# Mutational Analysis of Baculovirus Phosphatase Identifies Structural Residues Important for Triphosphatase Activity in Vitro and in Vivo†

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ABSTRACT: Baculovirus phosphatase (BVP) and mammalian capping enzyme (Mce1) are members of the RNA triphosphatase branch of the cysteine phosphatase superfamily. Although RNA triphosphatases have a core $\alpha/\beta$ fold similar to other cysteine phosphatases, there is little conservation of primary structure outside of the cysteine-containing P-loop motif, HCxxxxxR(S/T), that comprises the active site. However, there is extensive primary structure conservation between members of the RNA triphosphatase branch, whether from cellular or viral sources and whether they are bifunctional capping enzymes such as Mce1 or monofunctional RNA phosphatases such as BVP. To evaluate the functional significance of such sequence conservation, we performed a mutational analysis of 14 residues of BVP. We identified three side chains (Trp6, Lys25, and Arg153) as essential for triphosphatase activity in vitro, i.e., W6A, K25A, and R153A were <0.1% as active as wild-type BVP, and were unable to complement a yeast RNA triphosphatase null mutant in vivo. Six other BVP residues (Thr62, Tyr67, Tyr68, Lys82, Glu158, and Arg159) were deemed functionally important, i.e., Ala mutations reduced triphosphatase activity to <20% of wild-type. On the basis of the locations of the equivalent amino acids in the Mce1 crystal structure, we surmise that the essential/important BVP residues ensure proper conformation of the catalytic P-loop (e.g., Arg153 and Tyr68) or other elements of the tertiary structure. Our results highlight a conserved Trp6-Lys25 $\pi$-cation pair essential for BVP function.

RNA triphosphatase catalyzes the first step in the 5′ capping of eukaryotic mRNA—the hydrolysis of the $\gamma$ phosphate of the 5′-triphosphate terminus to form a diphosphate RNA end. The subsequent steps are catalyzed by RNA guanylyltransferase, which adds a GMP to the diphosphate RNA end to form GpppRNA, and RNA (guanine-N7)-methyltransferase, which transfers a methyl group from AdoMet to GpppRNA to form $m^7$GpppRNA (*1*). The RNA triphosphatases of metazoans and plants belong to the cysteine phosphatase superfamily that includes protein tyrosine phosphatases, dual specificity protein phosphatases, and phosphoinositide phosphatases (*1*−*6*). The cysteine phosphatases catalyze a two-step ping-pong phosphoryl transfer reaction via a cysteinyl−phosphoenzyme intermediate (*7*).

The cysteinyl phosphatase-type RNA triphosphatases are subdivided into "bifunctional" and "monofunctional" groups. The bifunctional enzymes, composed of an N-terminal RNA triphosphatase domain and a C-terminal RNA guanylyltransferase domain, are ubiquitous in metazoans and are responsible for forming the 5′ cap structure of cellular mRNAs (*2*−*6*). A single case of a bifunctional viral cysteine phosphatase fused to a guanylyltransferase has been reported recently for the infectious spleen and kidney necrosis virus (ISKNV), a large DNA virus of the iridovirus family that causes disease in mandarin fish (*8*). The monofunctional RNA triphosphatases, which have no guanylyltransferase activity, include the human PIR1 phosphatase (*9*) and BVP, a phosphatase encoded by the *Autographa californica* baculovirus (*10*, *11*).

The 168-amino-acid baculovirus BVP protein displays extensive sequence similarity to the triphosphatase domains of metazoan, plant, and ISKNV capping enzymes (Figure 1). Purified recombinant BVP hydrolyzes the $\gamma$ phosphate of triphosphate-terminated RNA. BPV also hydrolyzes ATP to ADP and GTP to GDP. The remarkable feature of BVP is that it also catalyzes the hydrolysis of ADP to AMP and GDP to GMP and the conversion of diphosphate-terminated RNA to monophosphate-terminated RNA (*10*, *11*). Thus, BVP is a triphosphatase and a diphosphatase. In contrast, the metazoan capping enzymes hydrolyze only the $\gamma$ phosphate. Kinetic analyses of the reaction of BVP with either triphosphate-terminated RNA or free nucleoside triphosphates showed that the $\gamma$ phosphate is hydrolyzed prior to the $\beta$ phosphate and that the enzyme acts distributively, i.e., that nucleoside monophosphate products do not begin to accumulate until the majority of the input nucleoside triphosphate has been first converted to nucleoside diphosphate (*10*).

Recently, we reported that BVP activity is not limited to 5′ phosphorylated RNA or nucleotide substrates, but also embraces the hydrolysis of inorganic phosphoanhydrides such as tripolyphosphate and pyrophosphate (*12*). Despite its broad specificity for hydrolysis of phosphoanydrides in vitro, BVP can act as an RNA triphosphatase in the cap synthetic pathway in vivo in yeast cells when it is fused to the C-terminus of the mammalian guanylyltransferase domain (*12*) or when fused to the N-terminus of the *Schizosaccharomyces pombe* guanylyltransferase (Hausmann, Martins, and

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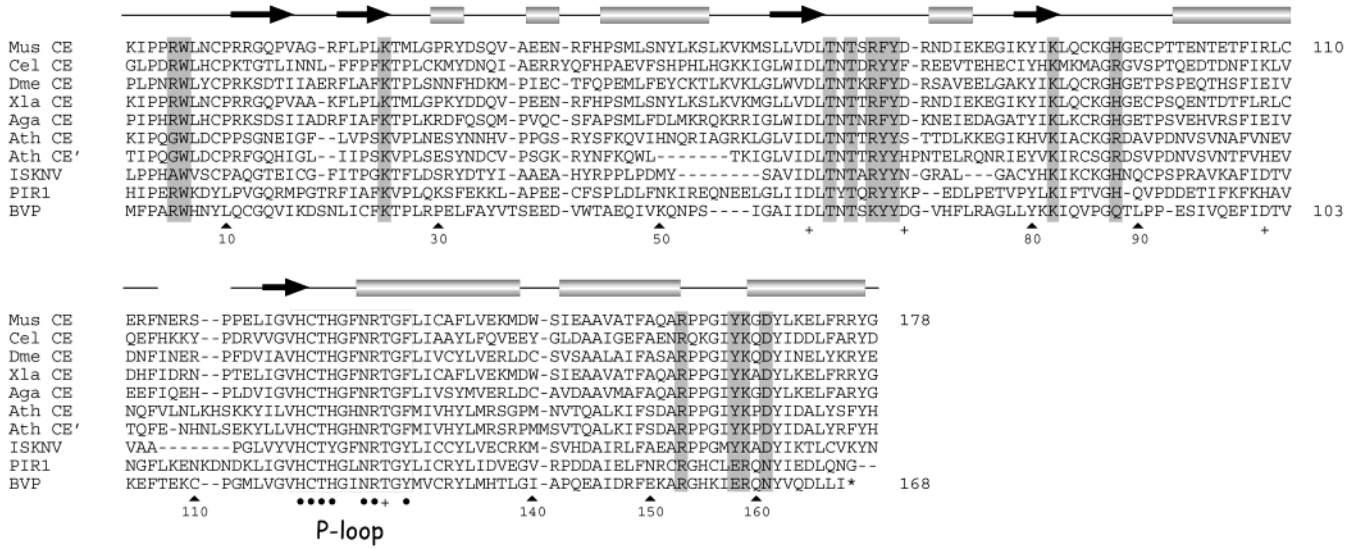


FIGURE 1: Structure-based sequence alignment of cysteine phosphatase-type RNA triphosphatases. The amino acid sequences of the N-terminal RNA triphosphatase domains of mouse capping enzyme (Mus CE), *C. elegans* capping enzyme (Cel CE), *D. melanogaster* capping enzyme (Dme CE), *X. laevis* capping enzyme (Xla CE), *Anopheles gambiae* capping enzyme (Aga CE), two capping enzymes of *A. thaliana* (Ath CE and CE′), and a capping enzyme of the ISKNV iridovirus (ISKNV) are aligned to human RNA phosphatase PIR1 and the baculovirus RNA phosphatase BVP. Gaps in the sequences are indicated by dashes. The cysteine phosphatase signature motif (the P-loop) is indicated. Secondary structure elements of the mammalian RNA triphosphatase (PDB code 1I9S) are shown above the sequence with α helices depicted as bars, $\beta$ strands as arrows, and loops as solid lines. The previously reported effects of single-alanine substitutions on BVP function in yeast are indicated below the BVP acid sequence, with viable mutants denoted by + and lethal mutants by ●. The 14 residues of BVP subjected to mutational analysis in the present study are highlighted in shaded boxes.

Shuman unpublished). We infer that the RNA diphosphatase activity of BVP does not preclude timely guanylylation of nascent pre-mRNAs.

The crystal structure of the mammalian RNA triphosphatase domain Mce1(1−210) revealed that the enzyme is a globular monomeric protein with a deep, positively charged active site pocket that can accommodate a 5′ triphosphate end (*2*). The structure consists of five $\beta$-strands that form a central twisted sheet flanked by two α-helices on one side and four α-helices on the other. The secondary structure elements are shown above the Mce1 sequence in Figure 1. The conserved $^{125}$HCTHGFNRT$^{133}$ phosphate-binding loop (the P loop, highlighted in Figure 1) contains the cysteine nucleophile (Cys126 in Mce1; Cys119 in BVP) that forms a phosphoenzyme intermediate (*2*). The cysteine is situated in a deep pocket formed by residues from loops joining several of the secondary structural elements and also from residues in the N- and C-terminal regions.

Structural, biochemical, and mutational studies show that despite sharing a HCxxxxxR(S/T) motif, a phosphoenzyme intermediate, and a core α/$\beta$ fold with other cysteine phosphatases, the mechanism of phosphoanhydride cleavage by mammalian capping enzyme and baculovirus BVP is different in key respects from that used by protein phosphatases to hydrolyze phosphomonoesters (*2*, *13*). The most significant difference is the apparent absence of a carboxylate general acid catalyst in mammalian RNA triphosphatase and BVP. Mutational analysis highlighted two P-loop residues (His128 and Asn131 in Mce1; His121 and Asn124 in BVP) that are conserved in the RNA phosphatase subfamily and are critical for activity in vivo and in vitro (*2*, *12*, *13*). These functional groups may contribute to the specificity of Mce1 and BVP for the hydrolysis of phosphoanhydrides.

To gain further insights into the mechanism and specificity of the RNA-specific branch of the cysteine phosphatase superfamily, we performed a structure-guided mutational analysis of 14 new positions of BVP that are conserved in capping enzymes and/or PIR1 (these are highlighted in shaded boxes in Figure 1). Most of the residues chosen for mutagenesis are either located near the entrance to the active site pocket in the Mce1 crystal structure or else are engaged in hydrogen bond interactions with the P-loop or neighboring secondary structure elements (*2*). We identified nine residues (Trp6, Lys25, Thr62, Tyr67, Tyr68, Lys82, Arg153, Glu158, and Arg159) as essential or important for triphosphatase activity. Structure−activity relationships were clarified by conservative substitutions and are interpreted in light of the Mce1 crystal structure.

## EXPERIMENTAL PROCEDURES

*Missense Mutants of BVP.* Missense mutations and silent diagnostic restriction sites were introduced into the BVP gene by PCR as described (*13*). The PCR products were digested with *Nde*I and *Bam*HI and inserted into pET16b. The presence of the desired mutations in the pET16-BVP plasmids was confirmed by DNA sequencing; the inserted restriction fragments were sequenced completely in order to exclude acquisition of unwanted mutations during amplification and cloning. The wild-type and mutated pET16-BVP plasmids were transformed into *Escherichia coli* BL21(DE3)-pLysS. One liter cultures of *E. coli* BL21(DE3)pLysS/pET16-BVP were grown at 37 °C in LB medium containing 0.1 mg/mL ampicillin and 30 $\mu$g/mL chloramphenicol until the $A_{600}$ reached ∼0.6. The cultures were adjusted to 0.1 mM IPTG, and incubation was continued for 3 h at 30 °C with constant shaking. The cells were harvested by centrifugation, and recombinant $His_{10}$-tagged BVP was purified from the soluble bacterial extract by Ni−agarose chromatography as

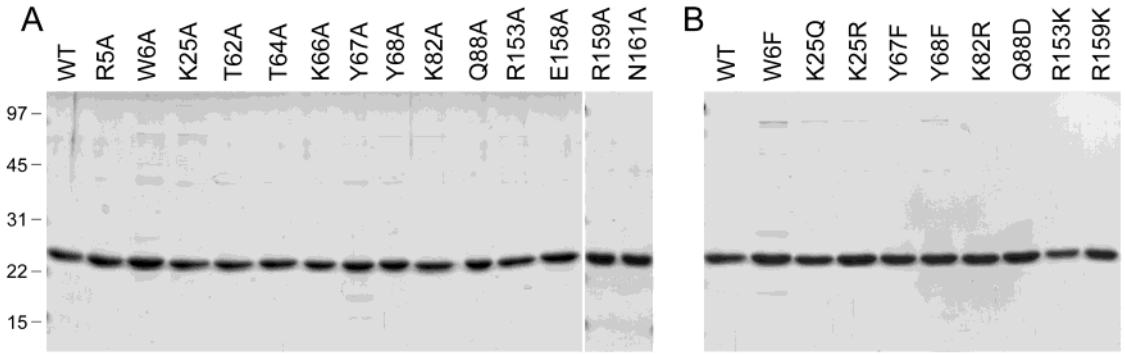


FIGURE 2: Purification of BVP mutants. Aliquots (4 $\mu$g) of the Ni−agarose preparations of wild-type (WT) BVP and the indicated BVP mutants were analyzed by electrophoresis through a 15% polyacrylamide gel containing 0.1% SDS. Polypeptides were visualized by staining with Coomassie blue dye. The positions and sizes (in kDa) of marker proteins are indicated. (A) Alanine mutants. (B) Conservative mutants.

described previously (*12*). The enzyme preparation was dialyzed against buffer containing 50 mM Tris HCl (pH 8.0) 200 mM NaCl, 2 mM DTT, 10% glycerol, and 0.1% Triton X-100 and stored at −80 °C.

*Determination of BVP Protein Concentration.* Aliquots (1× and 2×) of each phosphocellulose BVP preparation were analyzed by SDS−PAGE in parallel with 1, 2, 3, and 4 $\mu$g of bovine serum albumin. The gels were fixed and stained with Coomassie Blue dye. The staining intensity of the BVP polypeptide was quantitated with a Digital Imaging and Analysis System (Alpha Innotech Corporation). BVP concentrations were calculated by interpolation to the BSA standard curve.

*ATPase Assay.* Reaction mixtures (10 $\mu$L) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 100 $\mu$M [$\gamma$-$^{32}$P]ATP, and enzyme were incubated for 15 min at 30 °C. The reactions were quenched by adding 2.5 $\mu$L of 5 M formic acid. Aliquots of the reaction mixtures were applied to polyethyleneimine cellulose TLC plates that were developed in 0.5 M LiCl, 1 M formic acid. [$\gamma$-$^{32}$P]ATP and $^{32}P_i$ were quantitated by scanning the TLC plate with a phosphorimager.

*Yeast Vectors Encoding Mce1(211−597)-BVP Fusion Proteins.* The yeast *CEN TRP1* plasmid pYX-InvMce1-BVP encoding a chimeric Mce1(211−597)-BVP fusion protein (*Inv*Mce1-BVP) driven by the yeast *TPI1* promoter was constructed as described previously (*12*). Mutated versions of the BVP gene in the pET16-BVP plasmids were PCR-amplified using the T7 and S2 primers (*12*). The PCR products were digested with *Stu*I and *Bam*HI and then inserted into pYX-InvMce1 in place of the "wild-type" *Stu*I-*Bam*HI fragment. The inserts of pYX-InvMce1-BVP plasmids were sequenced completely to confirm the in-frame fusion and to exclude the introduction of unwanted coding changes during amplification and cloning.

*Assay of in Vivo Activity of BVP by Plasmid Shuffle.* The yeast *cet1*Δ strain YBS20 (*trp1 ura3 leu2 cet1::LEU2* p360-Cet1[*CEN URA3 CET1*]) was transformed with pYX-InvMce1-BVP plasmids. Trp$^+$ isolates were selected and then streaked on agar plates containing 0.75 mg/mL of 5-fluoroorotic acid (5-FOA) (*16*). Growth was scored after 7 days of incubation at 23, 30, and 37 °C. Lethal mutants (scored as −) were those that failed to form colonies on 5-FOA at any temperature. Individual colonies of the viable strains were picked from the 5-FOA plate at permissive temperature and patched on SC(-Trp) agar. Two isolates of each mutant were streaked on YPD agar at 23, 30, and 37 °C. Growth was assessed as follows: ++ indicates colony size indistinguishable from strains bearing wild-type *InvMCE1-BVP*; + denotes reduced colony size. Temperature-sensitive (ts) mutants were those that did not form colonies at 37 °C after 4 days. All of the pYX-InvMce1-BVP plasmids were capable of complementing the growth of a *ceg1*Δ strain in a plasmid shuffle assay.

## RESULTS

*Effects of Alanine Mutations on BVP Phosphohydrolase Activity.* Alanine was introduced in lieu of 14 individual amino acids of BVP. The residues mutated were Arg5, Trp6, Lys25, Thr62, Thr64, Lys66, Tyr67, Tyr68, Lys82, Gln88, Arg153, Glu158, Arg159, and Asn161 (Figure 1). Wild-type BVP and the BVP−Ala mutants were expressed in bacteria as His-tagged fusions and purified from soluble bacterial lysates by Ni−agarose chromatography. SDS−PAGE analysis showed that the Ni−agarose preparations were highly enriched with respect to the ~24 kDa BVP polypeptide and that the extents of purification were similar (Figure 2A). Recombinant wild-type BVP catalyzed the release of $^{32}P_i$ from [$\gamma$-$^{32}$P]ATP. The extent of ATP hydrolysis during a 15 min reaction was linear with input enzyme at limiting BVP concentrations, and the reaction proceeded to completion at saturating enzyme levels (not shown). Wild-type BVP released 2 pmol of $^{32}P_i$ per ng of protein. The specific ATPase activities of the BVP−Ala mutants were determined by enzyme titration. The findings are summarized in Figure 3, where the ATPase activities of the mutants are expressed as the percent of the wild-type specific activity. For the purpose of stratifying mutational effects, we set a 5-fold activity decrement as the working criterion of significance for alanine substitution. Side chains are deemed "important" when alanine substitution reduces specific activity to 6 to 20% of the wild-type value. Our operational definition of an "essential" residue is one at which alanine substitution reduces specific activity to ≤5% of the wild-type activity.

Mutants W6A, K25A, and R153A were effectively inert in ATP hydrolysis at a level of sensitivity of <0.1% of the wild-type specific activity. Thus Trp6, Lys25, and Arg153 are essential by the criteria discussed above. Mutants T62A, Y67A, Y68A, K82A, E158A, and R159A displayed between 7% and 18% of wild-type ATPase activity. Therefore Thr62, Tyr67, Tyr68, Lys82, Glu158, and Arg159 are classified as functionally important, albeit not essential. The activities of

| *BVP Mutant* | *ATPase Activity (% of WT)* | *$^{32}$P–BVP (% of WT)* | *Complementation of cet1Δ* |
|---|---|---|---|
| R5A | 95 | 8 | ++ (ts) |
| W6A | <0.1 | 1 | – |
| W6F | 2 | 2 | – |
| K25A | <0.1 | 1 | – |
| K25Q | <0.1 | 1 | – |
| K25R | 11 | 10 | ++ (ts) |
| T62A | 17 | 18 | ++ (ts) |
| T64A | 36 | 78 | ++ (ts) |
| K66A | 140 | 100 | ++ |
| Y67A | 18 | 15 | – |
| Y67F | 97 | 130 | ++ |
| Y68A | 18 | 74 | – |
| Y68F | 12 | 11 | – |
| K82A | 7 | 83 | + (ts) |
| K82R | 17 | 100 | ++ (ts) |
| Q88A | 180 | 92 | ++ |
| Q88D | 55 | 55 | ++ |
| R153A | <0.1 | 2 | – |
| R153K | <0.1 | 1 | – |
| E158A | 13 | 19 | ++ (+) |
| R159A | 18 | 17 | – |
| R159K | 28 | 22 | ++ |
| N161A | 70 | 47 | ++ |

FIGURE 3: Mutational effects on BVP phosphohydrolase activity and complementation of *cet1*Δ. The ATPase specific activities of the BVP mutants were normalized to that of wild-type BVP (100%). The extents of phosphoenzyme formation by the BVP mutants (see Figure 4) were normalized to that of wild-type BVP (100%). The in vivo activity of the mutated *BVP* alleles in complementation of the yeast *cet1*Δ deletion was tested by plasmid shuffle and growth was scored as described under Experimental Procedures. The growth score was the same at all three temperatures tested unless qualified by scores in parentheses as follows: ts indicates growth at 23 and 30 °C, but not at 37 °C; + indicates smaller colony size at 37°C.

mutants R5A, K66A, Q88A, and N161A were within a factor of 2 of wild-type BVP activity. Also, the modest effects of the T64A change (36% of wild-type activity) did not meet our criterion of significance. Thus, Arg5, Lys66, Thr64, and Asn161 were deemed unimportant and were not subjected to further mutational analysis.

*Effects of Conservative Mutations on BVP Phosphohydrolase Activity.* Alanine substitution eliminates the side chain beyond the $\beta$ carbon but does not reveal the properties of the missing side chain that are essential or important for activity. This was addressed by introducing conservative substitutions at the three newly defined essential residues and at four of the positions deemed important. The purity of the conservatively substituted recombinant BVP proteins was confirmed by SDS−PAGE (Figure 2B). The specific ATPase activities of the conservative mutants were determined by enzyme titration and are shown in Figure 3. The instructive findings are summarized below.

The W6F mutant was 2% as active as wild-type BVP. The failure to restore activity by introducing an alternative aromatic amino acid underscored the strict requirement for a tryptophan at this position. Lys25 was replaced conservatively by glutamine and arginine. Whereas the K25Q mutant was as defective in ATP hydrolysis as K25A, the K25R change restored activity to 11% of wild-type. These findings suggested that the essential function of the side chain at position 25 depends on its positive charge. In contrast, the introduction of a lysine in place of essential side chain Arg153 had no salutary effect, i.e., R153K was catalytically inert, just like R153A. We surmise that Arg153 makes essential bidentate interactions that cannot be achieved by lysine.

Distinctive structure−activity relationships were seen at vicinal residues Tyr67 and Tyr68. Whereas ATPase activity was restored fully when a phenylalanine was introduced at Tyr67 (97% activity for Y67F vs 18% for Y67A), the phenylalanine substitution at position 68 had no salutary effects and actually reduced BVP activity compared to Y68A (12% activity for Y68F vs 18% for Y68A). We infer that the aromatic ring of Tyr67 suffices for full activity and that potential hydrogen-bonding interactions of the hydroxyl are not important. In contrast, the hydroxyl of Tyr68 is important for ATP hydrolysis. Arg159 was substituted conservatively by lysine; the activity of R159K (28% of wild-type) was modestly higher than that of R159A (18%), such that R159K activity exceeded our threshold criterion for a significant mutational effect.

*Mutational Effects on Phosphoenzyme Formation.* The recombinant mutant BVP proteins were assayed for phosphoenzyme formation during a brief reaction with [$\gamma$-$^{32}$P]-ATP on ice at pH 5.5, conditions shown previously to be optimal for capturing the reaction intermediate (*13*). Label transfer to the BVP polypeptide was detected by SDS−PAGE and autoradiography (Figure 4). The relative yields of $^{32}$P−BVP polypeptide were quantitated by scanning the gels with a phosphorimager and are expressed in Figure 3 as the percent of the wild-type value. There was good concordance in most cases between the mutational effects on steady-state ATP hydrolysis at pH 7.5 and the yield of phosphoenzyme at pH 5.5. For example, the W6A, W6F, K25A, K25Q, R153A, and R153K mutations that abolished ATP hydrolysis also eliminated phosphoenzyme formation, while the conservative K25R change that restored ATPase activity to 11% of wild-type also partially revived phosphoenzyme formation.

T62A, Y67A, E158A, Y68F, R159A, and R159K, which were partially defective in ATP hydrolysis, formed lower levels of phosphoenzyme than did wild-type BVP (Figure 4). Mutations T64A, K66A, Q88A, Y67F, and N161A, which had little impact on ATP hydrolysis, similarly had little effect on phosphoenzyme formation. These data provide additional correlative evidence that the covalent BVP thiophosphate adduct is a genuine reaction intermediate and that most mutations affecting BVP ATPase activity impact on the first step of the two-step ping-pong reaction.

The exceptional cases included mutations Y68A, K82A, and K82R, which reduced ATP hydrolysis in the steady-state (to 18%, 7%, and 17% of wild-type activity, respectively) but had little or no impact on the extent of phosphoenzyme formation (Figure 4). These mutations may selectively impair the second step in the proposed reaction pathway, in which the phosphoenzyme is hydrolyzed to release $P_i$. R5A was the lone instance in which steady-state ATPase activity was unaffected while the yield of phosphoenzyme was reduced significantly (Figure 4).

*Mutational Effects on Capping Activity in Yeast. Inv*Mce1-BVP is a chimeric capping enzyme consisting of BVP fused to the C-terminus of the guanylyltransferase domain of

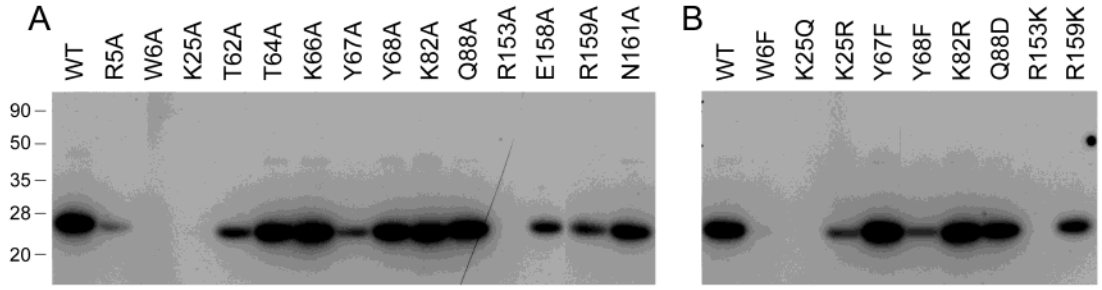


FIGURE 4: Mutational effects on BVP phosphoenzyme formation. Reaction mixtures (10 $\mu$L) containing 50 mM Tris-acetate (pH 5.5), 5 mM DTT, 10 $\mu$M [$\gamma$-$^{32}$P]ATP, and 2 $\mu$g of wild-type or mutant BVP as specified were incubated on ice for 15 s. After quenching with SDS, the mixtures were analyzed by SDS−PAGE and autoradiography. (A) Alanine mutants. (B) Conservative mutants. The relative yields of $^{32}$P−BVP polypeptide were quantitated by scanning the gels with a phosphorimager.

mammalian capping enzyme. We reported previously that *Inv*MCE1-BVP complemented the *S. cerevisiae cet1*Δ mutant (*12*). By testing a collection of 28 biochemically characterized BVP mutants (mostly located in the P-loop) for capping function in yeast, we found that (i) any mutation that reduced triphosphatase activity in vitro to ≤2% of wild-type abrogated BVP activity in vivo, (ii) mutants that retained at least one-fourth of wild-type activity in vitro were functional in vivo, and (iii) whereas a threshold level of triphosphatase activity was required for BVP function in yeast, the retention of triphosphatase activity in the range of 10% of wild-type did not inevitably suffice for in vivo function (*12*). The key point was that *Inv*Mce1 fusions and yeast genetics can be used to assess structure−activity relationships in vivo for heterologous RNA triphosphatases, including the monofunctional class of cysteinyl phosphatase-type enzymes such as BVP, for which a physiological substrate is not clearly defined.

To correlate the new mutational effects seen here on BVP activity in vitro with in vivo activity in cap synthesis, we introduced the new Ala and conservative mutations into *Inv*MCE1-BVP and tested them by plasmid shuffle in yeast strains deleted for endogenous capping enzymes. Control experiments showed that all of the *Inv*MCE1-BVP alleles were able to complement the guanylyltransferase deletion *ceg1*Δ, confirming that the chimeric *Inv*Mce1-BVP proteins were all produced in vivo at levels sufficient for cap formation by the guanylyltransferase component (data not shown). The mutational effects on triphosphatase function in capping (*cet1*Δ complementation) are summarized in Figure 3.

All the mutations in essential side chains that abolished triphosphatase activity in vitro were lethal in vivo, e.g., *W6A*, *W6F*, *K25A*, *K25Q*, *R153A*, and *R153K*. Six other mutations (*Y67A*, *Y68A*, *Y68F*, and *R159A*) were lethal in vivo, even though the mutant recombinant BVP enzymes displayed 12−18% residual triphosphatase activity in vitro compared to wild-type BVP (Figure 4). Factors such as mutational effects on protein folding and stability or substrate binding affinity in the yeast milieu may come into play to account for the lethality of latter group of mutants. Conservative mutations *K25R*, *Y67F*, and *R159K* revived the in vivo function of BVP compared to the lethal alanine mutants, just as they elicited either full or partial restoration of phosphohydrolase activity in vitro. In the case of Arg159 mutations, the shift from lethality of the *R159A* allele to normal growth of the *R159K* strain correlated with a change from 18% to 28% of wild-type activity in vitro, which underscores the theme that the growth readout requires a threshold level of BVP activity.

It is noteworthy that *K25R* cells were viable at 23−30 °C, but unable to grow at 37 °C, suggesting that either (i) the mutant triphosphatase domain of *Inv*Mce1-BVP−K25R is thermosensitive or (ii) the threshold level of triphosphatase activity required for yeast growth is temperature-dependent. Similar interpretations apply to mutations *T62A*, *T64A*, and *K82R*, which reduced BVP phosphohydrolase activity in vitro and elicited temperature-sensitive (ts) growth phenotypes in vivo, i.e., the mutant *Inv*MCE1-BVP yeast strains grew normally at 23−30 °C, but failed to grow at 37 °C (Figure 3). The *K82A* mutation, which reduced triphosphatase activity to 7% of wild-type, resulted in slowed growth at the permissive temperature of 23−30 °C and no growth at 37 °C. The *E158A* mutation resulted in slow growth at 37 °C.

*Effect of Changing Gln88 to Asp.* The hydrolysis of phosphomonoesters by the classical protein phosphatases of the cysteine phosphatase superfamily depends on a conserved aspartate general acid that is located on a flexible loop connecting a central $\beta$-strand and an adjacent α-helix (*7*, *14*). The general acid loop undergoes a conformational change upon substrate binding that closes off the active site pocket and brings the aspartate (which is protonated in the ground state) into the active site, where it then serves as a proton donor to expel the hydroxyl amino acid leaving group (Ser, Thr, or Tyr). The structure of Mce1 reveals a similarly positioned loop between the $\beta$4 strand and α5 helix (residues 88−99). The conformation of this loop in Mce1 resembles that of the general acid loop in the unliganded forms of the protein tyrosine phosphatases. However, a structural alignment shows that there is no conserved aspartate or glutamate residue within this loop region of Mce1 (or BVP) to serve as a candidate general acid (*2*). We have proposed that Mce1 and BVP can cleave phosphoanhydride bonds without a general acid catalyst because the relatively low p$K_a$ of the 5′ diphosphate leaving group circumvents the need for proton transfer (*2*, *13*).

We considered the possibility that the triphosphatase reaction might nonetheless be accelerated if a proton donor were introduced into the loop at a position corresponding to the essential aspartate of the protein phosphatases. Structural alignment suggests that Gln88 of BVP occupies that position; thus, we replaced Gln88 with Asp and purified the recombinant Q88D protein (Figure 2B). Steady-state ATP hydrolysis by Q88D was 55% of the wild-type specific activity (Figure 3), indicating that the presence of the carboxylate does not upgrade the activity of BVP. The Q88D protein formed near wild-type levels of the phosphoenzyme inter-

mediate (Figure 4) and was functional in RNA capping when expressed in yeast (Figure 3). We surmise that BVP activity is not limited by the absence of an aspartate side chain in the equivalent of the general acid loop of the protein phosphatases.

## DISCUSSION

Mce1 and BVP belong to a distinct branch of the cysteine phosphatase superfamily that has evolved to hydrolyze phosphoanhydride bonds. Although the tertiary structure is conserved between the RNA 5′-phosphatases and other cysteine phosphatases, there is almost no conservation of primary structure outside of the cysteine-containing P-loop segment that comprises the active site (*2*). Nevertheless, there is considerable primary structure conservation among the members of the RNA phosphatase family, whether from cellular or viral sources and whether they are bifunctional capping enzymes or monofunctional RNA phosphatases (Figure 1). Having already identified via mutagenesis residues within the P-loop of BVP that are essential for BVP activity in vivo and in vitro, we set out here to evaluate the functional significance of sequence conservation elsewhere in the protein. Out of 14 residues targeted for mutation, we identified three side chains that are essential for activity (Trp6, Lys25, and Arg153) and six others that we deemed functionally important (Thr62, Tyr67, Tyr68, Lys82, Glu158, and Arg159). On the basis of the locations of the equivalent residues in the crystal structure of Mce1, we surmise that the essential and important residues are not likely to participate directly in catalysis but, rather, ensure either proper conformation of the P-loop or the overall tertiary structure of the enzyme. The mutational effects at specific residues are interpreted in light of the crystal structure as outlined below.

*Arg153 Stabilizes the P-loop in an Active Conformation.* Arg153 of BVP (corresponding to Arg160 of Mce1) is strictly conserved in all other RNA triphosphatases (Figure 1). In fact, this arginine is the only side chain outside the P-loop that is conserved in the RNA triphosphatase, protein phosphatase, and phoshoinositide phosphatase branches of the cysteine phosphatase superfamily (*2*, *7*, *14*). In the Mce1 crystal structure, the N$\epsilon$ and NH2 nitrogens of this invariant arginine form hydrogen bonds to the carbonyl oxygens of two residues of the P-loop (His128 and Gly129 in Mce1, equivalent to His121 and Gly122 in BVP) on the "back" side of the loop facing away from the oxyanion hole formed by the backbone amides of the P-loop pointing into the active site. In addition, the NH1 guanidinium nitrogen of the arginine forms a hydrogen bond to the backbone carbonyl of a residue in the first $\beta$ strand (Pro14 in Mce1, equivalent to Leu10 in BVP). Our mutational data testify to the critical role of the bidentate hydrogen bond from the arginine to the P-loop in stabilizing the active conformation of BVP (i.e., R153K was just as defective as R153A in vitro and in vivo). The corresponding R160A mutation in Mce1 elicited a tight temperature-sensitive growth defect in vivo in yeast (*2*). The equivalent mutations of this arginine in *Yersinia* protein tyrosine phosphatase, R440A and R440K, reduced $k_{cat}$ by three and 2 orders of magnitude, respectively (*15*).

*Trp6 and Lys25 Form an Essential $\pi$-Cation Pair*. Trp6 and Lys25 of BVP (corresponding to Trp10 and Lys28 of Mce1) are conserved in all members of the RNA triphosphatase family (Figure 1), and both side chains are essential for BVP activity. The tryptophan and lysine side chains are in close proximity and engaged in a $\pi$-cation stack in the Mce1 crystal structure (*2*). The LysN$\zeta$ makes additional hydrogen bonds to two backbone carbonyl oxygens (to Trp10 and Cys13 in Mce1, corresponding to Trp6 and Tyr9 of BVP). The effects of conservative mutations on BVP function highlight the critical role of the $\pi$-cation stack (K25Q being inert) and the specific requirements for tryptophan and lysine as its components (with W6F and K25R having 2 and 11% activity, respectively). The $\pi$-cation pair is buried within the hydrophobic core of the triphosphatase. The tryptophan aromatic ring interacts on the face opposite the lysine with a leucine side chain (Leu31 in Mce1; Leu28 in BVP) conserved in every other RNA phosphatase family member. It is likely that this cation−aromatic−hydrophobic sandwich stabilizes the first two strands of the central $\beta$ sheet of BVP and nearby surface loops that flank the entrance to the active site pocket. Neither Trp6 nor Lys25 is in a position to interact directly with the substrate or with catalytic side chains of the P-loop.

*The $^{62}$TxTxKYY$^{68}$ Loop*. The BVP sequence $^{58}$IIDLTNTSKYY$^{68}$ is the most highly conserved segment of primary structure in the RNA triphosphatase enzymes outside of the P-loop (Figure 1). Previous studies had shown that the invariant Asp side chain at the proximal margin of this element is not important for the activity of either BVP or Mce1 in vitro or in vivo (*2*, *13*). The proximal residues of this element comprise the third strand in the central $\beta$ sheet and the distal residues ($^{68}$TNTSRFY$^{74}$ in Mce1; corresponding to $^{62}$TNTSKYY$^{68}$ in BVP) form a surface loop that lines the entrance to the active site pocket. Here we found that Thr62, Tyr67, and Tyr68 are important for BVP triphosphatase activity in vitro. The first threonine of the loop in the Mce1 structure (Thr68, equivalent to BVP Thr62) makes a bifurcated H-bond from O$\gamma$ to the backbone amide and carbonyl of the second threonine (Thr70; Thr64 in BVP) and makes van der Waals contacts to the phenyl ring of Phe73 (Tyr67 in BVP). We presume that these interactions contribute to the stability of the loop and account for the mutational effects seen at BVP Thr62 and Tyr67. The conservative Y67F mutation of BVP restores full activity. Insofar as Phe is naturally present at this position in Mce1 and in the *Drosophila*, *Anopheles*, and *Xenopus* capping enzymes (Figure 1), we surmise that aromaticity is the relevant property and the hydrophobic interaction with threonine is functionally important. This is another instance (like the Trp-Lys pair noted above) in which interacting side chains are conserved in the RNA triphosphatase family and Ala-mutation at one residue of the pair effectively phenocopies mutations of the second residue (e.g., T62A and Y67A are 17−18% as active as wild-type BVP in vitro). Although T62A and Y67A have similar catalytic activities, the in vivo mutation effect is more severe for *Y67A* (lethal) than *T62A* (ts). In the Mce1 crystal, the aromatic side chain makes an additional van der Waals contact with C$\gamma$ of the second threonine in the loop (Thr64 in BVP). The T64A mutation, though not catalytically significant by our criteria, did confer a ts growth phenotype in yeast. Thus, the lethality of *Y67A* may reflect the combined effects of the loss of stabilizing contacts with several residues of the loop.

The distal tyrosine of the loop (Tyr74 in Mce1; Tyr68 in BVP) is strictly conserved in all of the RNA triphosphatases (Figure 1). In the Mce1 structure, the Tyr side chain is buried in the interior of the protein, where the phenolic OH forms a hydrogen bond to N$\epsilon$ of the first histidine of the P-loop (His125 in Mce1; His118 in BVP). The hydrogen bonding capacity of the tyrosine is functionally relevant, insofar as the Y68F protein was no more active than Y68A, and both *Y68A* and *Y68F* were lethal in vivo. The Tyr68 mutations phenocopy the in vivo lethality of mutations in His118, the side chain with which Tyr68 interacts (*12*). However, whereas the His118A protein is apparently inert in ATP hydrolysis (*13*), the Y68A and Y68F proteins retain 12−18% of wild-type activity. We infer that Tyr68 plays a noncatalytic role in stabilizing the P-loop conformation via His118, which is in turn predicted (according to the Mce1 structure) to H-bond via N$\delta$ to the backbone carbonyl of the catalytic P-loop cysteine (*2*).

*Lys82*. The Lys82 side chain of BVP (Lys88 in Mce1) is conserved among the RNA triphosphatase enzymes (Figure 1). In the Mce1 structure, this lysine is located in the fourth strand of the $\beta$ sheet. The side chain is on the protein surface and N$\zeta$ makes a hydrogen bond to the backbone carbonyl of the second threonine of the TxTxKYY loop (Thr70 in Mce1; Thr64 in BVP). Substituting the BVP lysine with alanine results in significant loss of NTPase activity in vitro (7% of wild-type) and growth defects in yeast (slowed growth at permissive temperature and no growth at 37 °C); yet there was little effect on formation of the phosphoenzyme adduct in vitro. Replacement by arginine only partially ameliorated the defects of K82A. We surmise that the conserved lysine tethers the TxTxKYY loop to $\beta$4 and thereby stabilizes the enzyme fold.

*Arg159*. Position 159 of BVP is conserved as a basic residue in all of the RNA triphosphatases and is either an arginine in BVP and PIR1 or a lysine in the RNA capping enzymes (Figure 1). The equivalent Lys166 residue of Mce1 is located on the enzyme surface with the basic side chain at the rim of the active site pocket (*2*). The side chain makes no contacts with other constituents of the protein, suggesting a potential contribution by Lys166 in substrate binding. This speculation is consistent with the BVP mutational results, whereby the R159A protein has reduced ATPase activity and is nonfunctional in yeast. Introduction of a lysine at this position of BVP (thereby mimicking the natural lysine of Mce1) restored function in yeast and boosted the ATPase activity above our threshold of significance.

*Arg5 and Glu158*. BVP residues Arg5 and Glu158 correspond to Arg9 and Tyr165 in Mce1. The Mce1 structure shows that Arg9 and Tyr165 are located on the rim of the active site pocket and interact via hydrogen bond between a terminal guanidium nitrogen and the phenolic oxygen. Both side chains also coordinate a water molecule in the active site (*2*). Mce1 mutations R9A and Y165A had no effect on complementation of yeast *cet1*$\Delta$ (*2*). Assuming that BVP side chains Arg5 and Glu158 occupy similar positions in the tertiary structure, we predict that they would form an ion-pair. In agreement with the in vivo finding for Mce1, we see that neither Arg5 nor Glu158 is required for triphosphatase activity in yeast, although the R5A and E158A mutations did result in impaired growth at 37 °C. The Arg5 position of BVP is not conserved in all members of the RNA triphosphatase family but is substituted by glycine and alanine in the capping enzymes of *Arabidopsis* and ISKNV, respectively (Figure 1). We found that the BVP R5A mutant was fully active in steady-state ATP hydrolysis but had unexpectedly low activity in phosphoenzyme formation. It is conceivable that R5A activity is sensitive to the low-temperature and low-pH conditions used to capture the phosphoenzyme. The Glu158 position of BVP is conserved in PIR1, but it is a tyrosine in all of the capping enzymes. The E158A mutation of BVP reduced ATPase activity to 13% of wild-type. Whether this effect is direct (via Glu158 contacting substrate) or indirect (via altered electrostatics near the active site) remains to be clarified.

In summary, we have shown that residues outside of the signature P-loop that are characteristic of RNA triphosphatases are either essential or important for the triphosphatase activity of BVP. Although a structural role for these residues is inferred, a fuller understanding of the functional data will hinge on crystallizing of BVP with substrate bound at the active site.

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