μM pDNA, 100 μM ADP and 0.25, 0.5, 1.0 or 2.0 μM kinase). The kinetic profiles are shown in Fig. 2C. The data were fit by nonlinear regression to a one-phase association in Prism; the k_{obs} values increased with kinase concentration ($0.17 \pm 0.004 \text{ min}^{-1}$, $0.53 \pm 0.018 \text{ min}^{-1}$, $0.83 \pm 0.043 \text{ min}^{-1}$, and $1.57 \pm 0.05 \text{ min}^{-1}$ at 0.25, 0.5, 1.0 and 2.0 μM kinase, respectively) signifying that kinase•pDNA association, rather than reaction chemistry, was rate-limiting.

Dependence of kinase activity on the concentration of the 5'-OH polynucleotide

The above findings that the rate constants for the forward and reverse kinase reactions performed at 0.05 or 0.1 μM polynucleotide were proportional to kinase concentration even at enzyme excess prompted us to assess the affinity of the kinase for the 15-mer 5'-OH DNA phosphoacceptor under conditions of substrate excess. The extent of DNA phosphorylation during a 2-min reaction with 100 μM [$\gamma\text{-}^{32}\text{P}$]ATP is plotted as a function of HODNA concentration in Fig. 3. Nonlinear regression fitting of the data to the Michaelis-Menten equation yielded a K_m of $2.8 \pm 0.4 \mu\text{M}$ for the HODNA phosphoacceptor and a k_{cat} of $4.3 \pm 0.2 \text{ min}^{-1}$.

Structures of the kinase in complexes with GTP, CTP, UTP and dATP

To gain further insights to phosphate donor selection, we crystallized the kinase in the presence of 10 mM MgCl_2 plus 2 mM GTP, CTP, UTP or dATP. (By contrast, we were unable to grow crystals of the kinase in the presence of 10 mM MgCl_2 plus 2 mM inorganic triphosphate, which is not an effective phosphate donor for the kinase reaction.) The

refinement statistics and model contents of the kinase•GTP•Mg²⁺, kinase•UTP•Mg²⁺, kinase•dATP•Mg²⁺ and kinase•CTP•Mg²⁺ structures at 2.14, 2.03, 2.02, and 2.49 Å resolution, respectively, are provided in Table 1.

Stereo views of the 2F_o-F_c maps for the NTP•Mg²⁺ ligands in the refined model are shown in Fig. 4. They reveal distinctive electron density over the nucleobase that discriminates adenine from guanine (via the exocyclic N2 atom of guanine) and the larger purines from the two pyrimidines. The guanosine and adenosine nucleosides are in an *anti* conformation (Fig. 4, left panels), as are the uridine and cytidine nucleosides, as gauged by the density over their respective pyrimidine O2' atoms (Fig. 4, right panels). The ribose O2' atoms in the GTP, UTP and CTP ligands are evident in the electron density maps; as expected, there is no density for an O2' atom in the dATP ligand (Fig. 4). Each nucleobase makes a π -cation stack on the Arg116 side chain.

The tertiary structure of the crystallized kinase was virtually identical when bound to ATP, GTP, UTP, CTP, or dATP, with rmsd values of 0.13 to 0.32 Å over 171 C α atoms. The NTP donor sites of all five kinase•NTP•Mg²⁺ structures are superimposed in a stereo view in Fig. 5. The triphosphate moieties of the NTPs are virtually identical in position and conformation. The Thr23 hydroxyl donates a hydrogen bond to the NTP α phosphates. The Lys21 side chain makes a bifurcated ionic interaction with the NTP β and γ phosphates and the NTP γ phosphates are engaged by Arg120. Ser22 coordinates the Mg²⁺ ion. There is virtually no positional variation in the Lys21, Ser22, Thr23 and Arg120 side chains in the kinase•NTP structures (Fig. 5). The Arg123 side chain near the NTP γ phosphates does vary with respect to the position of its terminal guanidinium moiety. The nucleobases vary slightly (e.g., guanine is shifted counterclockwise relative to adenine in the view in Fig. 5) but they are all coplanar and stacked over the Arg116 side chain. There are no additional enzymic contacts to the nucleoside in any of the kinase•NTP structures.

Mutational analysis

Previously, we initiated a structure-guided mutational analysis of the ATP phosphate donor site of the kinase by testing the effects of alanine substitutions in the context of the *CthPnkp*-(1-425) protein, comprising the kinase and phosphatase catalytic domains.¹⁴ The wild-type and mutant preparations were assayed for kinase activity in reaction mixtures containing an amount of wild-type protein that sufficed for near-quantitative radiolabeling of the input 5'-OH DNA oligonucleotide acceptor in the presence of 100 μ M [γ -³²P]ATP. We chose to conduct the screening assays in this fashion in order to highlight the most severe mutational effects on catalysis. We found that the kinase activity was ablated by subtraction of Lys21, whereas elimination of the Ser22 hydroxyl group reduced activity by an order of magnitude. Kinase activity was abolished by alanine substitution for Asp38, consistent with its postulated role in orienting and activating the polynucleotide 5'-OH nucleophile.¹⁴ By contrast, replacing lid residue Arg116 with alanine had little impact on the extent of DNA labeling. Mutating lid residue Arg120 to alanine also had little impact.

The relatively benign effects of subtracting the lid arginine residues was surprising to us and suggested that the contributions of their individual contacts with the ATP phosphate donor might either be functionally redundant or masked by the assay conditions employed (saturating enzyme, saturating ATP). For example, the contact of Arg120 with the ATP γ phosphate might be redundant with the contacts to the γ phosphate made by Arg123 (Fig. 1A). To address this issue we made single R120A and R123A changes and a double R120A-R123A mutation in the context of *CthPnkp*-(1-170). We assayed the extent of label transfer from 100 μ M [γ -³²P]ATP to 6 μ M 15-mer HO₂DNA acceptor as a function of input protein (Fig. 6). Specific activities of the wild-type and mutant kinases were derived by linear regression curve fitting in Prism and then normalized to the wild-type activity (defined as

100%). The R120A and R123A mutants were 42% and 77% as active as wild-type, respectively. The R120A-R123A double-mutant was 13% as active as wild-type (Fig. 6), suggesting that the lid Arg120 and Arg123 side chains do indeed make functionally redundant contacts to the γ phosphate oxygens.

It is noteworthy that the effects of singly subtracting the lid arginines that contact the γ phosphate on the *CthPnkp* kinase activity are much milder than those observed for other homologous P-loop phosphotransferases. A single R126A change in the lid of bacteriophage T4 Pnkp (corresponding to the R120A mutation in *CthPnkp*) reduced kinase specific activity to 3% of the wild-type value.²⁴ Changing the equivalent Arg132 residue of chicken muscle adenylate kinase (a prototypal nucleoside 5' monophosphate kinase²⁵) to methionine reduced k_{cat} by a factor of 8,000 without affecting K_m for the ATP phosphate donor.^{26,27} Replacing chicken adenylate kinase Arg138 (corresponding to Arg123 in *CthPnkp*) with methionine was even more deleterious, eliciting a 13,000-fold decrement in k_{cat} and increasing K_m for the AMP phosphate acceptor by 20-fold.^{26,28} By contrast, the R123A change in *CthPnkp* has little impact on kinase specificity activity (Fig. 6). The disparate effects of the loss of this structurally homologous distal lid arginine might reflect the different chemistries of the respective kinase reactions, whereby, in adenylate kinase, the distal lid arginine contacts the AMP phosphate nucleophile that attacks the ATP γ phosphorus.^{29,30} By contrast, the 5'-OH nucleophile in the polynucleotide kinase reaction is coordinated and activated by an essential aspartate: Asp38 in *CthPnkp* and Asp35 in T4 Pnkp.^{14,31-33}

The *CthPnkp*-(1-170) Arg116A mutant was 83% as active as the wild-type kinase (Fig. 6). Given the role of the Arg116 guanidinium as a stacking platform for the NTP nucleobase, we queried whether its loss had any impact on the K_m for ATP. Wild-type and R116A kinase preparations purified to apparent homogeneity through a gel-filtration step were assayed for label transfer from [$\gamma^{32}\text{P}$]ATP to the a 15-mer HO DNA acceptor under conditions of substrate excess (0.25 μM kinase; 10 μM HO DNA). The extents of DNA phosphorylation during a 2-min reaction are plotted as a function of [$\gamma^{32}\text{P}$]ATP concentration in Fig. 7. Nonlinear regression fitting of the data to the Michaelis-Menten equation yielded steady-state parameters for the wild-type kinase ($K_m = 4.0 \pm 0.7 \mu\text{M}$ ATP; $k_{\text{cat}} = 4.6 \pm 0.2 \text{ min}^{-1}$) and R116A mutant ($K_m = 40 \pm 6 \mu\text{M}$ ATP; $k_{\text{cat}} = 4.4 \pm 0.3 \text{ min}^{-1}$). These results signify that the π -cation interaction of Arg116 with the adenine nucleobase enhances affinity for the ATP phosphate donor by 10-fold, but has little effect on the rate of phosphoryl transfer chemistry.

Discussion

The present study provides new insights to the mechanism by which *CthPnkp* heals 5'-OH ends, especially the contributions of the arginine residues that emanate from the lid that overlies the NTP phosphate donor site. Prompted by the paucity of enzymic contacts to the nucleoside of ATP in our initial crystal structure of the kinase, we interrogated the NTP specificity of *CthPnkp*. We found that the kinase utilizes any of the NTPs tested as donor substrates for phosphorylation of a polynucleotide 5'-OH end in the forward kinase reaction. Moreover, the kinase uses any of the NDPs tested as phosphate acceptors in the reverse kinase reaction, in which a polynucleotide 5'- PO_4 is transferred to an NDP to generate the cognate NTP. The apparent K_m values for rNDP acceptors in the reverse reaction varied over a ~ 3 -fold range, with GDP and ADP being superior to CDP and UDP. (This could reflect the size of the nucleobase π systems available for interaction with Arg116). Whereas the *CthPnkp* kinase is not stringently specific for the nucleobase (or the ribose sugar), the enzyme requires a nucleoside, insofar as tripolyphosphate and pyrophosphate do not serve as donor and acceptor in the forward and reverse kinase reactions, respectively.

We focused on the role of Arg116, the sole contact between the kinase and the nucleobase of the NTP phosphate donor. By solving new crystal structures of the kinase in complexes with GTP, dATP, CTP and UTP, we showed that the purine and pyrimidine nucleobases are in *anti* conformation and are coplanar in a π -cation stack over the Arg116 guanidinium. Subtracting this interaction by replacing Arg116 with alanine caused a 10-fold increase in K_m for ATP, but had little effect on k_{cat} . These findings illuminate the basis for non-specific donor nucleotide utilization by a P-loop phosphotransferase.

It is worthwhile to compare the phosphate donor specificity of *Cth*Pnkp kinase to other polynucleotide kinases and P-loop phosphotransferases/phosphohydrolases. The kinase domains of bacteriophage T4 Pnkp and *Cth*Pnkp are structurally homologous. The crystal structure³⁴ of T4 kinase bound to ADP revealed that the adenine base makes a π -cation stack over Arg122, which is the counterpart of Arg116 in *Cth*Pnkp. This is the sole contact between T4 Pnkp and the adenosine nucleoside, presumably explaining why T4 kinase catalyzes reversible transfer of the γ phosphate from any NTP to a polynucleotide 5'-OH end. The effects of subtracting Arg122 on the affinity of T4 Pnkp for the nucleotide substrates have not been reported.

Plant tRNA ligase AtRNL has a central kinase domain that uses any NTP or dATP as a phosphate donor for converting 5'-OH RNA ends to 5'-PO₄ ends.³⁵ Although there is no structure available for AtRNL, its kinase module belongs to the P-loop superfamily and it includes a putative counterpart of the lid: ⁸⁰⁰RVLQR⁸⁰⁴, corresponding to ¹¹⁶RNKNR¹²⁰ in *Cth*Pnkp.³⁶ By contrast, yeast tRNA ligase Trl1 strongly prefers GTP over ATP as the phosphate donor for its 5'-OH kinase reaction.³⁷ Given that Trl1 also has a lid counterpart (⁵⁰⁷RNSKR⁵¹¹) that includes the nucleobase-stacking arginine, we presume that the Trl1 kinase (unlike the *Cth* and T4 kinases) does make additional contacts with the guanine base that account for this specificity.

Mammalian Pnkp is specific for 5'-OH DNA as the phosphoacceptor in the kinase reaction. It also catalyzes the reverse kinase reaction with a 5'-PO₄ DNA donor and various rNDPs or dNDPs (but not PP_i) as phosphate acceptors.³⁸ The crystal structure of mammalian Pnkp with ADP•Mn²⁺ in the kinase active site is available.³⁹ When superimposed on the *Cth*Pnkp kinase•ADP•Mg²⁺ structure (pdb 4GP6), the ADP•metal ligands are virtually identical in position and conformation and in their key contacts with the P-loop lysine, serine, and threonine side chains. Yet, mammalian kinase does not have an equivalent of the *Cth*Pnkp kinase Arg116 residue (it being asparagine instead) and there is no π -cation or π - π interaction of the enzyme with the adenine nucleobase. Rather, the mammalian kinase forms hydrogen bonds from a main-chain carbonyl to adenine N6 and from a main-chain NH to adenine N1.³⁹ Conversely, there are no counterparts of the mammalian kinase adenine-binding elements in *Cth*Pnkp. The mammalian Pnkp structure suggests that mammalian kinase ought to be selective for adenine nucleotides, which runs counter to the earlier report that the enzyme uses multiple NDPs in the reverse reaction. Finer biochemical analysis of the NTP donor specificity of mammalian DNA kinase, coupled with attempts to co-crystallize mammalian Pnkp with alternative NTPs or NDPs in the kinase active site, could clarify the issue.

Archaeal and human Clp1 are homologous 5'-OH RNA kinases implicated in RNA repair.^{23,40,41} The archaeal Clp1 kinase reaction is reversible and any rNDP suffices as the phosphoacceptor substrate.²³ By contrast, human Clp1 favors ATP over GTP as the phosphate donor.⁴⁰ The yeast Clp1 homolog binds ATP•Mg²⁺ at its P-loop, yet yeast Clp1 is devoid of kinase or ATPase activity.⁴² Based on the crystal structure of a yeast Clp1•ATP•Mg²⁺ complex⁴², one predicts that archaeal and human Clp1 kinase lack a counterpart of the lid module found in *Cth*Pnkp, T4 Pnkp, and mammalian Pnkp. Whereas

CthPnkp and T4 Pnkp have an open nucleoside site on the enzyme surface, yeast Clp1 envelops the nucleobase and ribose within a pocket formed by an accessory domain not found in the bacterial and phage kinases. Gauging whether the same nucleoside binding mode applies to the kinase-active human and archaeal Clp1 proteins must await their structure determination.

Adenylate kinases have a lid domain with a conserved RxxxR motif that engages the ATP substrate. The proximal arginine in the lid motif (Arg119 in *E. coli* adenylate kinase and Arg128 in yeast adenylate kinase, corresponding to Arg116 in *CthPnkp*) makes a π -cation stack on the adenine nucleobase of the phosphate donor.^{29,30} Preference for ATP as the donor in the adenylate kinase reaction is thought to be conferred by a single hydrogen bond from the adenine N7 atom to a main-chain carbonyl oxygen of the enzyme.^{25,29,30}

The P-loop ATPase *E. coli* UvrD exemplifies the superfamily I clade of DNA helicases. (UvrD is a phosphohydrolase rather than a phosphotransferase.) Crystal structures of UvrD with adenosine nucleotides in the active site highlight a combination of nucleobase-unspecific and adenine-specific enzymic contacts.¹⁶ To wit, the adenine base is sandwiched within a cation- π - π stack with an arginine guanidinium on one side of the adenine and a tyrosine on the other. A glutamine side chain makes two hydrogen bonds to the adenine ring nitrogens: one from Gln-N ϵ to adenine N7 and another from Gln-O ϵ to adenine N6.¹⁶ This glutamine is conserved in many ATPase/dATPase helicases; in the case of mycobacterial UvrD1, the glutamine acts as a substrate filter, such that when it is replaced by alanine, UvrD1 gains a new activity as a UTPase/UTPase.¹⁷

In conclusion, diverse P-loop phosphotransferases with different NTP donor and 5'-OH acceptor specificities have evolved to contend with the 5' end-healing problem during RNA and DNA repair. Whereas some polynucleotide kinases are fastidious with respect to the nucleobase or the pentose sugar of the NTP substrate, others are not. The arginine π -cation interaction with all nucleobases seen in our suite of *CthPnkp*•NTP•Mg²⁺ structures is a seemingly effective strategy to enhance NTP affinity without imposing stringent NTP selectivity.

Acknowledgments

We thank NSLS staff members Annie Heroux and Neil Whalen for their assistance with data collection. This research was supported by NIH grant GM42498. SS is an American Cancer Society Research Professor.

References

1. Nandakumar J, Schwer B, Schaffrath R, Shuman S. RNA repair: an antidote to cytotoxic eukaryal RNA damage. *Mol Cell*. 2008; 31:278–286. [PubMed: 18657509]
2. Popow J, Schleiffer A, Martinez J. Diversity and roles of (t)RNA ligases. *Cell Mol Life Sci*. 2012; 69:2657–2670. [PubMed: 22426497]
3. Martins A, Shuman S. An end-healing enzyme from *Clostridium thermocellum* with 5' kinase, 2',3'' phosphatase, and adenylyltransferase activities. *RNA*. 2005; 11:1271–1280. [PubMed: 15987807]
4. Chan CM, Zhou C, Huang RH. Reconstituting bacterial RNA repair and modification in vitro. *Science*. 2009; 326:247. [PubMed: 19815768]
5. Jain R, Shuman S. Bacterial Hen1 is a 3' terminal RNA ribose 2'O-methyltransferase component of a bacterial RNA repair cassette. *RNA*. 2010; 16:316–323. [PubMed: 20007328]
6. Keppetipola N, Shuman S. Mechanism of the phosphatase component of *Clostridium thermocellum* polynucleotide kinase-phosphatase. *RNA*. 2006; 12:73–82. [PubMed: 16301605]
7. Keppetipola N, Shuman S. Distinct enzymic functional groups are required for the phosphomonoesterase and phosphodiesterase activities of *Clostridium thermocellum* polynucleotide kinase/phosphatase. *J Biol Chem*. 2006; 281:19251–19259. [PubMed: 16675457]

8. Keppetipola N, Shuman S. Characterization of the 2',3' cyclic phosphodiesterase activities of *Clostridium thermocellum* polynucleotide kinase-phosphatase and bacteriophage λ phosphatase. *Nucleic Acids Res.* 2007; 35:7721–7732. [PubMed: 17986465]
9. Keppetipola N, Nandakumar J, Shuman S. Reprogramming the tRNA splicing activity of a bacterial RNA repair enzyme. *Nucleic Acids Res.* 2007; 35:3624–3630. [PubMed: 17488852]
10. Smith P, Wang LK, Nair PA, Shuman S. The adenylyltransferase domain of bacterial Pnkp defines a unique RNA ligase family. *Proc Natl Acad Sci USA.* 2012; 109:2296–2301. [PubMed: 22308407]
11. Wang P, Chan CM, Christensen D, Zhang C, Selvadurai K, Huang RH. Molecular basis of bacterial protein Hen1 activating the ligase activity of bacterial protein Pnkp for RNA repair. *Proc Natl Acad Sci USA.* 2012; 109:13248–13253. [PubMed: 22847431]
12. Chan CM, Zhou C, Brunzelle JS, Huang RH. Structural and biochemical insights into 2'-O-methylation at the 3'-terminal nucleotide of RNA by Hen1. *Proc Natl Acad Sci USA.* 2009; 106:17699–17704. [PubMed: 19822745]
13. Jain R, Shuman S. Active site mapping and substrate specificity of bacterial Hen1, a manganese-dependent 3' terminal RNA ribose 2'-O-methyltransferase. *RNA.* 2011; 17:429–438. [PubMed: 21205839]
14. Wang LK, Das U, Smith P, Shuman S. Structure and mechanism of the polynucleotide kinase component of the bacterial Pnkp-Hen1 RNA repair system. *RNA.* 2012; 18:2277–2286. [PubMed: 23118415]
15. Wang LK, Smith P, Shuman S. Structure and mechanism of the 2',3' phosphatase component of the bacterial Pnkp-Hen1 RNA repair system. *Nucleic Acids Res.* 2013 in press.
16. Lee JY, Yang W. UvrD helicase unwinds DNA one base pair at a time by a two-part power stroke. *Cell.* 2006; 127:1349–1360. [PubMed: 17190599]
17. Sinha KM, Glickman MS, Shuman S. Mutational analysis of Mycobacterium UvrD1 identifies functional groups required for ATP hydrolysis, DNA unwinding, and chemomechanical coupling. *Biochemistry.* 2009; 48:4019–4030. [PubMed: 19317511]
18. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr.* 2004; D60:2126–2132.
19. Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCoy AJ, Moriarty NW, Read RJ, Sacchettini JC, Sauter NK, Terwilliger TC. PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr.* 2002; D58:1948–1954.
20. Das U, Shuman S. Mechanism of RNA 2',3'-cyclic phosphate end-healing by T4 polynucleotide kinase-phosphatase. *Nucleic Acids Res.* 2013; 41:355–365. [PubMed: 23118482]
21. van de Sande JH, Kleppe K, Khorana HG. Reversal of bacteriophage T4 induced polynucleotide kinase action. *Biochemistry.* 1973; 12:5050–5055. [PubMed: 4366306]
22. Zhu H, Yin S, Shuman S. Characterization of polynucleotide kinase/phosphatase enzymes from mycobacteriophages Omega and Cjw1 and vibriophage KVP40. *J Biol Chem.* 2004; 279:26358–26369. [PubMed: 15056675]
23. Jain R, Shuman S. Characterization of a thermostable archaeal polynucleotide kinase homologous to human Clp1. *RNA.* 2009; 15:923–931. [PubMed: 19299550]
24. Wang LK, Shuman S. Mutational analysis defines the 5' kinase and 3' phosphatase active sites of T4 polynucleotide kinase. *Nucleic Acids Res.* 2002; 30:1073–1080. [PubMed: 11842120]
25. Yan H, Tsai MD. Nucleoside monophosphate kinases: structure, mechanism, and substrate specificity. *Adv Enzymol Rel Areas Mol Biol.* 1999; 73:103–134.
26. Tsai MD, Yan H. Mechanism of adenylylase kinase: site-directed mutagenesis versus X-ray and NMR. *Biochemistry.* 1991; 30:6806–6818. [PubMed: 2069947]
27. Dahnke T, Shi Z, Yan H, Jiang RT, Tsai MD. Mechanism of adenylylase kinase. Structural and functional roles of the conserved arginine-97 and arginine-132. *Biochemistry.* 1992; 31:6318–6328. [PubMed: 1627570]
28. Yan H, Shi Z, Tsai MD. Mechanism of adenylylase kinase. Structural and functional demonstration of arginine-138 as a key catalytic residue that cannot be replaced by lysine. *Biochemistry.* 1990; 29:6385–6392. [PubMed: 2119801]

29. Müller CW, Schulz GE. Structure of the complex between adenylate kinase from *Escherichia coli* and the inhibitor Ap₅A refined at 1.9 Å resolution: a model for a catalytic transition state. *J Mol Biol.* 1992; 224:159–177. [PubMed: 1548697]
30. Abele U, Schulz GE. High-resolution structures of adenylate kinase from yeast ligated with inhibitor Ap₅A, showing the pathway of phosphoryl transfer. *Protein Sci.* 1995; 4:1262–1271. [PubMed: 7670369]
31. Wang LK, Shuman S. Domain structure and mutational analysis of T4 polynucleotide kinase. *J Biol Chem.* 2001; 276:26868–26874. [PubMed: 11335730]
32. Wang LK, Lima CD, Shuman S. Structure and mechanism of T4 polynucleotide kinase – an RNA repair enzyme. *EMBO J.* 2002; 21:3873–3880. [PubMed: 12110598]
33. Eastberg JH, Pelletier J, Stoddard BL. Recognition of DNA substrates by bacteriophage T4 polynucleotide kinase. *Nucleic Acids Res.* 2004; 32:653–660. [PubMed: 14754987]
34. Galburt EA, Pelletier J, Wilson G, Stoddard BL. Structure of a tRNA repair enzyme and molecular biology workhorse: T4 polynucleotide kinase. *Structure.* 2002; 10:1249–1260. [PubMed: 12220496]
35. Remus BS, Shuman S. A kinetic framework for tRNA ligase and enforcement of a 2'-phosphate requirement for ligation highlights the design logic of an RNA repair machine. *RNA.* 2013 in press.
36. Wang LK, Schwer B, Englert M, Beier H, Shuman S. Structure-function analysis of the kinase-CPD domain of yeast tRNA ligase (Trl1) and requirements for complementation of tRNA splicing by a plant Trl1 homolog. *Nucleic Acids Res.* 2006; 34:517–527. [PubMed: 16428247]
37. Westaway SK, Belford HG, Apostol BL, Abelson J, Greer CL. Novel activity of a yeast ligase deletion polypeptide: evidence for GTP-dependent tRNA splicing. *J Biol Chem.* 1993; 268:2435–2443. [PubMed: 8428918]
38. Pfeiffer BH, Zimmerman SB. Deoxyribonucleic acid kinase from nuclei of rat liver: mechanism, reversal, and inhibitors of the reaction. *Biochemistry.* 1979; 18:2960–2963. [PubMed: 37889]
39. Garces F, Pearl LH, Oliver AW. The structural basis for substrate recognition by mammalian polynucleotide kinase 3' phosphatase. *Mol Cell.* 2011; 44:385–396. [PubMed: 22055185]
40. Weitzer S, Martinez J. The human RNA kinase hClp1 is active on 3' transfer RNA exons and short interfering RNAs. *Nature.* 2007; 447:222–226. [PubMed: 17495927]
41. Ramirez A, Shuman S, Schwer B. Human RNA 5'-kinase (hClp1) can function as a tRNA splicing enzyme in vivo. *RNA.* 2008; 14:1737–1745. [PubMed: 18648070]
42. Noble CG, Beuth B, Taylor IA. Structure of a nucleotide-bound Clp1-Pcf11 polyadenylation factor. *Nucleic Acids Res.* 2007; 35:87–99. [PubMed: 17151076]

Figure 1. NTP donor specificity of the kinase reaction

(A) Stereo view of the phosphate donor site of the A protomer of the kinase•ATP•Mg²⁺ substrate complex (pdb 4GP7). Amino acids and ATP are shown as stick models with beige and gray carbons, respectively. Mg²⁺ is depicted as a magenta sphere in the center of an octahedral coordination complex. Waters in the metal coordination complex are denoted by red spheres. Atomic contacts are indicated by dashed lines. (B) Kinase reactions were performed as described in Experimental Procedures. The reaction mixtures included 100 μM NTPs as specified, 0.05 μM 10-mer H_2O RNAp phosphoacceptor (5'- H_2O AUCACGCUUpCp) labeled with ³²P at the penultimate phosphate, and 0.25 μM kinase (Pnk +). The ³²P-labeled RNAs were resolved by PAGE and visualized by autoradiography. The radiolabeled species corresponding to 5'-OH substrate RNA and the 5'-PO₄ product RNA are indicated at *left*. (C) Kinetics. Reaction mixtures containing 50 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, 5 mM DTT, 100 μM ATP, 0.05 μM ³²P-labeled 10-mer H_2O RNAp phosphoacceptor, and 0.25, 0.5, 1.0 or 2.0 μM kinase as indicated were incubated at 45°C. Aliquots (10 μl) were withdrawn at times specified and quenched immediately with formamide/EDTA. The time 0 samples were withdrawn prior to adding kinase. Product formation is plotted versus reaction time. Each datum is the average of three independent time-course experiments (±SEM). The data were fit by non-linear regression to a one-phase association function in Prism.

Figure 2. NDP acceptor specificity of the reverse kinase reaction

(A) Reverse kinase reactions were performed as described in Experimental Procedures. The reaction mixtures included 0.1 μM 5'-³²P labeled 15-mer DNA oligonucleotide (pCCGACCAACGAAGGT), 100 μM ADP, UDP, GDP, CDP or dADP as specified, and 0.25 μM kinase (Pnk +). The radiolabeled products were resolved by TLC and visualized by autoradiography, which showed label transfer from the ³²P-DNA oligonucleotide to the added NDP acceptor to form the respective ³²P-labeled NTPs. The extents of label transfer to the NDPs are indicated below the lanes. (B) NDP dependence. Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, 5 mM DTT, 0.1 μM 5'-³²P-labeled 15-mer oligonucleotide, 0.125 μM kinase and NDP as indicated were incubated at 45°C for 30 min. The products were analyzed by TLC. The extent of ³²P-NTP formation is plotted as a function of NDP concentration. Each datum is the average of three independent titration experiments (±SEM). Nonlinear regression fitting of the data to the Michaelis-Menten equation was performed in Prism. (C) Kinetics. Reaction mixtures containing 50 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, 5 mM DTT, 100 μM ADP, 0.1 μM of a 15-mer 5'-³²P labeled 15-mer oligonucleotide, and 0.25, 0.5, 1.0 or 2.0 μM kinase as indicated were incubated at 45°C. Aliquots (10 μl) were withdrawn at times specified and quenched immediately with formic acid. The time 0 sample was withdrawn prior to adding kinase. The products were analyzed by TLC. The extents of label transfer to form ³²P-ATP are plotted versus reaction time. Each datum is the average of three independent time course experiments (±SEM). The data were fit by non-linear regression to a one-phase association function in Prism.

Figure 3. Dependence of kinase activity on 5'-OH polynucleotide concentration

Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.0), 10 mM MgCl_2 , 5 mM DTT, 100 μM [$\gamma\text{-}^{32}\text{P}$]ATP, 0.25 μM kinase, and 15-mer 5'-OH DNA oligonucleotide phosphoacceptor (HOCCGACCAACGAAGGT) as specified were incubated for 2 min at 45°C. The reactions were initiated by adding kinase to pre-warmed reaction mixtures and quenched with formamide, EDTA. The extents of DNA phosphorylation are plotted as a function of HO DNA concentration. Each datum is the average of three HO DNA titration experiments $\pm$ SEM. Nonlinear regression curve fitting to the Michaelis-Menten equation was performed in Prism.

Figure 4. Nucleotide electron density in the kinase•NTP•Mg²⁺ complexes
 Stereo views of the 2F_o-F_c electron density maps of the nucleotides in the kinase•GTP•Mg²⁺, kinase•dATP•Mg²⁺, kinase•UTP•Mg²⁺, and kinase•CTP•Mg²⁺ complexes, contoured at 1.5 σ . The NTPs are shown as stick models with gray carbons. Magnesium ions are depicted as magenta spheres. The Arg116 side chain underlying the nucleobase is shown as a stick model with beige carbons.

Figure 5. Superposition of five kinase•NTP structures

A stereo view of the superimposed kinase•NTP•Mg²⁺ complexes with ATP (from pdb 4GP7), GTP, CTP, UTP and dATP. NTPs in the phosphate donor site are shown as stick models with gray carbons. Magnesium ions are depicted as magenta spheres. Selected Pnkp side chains are shown as stick models with beige carbons.

Figure 6. Structure-guided mutagenesis

Kinase reactions were performed as described in Experimental Procedures. The reaction mixtures included 100 μ M [γ - 32 P]ATP, 60 pmol of a 15-mer 5'-OH DNA oligonucleotide phosphoacceptor (HOCCGACCAACGAAGGT), and wild-type or mutant kinase as indicated. The extents of DNA phosphorylation are plotted as a function of input protein. Each datum is the average of three titration experiments $\pm$ SEM.

Figure 7. Steady-state kinetics

Reaction mixtures (10 μ l) containing 50 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 5 mM DTT, 100 pmol of a 15-mer 5'-OH DNA oligonucleotide phosphoacceptor ($H_2OCCGACCAACGAAGGT$), 0.25 μ M wild-type or R116A kinase, and [$\gamma^{32}P$]ATP as specified were incubated for 2 min at 45°C. The reactions were initiated by adding kinase to pre-warmed reaction mixtures and quenched with formamide, EDTA. The extents of DNA phosphorylation are plotted as a function of ATP concentration. Each datum is the average of three ATP titration experiments $\pm$ SEM. Nonlinear regression curve fitting to the Michaelis-Menten equation was performed in Prism.

Table 1

Crystallographic data and refinement statistics

	UTP	GTP	CTP	dATP
Space group	P2 ₁ /2 ₁	P2 ₁ /2 ₁	P2 ₁ /2 ₁	P2 ₁ /2 ₁
Unit cell dimensions (Å)@ 130K	a = 45.31 b = 66.78 c = 119.55	a = 45.31 b = 66.78 c = 119.55	a = 45.31 b = 66.78 c = 119.55	a = 45.31 b = 66.78 c = 119.55
Diffraction data quality				
Resolution (Å)	46.1 - 2.03 (2.08 - 2.03)	39.6 - 2.14 (2.26 - 2.14)	58.0 - 2.48 (2.58 - 2.48)	45.5 - 2.02 (2.07 - 2.02)
Radiation source	NSLS X25	NSLS X25	NSLS X25	NSLS X25
Wavelength, Å	1.10	1.10	1.10	1.10
Processing software	Mosflm/Scala	Mosflm/Scala	Mosflm/Scala	Mosflm/Scala
R _{sym} , %	5.9 (19.9)	9.1 (18.6)	7.6 (18.8)	4.8 (15.4)
Unique reflections	24709 (1787)	20889 (2998)	13156 (1445)	24363 (1760)
Mean redundancy	6.1 (5.6)	11.9 (12.0)	6.0 (6.2)	5.9 (5.2)
Completeness, %	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	99.7 (99.2)
Mean I/σ	17.7 (7.3)	17.4 (9.8)	17.4 (8.4)	22.5 (9.6)
Refinement and model statistics (1.34F>σF)				
Resolution (Å)	44.5 - 2.03 (2.08 - 2.03)	37.5 - 2.14 (2.20 - 2.14)	44.5 - 2.49 (2.62 - 2.49)	44.5 - 2.02 (2.07 - 2.02)
Completeness, %	99.8 (98.6)	99.8 (97.0)	98.8 (92.0)	98.9 (90.2)
R _{free} /R _{work} , %	22.4/17.0 (27.3/19.4)	20.4/16.8 (21.0/16.2)	22.7/18.1 (28.7/20.2)	21.4/15.3 (23.7/15.1)
RMSD bonds/angles	0.009 Å/1.26°	0.005 Å/1.00°	0.006 Å/1.09°	0.010 Å/1.28°
Ramachandran plot	99.1 % favored, no outliers	99.7 % favored, no outliers	99.7 % favored, no outliers	98.8 % favored, no outliers
B-factors, Å ²				
Wilson/Overall	22.9/29.2	21.9/24.3	27.9/36.4	22.0/27.2
Protein/NTP				
Protomer A	29.1/31.3	23.5/26.0	35.7/31.9	25.4/26.1

	UTP	GTP	CTP	dATP
Protomer B	29.6/20.7	24.7/24.4	37.6/33.6	28.0/24.6
Mean TLS anisotropy [‡]	0.67 over 7 groups			
Model contents				
Protein residues	342	342	341	342
Heteroatoms	2 UTP, 2 Mg ²⁺ , 1Na ⁺	2 GTP, 2 Mg ²⁺	2 CTP, 2 Mg ²⁺	2 dATP, 2 Mg ²⁺ , 1Na ⁺
Waters	266	180	126	279
PDB ID	4JST	4JSY	4JT2	4JT4

Standard definitions are used for all parameters. Figures in parentheses refer to data in the highest resolution bin. The refinement and geometric statistics come from PHENIX.

* *R*_{sym} output as *R*_{merge} by SCALA.

¶ *R*_{free} sets consisted of 8% of data chosen at random against which structures were not refined.

‡ TLS refinement was carried out only for the CTP