Mutational analysis of the 5′-OH oligonucleotide phosphate acceptor site of T4 polynucleotide kinase

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Received October 15, 2009; Revised and Accepted November 6, 2009

ABSTRACT

T4 polynucleotide kinase/phosphatase (Pnkp) exemplifies a family of bifunctional enzymes with 5′-kinase and 3′-phosphatase activities that function in nucleic acid repair. The N-terminal kinase domain belongs to the P-loop phosphotransferase superfamily. The kinase is distinguished by a tunnel-like active site with separate entrances on opposite sides of the protein for the NTP phosphate donor and a 5′-OH single-stranded oligonucleotide phosphate acceptor. Here, we probed by mutagenesis the roles of individual amino acids that comprise the acceptor binding site. We thereby identified Glu57 as an important residue, by virtue of its participation in a salt bridge network with two catalytic residues identified previously: Arg38, which binds the 3′-phosphate of the terminal 5′-OH nucleotide, and the putative general base Asp35 that contacts the nucleophilic 5′-OH group. The 5′-OH nucleoside fits into a pocket lined by aliphatic amino acids (Val131, Pro132 and Val135) that make van der Waals contacts to the nucleobase. Whereas subtraction of these contacts by single alanine substitutions for Val131 or Val135 and glycine for Pro132 had modest effects on kinase activity, the introduction of bulkier phenylalanines for Val131 and Val135 were deleterious, especially V131F, which severely impeded both substrate binding (increasing $K_m$ by 15-fold) and catalysis (decreasing $k_{cat}$ by 300-fold).

INTRODUCTION

T4 polynucleotide kinase/phosphatase (Pnkp) exemplifies a family of repair enzymes that heal broken termini in RNA or DNA by converting 3′-PO4/5′-OH ends into 3′-OH/5′-PO4 ends, which are then sealed by RNA or DNA ligases. During T4 infection, Pnkp thwarts an RNA-based innate immune response in which the bacterium blocks viral protein synthesis by inducing site-specific breakage of host-cell tRNAs, to which the phage responds by repairing the broken tRNAs using Pnkp and a phage-encoded RNA ligase (1). T4 Pnkp catalyzes two reactions in this pathway: (i) the transfer of the γ phosphate from ATP to the 5′-OH terminus of RNA and (ii) the hydrolytic removal of a 3′-PO4 terminus from RNA (2–6).

T4 Pnkp is a homotetramer of a 301-aa polypeptide, which consists of an N-terminal kinase domain of the P-loop phosphotransferase superfamily and a C-terminal phosphatase domain of the DxD acylphosphatase superfamily. The homotetramer is formed via pairs of phosphatase–phosphatase and kinase–kinase homodimer interfaces (7–10). Essential constituents of the separate active sites for the 5′ kinase and 3′ phosphatase activities have been identified by alanine-scanning mutagenesis, guided initially by phylogenetic conservation of primary structure among Pnkp homologs (11,12) and subsequently by crystal structures of T4 Pnkp (10). Of the 20 amino acids within the kinase domain that were targeted for mutagenesis, six were found to be essential: Lys15, Ser16, Asp35, Arg38, Asp85 and Arg126.

The kinase domain consists of a central four-stranded parallel β-sheet flanked by three α helices on each side. The kinase active site is composed of (i) a classical P-loop motif (9GxxGxGKS16) that coordinates the β phosphate of the NTP donor via the main-chain amide nitrogens of the P-loop and the Lys15 side chain; (ii) essential side chain Arg126, which also coordinates the NTP β phosphate; (iii) essential side chain Arg38, which coordinates the 3′ phosphate of the 5′-OH acceptor nucleotide (Figure 1A), plus (iv) essential side chain Asp35, a putative general base that activates the 5′-OH for direct nucleophilic attack on the NTP γ phosphate. The active site is located within a tunnel through the heart of the enzyme. The tunnel entrance on the P-loop side admits the NTP phosphate donor and controls release of the NDP reaction product. The tunnel opening on the opposite face allows ingress of the 5′ end of a single-stranded polynucleotide to the kinase active site (7,8).

Further insights to the anatomy of the phosphate acceptor site were attained by Eastberg et al. (9), who...
soaked short 5'-OH DNA oligonucleotides (a 3mer and two different 5mers) into preformed Pnkp-ADP crystals and solved the structures of the substrate complexes. The observed electron density allowed modeling of up to 3 nt from the 5'-OH acceptor end. Figure 1 depicts the interactions of the kinase with HOGpTpC. The image in Figure 1B is a stereo view of the phosphate acceptor tunnel of the kinase domain with the protein rendered as a surface model and the 5'-GTC trinucleotide as a stick model. The deep end of the tunnel is demarcated by Asp35, which coordinates the 5'-OH. Also labeled are Tyr52, which contacts the second and third nucleosides, and Val131 and Pro132, which contact the first nucleobase. The figure highlights the narrow tunnel aperture, which allows ingress of single-stranded nucleic acid, but would seem to exclude a nucleic acid duplex. When bound in the tunnel, the 5'-OH of the terminal HOGp nucleotide is oriented appropriately for attack on ATP modeled in the phosphate donor site. The image in Figure 1A shows that the enzyme-oligonucleotide interface consists of a network of hydrogen bonds and van der Waals contacts between amino acid side chains and the bases, sugars and phosphates of the HOGpTpC trinucleotide.

The structures of the acceptor complexes raised interesting questions about the functional architecture of the acceptor binding site, which, in principle, has to
accomplish several tasks, including: accommodation of 5′-OH single-stranded nucleic acids with relatively little sequence bias; insistence on ingress of the 5′ ends of single-stranded nucleic acids while rejecting a 3′ oligonucleotide terminus; and exclusion of water as a futile nucleophile. The factors that aid these functions might include: shape complementarity between the tunnel surface and the 5′-OH oligonucleotide; and division of the tunnel into polar versus hydrophobic patches to orient the phosphates and bases, respectively. The enzyme might also rely on specific atomic contacts between the protein and nucleic acid atoms to bind and orient the phosphoacceptor. The aim of the present study was to examine by mutagenesis the contributions of the amino acids that line the tunnel to the 5′-kinase activity.

As it is well established that a mononucleoside 3′-phosphate (HONp) is the minimal phosphate acceptor for T4 Pnkp (2, 4), we assume that the contacts required for phosphoryl transfer chemistry to nucleic acid (and for kinase activity (11, 12). However, Thr86, which also donates a hydrogen bond to the +1 phosphate and contributes to the acceptor binding site via a van der Waals contact from Cβ to the acceptor phosphate (Figure 1A), can be replaced by alanine without affecting kinase function (11). It is conceivable that the phosphate contact of the Thr86 Oγ is functionally redundant with the hydrogen bond donated to the +1 phosphate oxygen from the Thr86 main chain amide (Figure 1A). Asp85, which is essential for kinase activity (11), contributes to the acceptor binding site via a van der Waals contact from Cβ to the +1 phosphate; it also interacts with Arg34, which donates a hydrogen bond to the +2 phosphate (HONpNpN) (Figure 1A). We suspect that neither of these contacts accounts for the complete loss of activity when Asp85 is replaced with alanine, insofar as Arg34 is dispensable for 5′-kinase activity when Asp85 is replaced with alanine, insofar as Arg34 and Asp35, which coordinate the -OH and phosphate of the +1 nt, are essential for kinase activity (11, 12). Indeed, prior mutational studies support this view, insofar as Arg34 is dispensable for 5′-kinase activity with an alanine mutant. The Asp85 is essential for the local structure of the kinase active site, via hydrogen bonding of its carboxylate oxygens with the Arg34 main chain amide nitrogen and the Gln64 side chain Nε (7, 9).

Here, we interrogated by mutagenesis the functions of the other constituents of the phosphate acceptor site (Figure 1A). Our findings highlight the importance of Glu57 in positioning the catalytic Arg38 residue and the sterical constraints on the hydrophobic pocket that accommodates the 5′ nucleoside base.

MATERIALS AND METHODS

Pnkp mutants

Missense mutations were introduced into the Pnkp ORF by using the two-stage polymerase chain reaction (PCR)-based overlap extension method as described previously (11, 12). The PCR products were digested with NdeI and BamHI and then inserted into pET16b. The inserts were sequenced completely to confirm the desired mutations and exclude the acquisition of unwanted changes. The pET-Pnkp plasmids were introduced into Escherichia coli BL21(DE3). Recombinant protein production was induced by adjusting exponentially growing cultures (100 ml) to 0.3 mM IPTG and incubating them at 17°C for 15 h with continuous shaking. The wild-type and mutant His10-Pnkp proteins were purified from soluble bacterial lysates by Ni-agarose chromatography as described previously (11, 12). Protein concentrations were determined by using the BioRad dye reagent with bovine serum albumin as the standard.

3′ Phosphatase assay

Reaction mixtures (25 μl) containing 100 mM imidazole (pH 6.0), 10 mM MgCl2, 5 mM DTT, 0.1 mg/ml bovine serum albumin (BSA), 1.6 mM 3′ dTMP (Sigma), and 0, 4.7, 9.4, 18.8, 37.5 or 75 ng of wild-type or mutant Pnkp as specified were incubated for 20 min at 37°C. The reactions were quenched by adding 75 μl of cold water and 1 ml of malachite green reagent (BIOMOL Research Laboratories). Phosphate release was determined by measuring A620 and interpolating the value to a phosphate standard curve. The phosphatase specific activity of each protein was determined from the slope of the titration curve. The results are shown in Figure 2B, where each datum is the average of three independent titration experiments ± SEM.

5′ Kinase assays

3′-CMP substrate. Reaction mixtures (10 μl) containing 70 mM Tris–HCl (pH 7.6), 10 mM MgCl2, 5 mM DTT, 25 μM [γ32P]ATP, 1 mM 3′ CMP (Sigma) and increasing amounts of wild-type or mutant Pnkp were incubated for 20 min at 37°C. The reactions were quenched by adding 5 μl of 5 M formic acid. Aliquots of the mixtures were applied to a polyethyleneimine–cellulose TLC plate, which was developed with 1 M formic acid, 0.5 M LiCl. The [γ32P]ATP substrate and [α32P]pCp product were visualized and quantified by scanning the gel with a Fujix BAS2500 phosphorimagor. The kinase specific activity of each protein was determined from the slope of the titration curve. The results are shown in Figure 2C, where each datum is the average of three independent titration experiments ± SEM.

Polynucleotide substrate. Reaction mixtures (10 μl) containing 70 mM Tris–HCl (pH 7.6), 10 mM MgCl2, 5 mM DTT, 25 μM [γ32P]ATP, 5 μM (50 pmol) of 5′-OH terminated 18-mer oligodeoxynucleotide d(ATTCCGAT AGTGACTACA), and increasing amounts of wild-type or mutant Pnkp were incubated for 20 min at 37°C. The reactions were quenched by adding 6 μl of 95% formamide/20 mM EDTA. The products were analyzed by electrophoresis through a 15-cm 15% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris–borate, 2.5 mM EDTA). The radiolabeled oligonucleotide products were visualized and quantified by scanning the
gel with a Fujix BAS2500 phosphorimager. The kinase specific activity of each protein was determined from the slope of the titration curve. The results are shown in Figure 3, where each datum is the average of three independent titration experiments ± SEM.

RESULTS AND DISCUSSION

Mutations in the polynucleotide kinase phosphate acceptor site

A goal of the present study was to extend the functional map of the kinase component of T4 Pnkp by mutating the amino acids that form the binding site for the 5'-OH terminated oligonucleotide phosphate acceptor. Alanines were introduced in lieu of the following residues highlighted in Figure 1: Tyr52, which makes stacking van der Waals interactions with the +2 base and hydrogen bonds to the +3 sugar; Glu57, which forms a salt bridge to the critical Arg38 side chain; Thr61, which makes a hydrogen bond and van der Waals contact to the +3 base; Asn89, which makes van der Waals contact to the +2 phosphate; Arg92, which makes a van der Waals contact to the +2 base and also a hydrogen bond to Thr61; and the Val131 and Val135 residues that make van der Waals contacts with the +1 base. Pro162, which contacts the +1 base, was changed to glycine. In order to gauge the steric constraints on the hydrophobic binding pocket for the +1 base, we also replaced Val131 and Val135 by bulkier phenylalanine side chains. In addition, we introduced alanines in lieu of two residues not depicted in Figure 1: Lys54, which is located near the entrance to the oligonucleotide acceptor site in the apoenzyme structure but is disordered in the oligonucleotide-bound enzyme; and Lys129, which donates a hydrogen bond from Nζ to the ribose O3' of the ATP phosphate donor.

Wild-type Pnkp and the 12 mutants were produced in bacteria as His10-tagged fusions and purified from soluble bacterial extracts by Ni-agarose chromatography (Figure 2A). Pnkp’s 3’-phosphatase activity was measured by the release of inorganic phosphate from deoxythymidine 3’ monophosphate (5,6). Wild-type Pnkp released 0.53 nmol of Pi from 3’dTMP per ng of protein in the

![Figure 3. Mutational effects on 5'-kinase activity with an oligonucleotide phosphate acceptor. 5' kinase specific activities with an 18-mer 5'-OH DNA oligonucleotide substrate (data as shown) were determined by enzyme titration as described under ‘Materials and methods’ section. Each datum in the bar graph is the average of three titration experiments ± SEM.](image-url)
linear range of enzyme-dependence, which translates into a
turnover number of $\sim 16\ s^{-1}$. The specific activities of the
Pnkp mutants were determined in parallel (Figure 2B).

The mutants had activity similar to wild-type Pnkp
(t.e. $\pm 60\%$), as follows: Y52A (135%), K54A (117%)
E57A (44%), T61A (163%), N89A (114%), R92A
(108%), K129A (86%), V131A (80%), V131F (41%),
P132G (105%), V135A (82%) and V135F (150%). Such
prep-to-prep variations in phosphatase-specific activities
among wild-type and ‘active’ Pnkp mutants were noted
previously (10). Here, as is our previous studies (10,12),
our operational criterion for a significant mutational effect
is one that elicits at least a 4-fold reduction in specific
activity compared to wild-type Pnkp. We construe the
retention of 3’-phosphatase activity above this threshold
to signify that none of the amino acid changes in the
kinase domain grossly affected the global folding of the
enzyme.

The wild-type and mutant Pnkp proteins were surveyed
for 5′ kinase activity, which was measured as the transfer
of $^{32}$P from 25 mM $[^{\gamma-32}$P]ATP to 1 mM 3′-CMP ($^{10}$CP)
to form $[^{5\prime-32}$P]pCP. Wild-type Pnkp phosphorylated
44 pmol of $^{10}$CP per nanogram of protein in the linear
range of enzyme-dependence, which translates into a
turnover number of $\sim 81\ min^{-1}$. The kinase-specific
activities of the 12 Pnkp mutants were determined in
parallel (Figure 2C). Most of the mutations elicited mild
to moderate activity decrements, among which E57A,
T61A, K129A and V131A met the 4-fold criterion of sig-
nificance. By contrast, the V131F change was uniquely
lowered $k_{\text{cat}}$ to 3.3 s$^{-1}$, corresponding to a 4400-fold
decrement in catalytic efficiency (Table 1). We surmise
that the Phe substitutions at Val135, and especially Val131,
impede positioning the terminal 5′-OH nucleophile in the active
site because of steric clash with the +1 base.

The E57A change increased $K_m$ to 157 $\mu$M $^{10}$CP and
lowered $k_{\text{cat}}$ to 3.3 s$^{-1}$ (Table 1), resulting a 150-fold
decrement in catalytic efficiency compared to wild-type Pnkp.

### Table 1. Mutational effects on 5′ kinase activity with $^{10}$CP acceptor

| Pnkp     | $K_m$ $^{10}$CP (μM) | $k_{\text{cat}}$ (min$^{-1}$) |
|----------|----------------------|-------------------------------|
| WT       | 31 ± 4               | 98 ± 16                       |
| Y52A     | 16 ± 3               | 69 ± 2                        |
| K54A     | 12 ± 1               | 80 ± 5                        |
| E57A     | 157 ± 22             | 3.3 ± 0.3                     |
| T61A     | 36 ± 4               | 21 ± 1.5                      |
| N89A     | 24 ± 6               | 50 ± 9                        |
| R92A     | 105 ± 7              | 74 ± 6                        |
| K129A    | 43 ± 9               | 33 ± 2                        |
| V131A    | 113 ± 13             | 32 ± 2                        |
| V131F    | 460 ± 46             | 0.33 ± 0.03                   |
| P132G    | 67 ± 7               | 56 ± 2                        |
| V135A    | 39 ± 6               | 33 ± 2                        |
| V135F    | 323 ± 28             | 23 ± 1.3                      |

Reaction mixtures (10 μL) containing 70 mM Tris–HCl (pH 7.6), 10 mM MgCl$_2$, 5 mM DTT, 25 μM $[^{\gamma-32}$P]ATP, wild-type or mutant Pnkp and varying concentrations of 3′-CMP were incubated for 20 min at 37 °C. The extents of $[^{\gamma-32}$P]pCP product formation were plotted as a function of 3′-CMP concentration. $K_m$ and $k_{\text{cat}}$ were obtained by nonlinear regression curve fitting of the data for each experiment to the Michaelis-Menten equation in Prism. The values shown are averages of three independent 3′-CMP titration experiments ± SEM. Representative 3′-CMP titration profiles are shown in Supplementary Figure S1.

Mutational effects on 3′-mononucleotide binding
and catalysis

Mutational effects on the steady-state kinetic parameters of the kinase reaction were gauged by performing $^{10}$CP titration experiments with each enzyme preparation. (Exemplary titrations are shown in Supplementary Figure S1.) The results are compiled in Table 1. Wild-type Pnkp had a $K_m$ of 31 μM $^{10}$CP and a $k_{\text{cat}}$ of 98 min$^{-1}$. Our observed $K_m$ value was in the same range as that reported by Novogrodsky et al. (4) for the monodeoxynucleotide $^{10}$H(dCP)p ($K_m$ 15 μM). Instructive findings emerged concerning the effects of mutations in the three hydrophobic residues (Val131, Pro132 and Val135) that pack against the same surface of the +1 purine base in the crystal structure depicted in Figure 1. Changing Pro132 to Gly had little effect on $k_{\text{cat}}$
(56 min$^{-1}$), although it increased $K_m$ by 2-fold. The
V135A change had little effect on $K_m$ but reduced $k_{\text{cat}}$
by 3-fold (to 33 s$^{-1}$), whereas the V131A mutation increased $K_m$ and reduced $k_{\text{cat}}$ by ~3-fold each, causing
a net 11-fold decrement in catalytic efficiency ($k_{\text{cat}}/K_m$).
This hierarchy of side-chain removal effects correlates
with the distances from the respective side chain atoms
to the +1 pyrimidine base observed in the crystal struc-
ture of Pnkp with a different trinucleotide ($^{10}$H(TpGpC)
occupying the acceptor site (pdb ID 1RPZ) (9). In that
structure, Val131 C$_Y$ makes close van der Waals contacts
to the pyrimidine C5 (3.3 Å) and C6 (3.4 Å) atoms, while
Val135 C$_Y$ is located farther away (3.9 Å) from the N3 and
O4 (N4 in cytidine) atoms. More drastic effects on kinase
activity were elicited by replacing the valines with
phenylalanine. The V135F change resulted in 10-fold
lower affinity for $^{10}$CP compared to wild-type Pnkp
while maintaining a reduced $k_{\text{cat}}$, so that the catalytic ef-
ciency of V135F was 44-fold lower than wild type. The
V131F mutant displayed a $K_m$ of 460 μM and a $k_{\text{cat}}$ of
0.33 s$^{-1}$, corresponding to a 4400-fold decrement in
catalytic efficiency (Table 1). We surmise that the Phe
substitutions at Val135, and especially Val131, impede
positioning the terminal 5′-OH nucleophile in the active
site because of steric clash with the +1 base.

The E57A change increased $K_m$ to 157 μM $^{10}$CP and
lowered $k_{\text{cat}}$ to 3.3 s$^{-1}$ (Table 1), resulting a 150-fold
decrement in catalytic efficiency compared to wild-type Pnkp.
Glu57 makes no direct contact with the kinase substrate;
rather it makes a salt bridge to the catalytically essential
Arg38 side chain, which coordinates the 3′-phosphate
moiety of the 5′-OH nucleotide (Figure 1). We surmise
that Glu57 is important to position Arg38 appropriately.

The Y52A and K54A mutations had little effect on $k_{\text{cat}}$
(69 s$^{-1}$ and 80 s$^{-1}$, respectively), but they had the distinctive
effect of lowering $K_m$ for $^{10}$CP to (16 and 12 μM,
respectively) compared to wild-type Pnkp (Table 1).
Neither Tyr52 nor Lys54 makes direct contact with the
terminal 5′-OH nucleotide (Figure 1); rather, both are
located toward the periphery of the oligonucleotide
entry channel. Our results, showing higher affinity for
the $^{10}$CP acceptor when either Tyr52 or Lys54 are
absent, suggest that these side chains normally provide
an obstacle to the ingress of a 3′-mononucleotide to the
kinase active site.
Arg92 contacts the +3 nucleoside (Figure 1) and, at first glance, might not be expected to contribute to mononucleotide phosphorylation. Yet, the R92A change caused a 3-fold increase in $K_m$, with little impact on $k_{cat}$ (Table 1). We suspect this result reflects the role of Arg92 in stabilizing the structure of the polynucleotide acceptor tunnel, by tethering two of the component $\alpha$-helices via hydrogen bond donation from Arg92 to Thr61 Oγ (Figure 1) and Asn64 Oε. Mutating Thr61 to alanine had little impact on $K_m$, but reduced $k_{cat}$ by a factor of five (Table 1). In addition to the hydrogen bond depicted in Figure 1, Thr61 makes van der Waals contacts via Cγ to Phe 65 and Arg92. Mutating Asn89 had little effect on the kinetic parameters for HOCp phosphorylation, in keeping with its lack of direct contact with the terminal nucleoside in the crystal structure (Figure 1). Finally, the effects of mutating Lys129 were consistent with its role as a constituent of the ATP-binding site of Pnkp; the K129A change had little effect on affinity for the HOCp acceptor, but reduced $k_{cat}$ by a factor of three (Table 1).

**Mutational effects on kinase activity with an oligonucleotide substrate**

The wild-type and mutant Pnkp proteins were tested for 5′ kinase activity with a 5′-OH terminated 18-mer DNA oligonucleotide phosphate acceptor. Transfer of $^{32}$P from 25μM [γ-$^{32}$P]ATP to 5μM 5′-OH DNA was quantified as a function of input enzyme. Wild-type Pnkp phosphorylated ~12 pmol of 18-mer per nanogram of protein in the linear range of enzyme dependence (Figure 3), which translates into a turnover number of ~24 min$^{-1}$. The kinase specific activities of the 12 Pnkp mutants were determined in parallel. The mutational effects on polynucleotide phosphorylation (Figure 3) were generally similar to the results obtained with HOCp (Table 1). The V131F and E57A changes were the most deleterious, reducing DNA kinase specific activity to 1% and 2% of the wild-type value, respectively (Figure 3).

**Structure–activity relations at Glu57**

To elucidate structure activity relations at Glu57, which we deemed important for polynucleotide kinase activity on the basis of the present alanine scan, we introduced conservative substitutions with glutamine and aspartate, purified the recombinant E57Q and E57D Pnkp proteins (in parallel with new preparations of wild-type Pnkp and the E57A mutant), and surveyed them for activity by enzyme titration. The E57Q and E57D proteins were both defective in 5′-kinase activity. Their respective specific activities in HOCp phosphorylation (at 1mM 3′CMP substrate concentration) were 0.5% and 5.3% of wild-type, while E57A was 12% as active as wild type (normalized averages of three titration experiments; data not shown). The polynucleotide kinase specific activities of E57Q, E57D and E57A with the 18-mer DNA oligonucleotide phosphate acceptor were 0.1%, 4.6% and 2% of wild-type Pnkp, respectively (normalized averages of three titration experiments; data not shown). These results signify that an isosteric amide is unable to function in lieu of Glu57; indeed, the E57Q mutation was more deleterious to kinase activity than E57A or E57D.

**CONCLUDING REMARKS**

The relatively narrow tunnel of T4 Pnkp that comprises the 5′-OH polynucleotide phosphate acceptor site (Figures 1B and 4A) accounts for why the phage enzyme prefers to phosphorylate single-stranded nucleic acids or duplexes with 5′ single-stranded tails (13,14). Indeed, the in vivo substrates for Pnkp are broken tRNA stem–loops with a short 5′-OH single-strand segment (1,15). By contrast, the mammalian Pnkp enzyme, which is a DNA-specific kinase, has an ‘open’ nucleic acid binding site that can accommodate a duplex nucleic acid (16,17) (Figure 4B). A key difference when the T4 and mammalian kinase structures are superimposed is that mammalian Pnkp lacks the 22-aa segment of T4 Pnkp (colored green in Figure 4A), comprising the ends of two serial $\alpha$-helices and the connecting loop (38RQSIMAHEERDEYKYTKKKEGI59), that forms one of the lateral walls and the roof of the tunnel. The open fold of the mammalian Pnkp allows it to phosphorylate its preferred substrates: 5′-OH DNA termini at duplex nicks, gaps and recessed 5′ ends within a 3′-tailed duplex (13,16). Unlike T4 Pnkp, which readily phosphorylates mononucleotides, mammalian Pnkp requires a polynucleotide phosphate acceptor with a minimum chain length of ~8 deoxynucleotides (13). The inability of mammalian Pnkp to phosphorylate mononucleoside 3′-phosphates can be explained by the fact that it is missing a counterpart of T4 Pnkp Arg38 that coordinates the 3′-phosphate of the 5′-OH terminal nucleotide (18).

Here, we relied on the structures of T4 Pnkp bound to short oligonucleotides (9) to guide a mutational analysis of the phosphate acceptor tunnel and thereby gained new insights to which constituents are important and why. In particular, we identified Glu57 as an important residue, by virtue of its participation in a salt-bridge network with two catalytic residues identified previously: the phosphate-binding Arg38 and the putative general base Asp35 (Figure 1). Mammalian Pnkp lacks a counterpart of Glu57.

The 5′-OH nucleoside fits into a pocket lined by several aliphatic amino acids that make van der Waals contacts with the nucleobase (Figure 1). Whereas subtraction of these contacts by single alanine substitutions for Val131 or Val135 and glycine for Pro132 had only modest effects on kinase activity, the introduction of bulkier phenylalanines for Val131 and Val135 were deleterious, especially V131F, which severely impeded both substrate binding and catalysis.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.
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