Mechanism of Phosphoanhydride Cleavage by Baculovirus Phosphatase*

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Baculovirus phosphatase (BVP) is a member of the metazoan RNA triphosphatase enzyme family that includes the RNA triphosphatase component of the mRNA capping apparatus. BVP and other metazoan RNA triphosphatasases belong to a superfamily of phosphatases that act via the formation and hydrolysis of a covalent cysteinyl-phosphate intermediate. Here we demonstrate the formation of a BVP phosphoenzyme upon reaction with [γ32P]ATP and identify the linkage as a thio-phosphate based on its chemical lability. We surmise that the phosphate is linked to Cys119 of BVP because replacement of Cys119 by alanine or serine abrogates phosphoenzyme formation and phosphohydrolase activity. The catalytic cysteine is located within a conserved phosphate-binding loop (118HCTHGINRTGY128). We show that all of the non-aliphatic side chains of the phosphate-binding loop are functionally important, insofar as mutants H118A, H121A, N124A, R125A, and T126A, and Y128A were inactive insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive. The catalytic cysteine is situated within a conserved phosphate-binding loop (118HCTHGINRTGY128). We show that all of the non-aliphatic side chains of the phosphate-binding loop functionally important, insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive. The catalytic cysteine is located within a conserved phosphate-binding loop (118HCTHGINRTGY128). We show that all of the non-aliphatic side chains of the phosphate-binding loop are functionally important, insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive. The catalytic cysteine is situated within a conserved phosphate-binding loop (118HCTHGINRTGY128). We show that all of the non-aliphatic side chains of the phosphate-binding loop are functionally important, insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive. The catalytic cysteine is situated within a conserved phosphate-binding loop (118HCTHGINRTGY128). We show that all of the non-aliphatic side chains of the phosphate-binding loop are functionally important, insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive. The catalytic cysteine is located within a conserved phosphate-binding loop (118HCTHGINRTGY128). We show that all of the non-aliphatic side chains of the phosphate-binding loop functionally important, insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive. The catalytic cysteine is situated within a conserved phosphate-binding loop (118HCTHGINRTGY128). We show that all of the non-aliphatic side chains of the phosphate-binding loop are functionally important, insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive. The catalytic cysteine is located within a conserved phosphate-binding loop (118HCTHGINRTGY128). We show that all of the non-aliphatic side chains of the phosphate-binding loop are functionally important, insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive. The catalytic cysteine is situated within a conserved phosphate-binding loop (118HCTHGINRTGY128). We show that all of the non-aliphatic side chains of the phosphate-binding loop are functionally important, insofar as mutants H118A, H121A, N124A, R125A, T126A, and Y128A were inactive.

RNA triphosphatase catalyzes the hydrolysis of the γ-phosphate of triphosphate-terminated RNA to form a diphosphate end. Two distinct classes of eukaryotic RNA triphosphatases have been described. The RNA triphosphatases of metazoans and plants belong to a superfamily of phosphatases that includes protein-tyrosine phosphatases, dual specificity protein phosphatase enzyme superfamily (Fig. 1). Protein-tyrosine phosphatases and dual specificity protein phosphatases catalyze the dephosphorylation of phosphoproteins via a two-step pathway (21, 22). First, a cysteine thiolate nucleophile of the enzyme attacks the phosphomonoester (R-O-P) to form a covalent protein-cysteinyl-S-phosphate intermediate and liberate R-OH (Fig. 1). Then, the covalent intermediate is hydrolyzed to liberate inorganic phosphate. The cysteine within the signature motif is the active site of phosphohydrolyase activity. The attack of cysteine on a phosphomonoester to form the phosphoenzyme is driven by two principal enzymic catalytic substrates: (i) a conserved arginine in the phosphate-binding motif that makes a bidentate interaction with the nonbridging phosphate oxygens and thereby stabilizes the transition state, and (ii) a conserved aspartate, which is protonated in the ground state and acts as a general acid to expel the leaving group oxygen. The general acid is located within a flexible loop element that is distant from the active site pocket when the enzyme is in the unliganded state and moves into the active site upon binding of substrate or substrate analog (23–26). A mechanism of regulation of protein phosphatases through the flexible general acid loop, either positively by promoting closure of the loop or negatively by hindering its mobility, has been discussed (26).

Although several groups have reported that mutation of the

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† The abbreviations used are: BVP, baculovirus phosphatase; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.
active-site cysteine of metazoan RNA triphosphatases to alanine or serine abolishes RNA triphosphatase activity in vitro and in vivo (1, 4, 6, 10, 11), there is as yet no direct evidence for the formation of a phosphoenzyme during the RNA triphosphatase reaction.

Here we probe the catalytic mechanism of the baculovirus RNA triphosphatase BVP. The 168-amino acid BVP protein displays extensive sequence similarity to the triphosphatase domains of metazoan and plant capping enzymes. The similarity embraces the cysteine-containing signature motif and a single conserved aspartic acid (Fig. 1). Purified recombinant BVP hydrolyzes the γ-phosphate of triphosphate-terminated poly(A) or the triphosphate-terminated trinucleotide pppApCpC (10, 11). BPV also hydrolyzes ATP to ADP and GTP phosphorylated prior to the γ-phosphate and that the enzyme acts dispensably, 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. Human PIR1, like BVP, is also an RNA triphosphatase and diphosphatase (12). In contrast, the metazoan capping enzymes hydrolyze only the γ-phosphate of RNA. Thus, BVP and PIR1 comprise a functionally distinctive subfamily of cysteinyl phosphate intermediates.

We demonstrate here the formation of a covalent phosphoenzyme adduct during the reaction of BVP with [γ-32P]ATP. Suboptimal reaction conditions, i.e. low temperature and mildly acidic pH, were required to detect the phosphoenzyme. The lability of the phosphoenzyme adduct to treatment with iodine and 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. Human PIR1, like BVP, is also an RNA triphosphatase and diphosphatase (12). In contrast, the metazoan capping enzymes hydrolyze only the γ-phosphate of RNA. Thus, BVP and PIR1 comprise a functionally distinctive subfamily of cysteinyl phosphate intermediates.

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**Experimental Procedures**

**Missense Mutants of BVP**—Plasmid pET16-BVP encodes the 168-amino acid BVP polypeptide fused to an N-terminal leader peptide containing 10 tandem histidines (10). Expression of the protein is under the control of a T7 RNA polymerase promoter. Missense mutations and “silent” diagnostic restriction sites were introduced into the BVP gene by polymerase chain reaction using the two-stage overlap extension method (39). pET16-BVP was used as the template for the first-stage amplifications. The mutated DNA products of the second-stage amplification were digested with Ncol and BamHI and inserted into pET16b. The presence of the desired mutations was confirmed by DNA sequencing; the inserted restriction fragments were sequenced completely in order to exclude acquisition of unwanted mutations during amplification and cloning.

**Expression and Purification of Recombinant BVP**—The wild-type and mutated pET16-BVP plasmids were transformed into Escherichia coli BL21. BVP expression was induced by infection of the cells with λCE6, which carries the T7 RNA polymerase gene. Cultures (1 liter) of E. coli BL21/pET16-BVP were grown at 37°C in Luria-Bertani medium containing 0.2% maltose and 0.1 mg/ml ampicillin until the Optical Density (OD) reached 0.3–0.4. The cultures were supplemented with 10 mM MgSO4 and 0.4% glucose, and incubation was continued at 37°C until the OD reached ~0.8. The cultures were chilled on ice for 15 min and then infected by adding λCE6 stock to attain a multiplicity of 5. The cultures were then incubated for 4 h at 25°C with continuous shaking, after which the cells were harvested by centrifugation. The recombinant BVP proteins were purified from soluble bacterial lysates by nickel-agarose and phosphocellulose column chromatography as described previously (10).

**Determination of BVP Protein Concentration**—Aliquots of each phosphocellulose BVP preparation were analyzed by SDS-PAGE in parallel with 1, 2, 3, and 4 μg of bovine serum albumin. The gels were fixed and stained with Coomassie Blue dye. The staining intensity of the polypeptide bands was visualized with a FUJIX BAS2000 phosphorimager.

**Detection and Chemical Stability of a BVP Phosphoenzyme**—A reaction mixture containing 50 mM Tris acetate (pH 5.5), 1 mM DTT, 3 μM BVP, and 20 μM [γ-32P]ATP was incubated on ice for 45 s. The mixture was then made 2% in SDS. Aliquots (1.5 μl) were distributed to separate tubes and treated with the following reagents in a total volume of 15 μl: 0.1 mM NaOH, 1 mM hydroxylamine (pH 7.0), 1 mM glycine (pH 7.0), 1 mM Tris-HCl (pH 7.0), 0.1 mM Tris-HCl (pH 7.0), 1 mM Tris-HCl (pH 7.0), and 1 mM Tris-HCl (pH 7.0). Additional aliquots (1.5 μl) were further adjusted in a total volume of 15 μl of the following solutions: 1 mM HCl, 0.1 mM NaCl, 1 mM glycine (pH 2.0), 0.1 mM Tris formate (pH 3.0), 0.1 mM Tris acetate (pH 4.0, 5.0, or 6.0), 300 mM Tris-HCl (pH 7.0 or 8.0), 0.1 mM TBT (pH 9.0 or 10.0), 0.1 mM NaOH. The SDS concentration was maintained at 2% during the chemical treatments. The mixtures were incubated at 37°C for 30 min, and the samples were analyzed by electrophoresis through a 15% polyacrylamide gel containing 0.1% SDS. (The mixtures in which pH was varied widely from neutrality were adjusted to neutrality prior to SDS-PAGE). The gels were soaked for 30 min in a solution of 3% glycerol and 30% methanol and then dried using the Promega gel-drying kit according to the vendor’s instructions. [32P]BVP adducts were visualized by autoradiography and quantitated by scanning the gel with a FUJIX BAS2000 phosphorimager.
**RESULTS**

Formation of a Covalent Phosphoenzyme Adduct during the Reaction of BVP with ATP—Although hydrolysis of the γ phosphate of triphosphate-terminated RNAs or nucleoside triphosphatases by members of the cysteine phosphatase superfamily is now well documented, there has been no direct evidence presented for a phosphoenzyme intermediate analogous to that shown for the protein-tyrosine phosphatases and dual-specificity protein phosphatases (27, 28). Our initial efforts to trap a phospho intermediates by members of the cysteine phosphatase superfamily were frustrated by the instability of the phosphoenzyme (27, 28). We were unable to detect a phosphoenzyme when the incubations were performed at 30 °C at pH 7.5 (the optimal conditions for ATP hydrolysis in the steady state) because the substrate was hydrolyzed completely to 32P (data not shown). Decreasing the reaction temperature to 4 °C reduced ATP hydrolysis by BVP by a factor of 18, but did not facilitate detection of a phosphoenzyme at pH 7.5 (data not shown). We inferred that hydrolysis of a putative phosphoenzyme intermediate might be much faster than phosphoril transfer from ATP when the reactions were performed at pH 7.5. Thus, we varied the pH of the reaction mixture from 3.5 to 9.0. Label transfer from [γ-32P]ATP to BVP during a 10-s reaction at 4 °C was detected within a rather narrow window at mildly acidic pH, with optimal phosphatase at pH 5.5 (Fig. 2A). No label transfer was observed when BVP was incubated with [γ-32P]ATP (data not shown). Kinetic analysis of the reaction with [γ-32P]ATP at pH 5.5 showed that the phosphoenzyme accumulated immediately at 10 s, persisted until 2 min, and decayed from 5 to 30 min (Fig. 2B). The decay of the radiolabeled phosphoprotein was prevented by adding SDS to the reaction mixtures after 30 s of reaction with ATP (data not shown). Thus, the loss of signal did not reflect chemical lability of the phosphoprotein adduct. The finding that phosphoenzyme formation and ATP hydrolysis were both abrogated by mutating the Cys119 to alanine (see below) provided evidence that the phosphoenzyme was relevant to the catalytic mechanism of BVP.

Evidence for a Thiophosphate Adduct—The SDS-denatured 32P-BVP adduct was stable to 30 min exposure at 37 °C to 0.1 M NaOH, or to 1 M hydroxylamine at pH 7.0, but the 32P was released quantitatively from the BVP by 1 M iodoide at pH 7.0 (Fig. 3A). Stability to neutral hydroxylamine argues against an acylphosphate linkage, whereas hydrolysis by iodoide is strongly indicative of a thiophosphate linkage, presumably to Cys119. A control experiment showed that the phosphoramidate linkage of [35S]GMP to Lys260 of vaccinia virus capping enzyme was unaffected by treatment with 1 M iodoide (data not shown).

The stability of the denatured BVP phosphoenzyme as a function of pH is shown in Fig. 3B. The adduct was alkaline-stable, as noted above, but relatively labile from pH 2 to pH 5. Stability was restored at pH 0 to 1. This trough-like pH stability profile is characteristic of a thiophosphate linkage (29) and has been described previously for the phosphoenzyme of a mammalian protein-tyrosine phosphatase (27).

**Amino Acid Mutations**—Amino acids within the conserved phosphatase-binding motif of BVP (175–HCTHGINRTGY189). The residues mutated were His118, Thr120, His121, Asn124, Arg125, Thr126, and Tyr128. Wild-type BVP and the BVP-Ala mutants were expressed in bacteria as His-tagged fusions and purified from soluble bacterial lysates by nickel-agarose and phosphocellulose column chromatography. SDS-PAGE analysis showed that the phosphocellulose preparations were highly enriched with respect to the 24-kDa BVP polypeptide and that the extents of purification were similar (Fig. 4A). Recombinant wild-type BVP catalyzed the release of 32P from [γ-32P]ATP. The extent of ATP hydrolysis was linear with input enzyme at limiting concentrations, and the reaction proceeded to completion at saturating enzyme levels (data not shown). The specific ATPase activities of the BVP-Ala mutants were determined by enzyme titration. The findings are summarized in Fig. 5, where the ATPase activities of the mutants are expressed as the percentage of the wild-type specific activity.

Mutants H118A, H121A, N124A, R125A, T126A, and Y128A were effectively inert in ATP hydrolysis at a level of sensitivity <0.1% of the wild-type specific activity. These mutational effects were similar to that of the C119A mutation (10). The T120A mutant was 7% as active as wild-type BVP. Thus, all of the non-aliphatic side chains of the phosphate-binding loop were important for activity.

Alanine substitution eliminated the side chain beyond the β carbon, but did not reveal the properties of the missing side chain that were important for activity. This was addressed by introducing conservative substitutions at the seven newly de-
fined essential residues and at Cys119, which was shown previously to be essential for BVP activity (10). The purity of the conservatively substituted recombinant BVP proteins was confirmed by SDS-PAGE (Fig. 4B). The specific ATPase activities of the conservative mutants were determined by enzyme titration and are shown in Fig. 5. The instructive findings are summarized below.

The C119V and C119S proteins were catalytically inert. The failure to restore activity by introducing an isosteric hydroxyamino acid in lieu of cysteine underscored the strict requirement for a thiolate as the reactive nucleophile. Distinct conservative mutational effects were noted at the two threonine positions of the phosphate-binding loop, Thr120 and Thr126. The ATPase activity was fully restored when a valine was introduced at Thr120; indeed, the T120V mutant was twice as active as wild-type BVP. Valine is nearly isosteric to threonine, but lacks the potential for hydrogen bonding. Placing a serine at position 120 only partially restored activity, i.e. to 26% of the wild-type value, compared with 7% of wild-type activity for the T120A mutant. Given that hydrogen bonding by the hydroxyl was clearly not important, we suspect that the salutary effects of serine at position 120 reflected occupancy by Oγ of one of the two γ sites on the threonine/valine side chains. We surmise that two substituents on the β carbon of amino acid 120 are optimal for BVP activity. This contrasts with the situation at position 126, where the T126V mutation was just as deleterious as T126A. However, activity was restored to 65% of the wild-type level by introducing a serine, implying that the hydroxyl group is essential for catalysis, presumably by virtue of its capacity to form a hydrogen bond.

Asn124 was replaced conservatively by glutamine and aspartic acid. The N124Q and N124D mutants were as defective in ATP hydrolysis as N124A. These findings suggested that the essential function of Asn124 entails hydrogen bonding interactions with the amide moiety that are sensitive to its distance from the main chain.

His118 was changed to valine and asparagine. The H118V and H118N proteins were just as defective as H118A. His121 was mutated to glutamine and asparagine. H121Q was 2% as active as wild type BVP, whereas H121N was 8% as active. Asparagine and glutamine are partially isosteric with histidine, such that the amide nitrogens of Asn and Gln can be imposed on Nε and Nd of His, respectively. The mutational findings at His118 and His121 of BVP suggested that hydrogen bonding properties of both histidines are important. Interactions of Nε apparently do not suffice for catalysis at position 118. (We did not construct a H118Q mutant and therefore cannot draw a conclusion concerning Ne). At position 121, both Ne and Nδ appeared to be functionally important.

Arg125 is conserved in all members of the cysteine phosphatase superfamily. A lysine in lieu of arginine restored phosphohydrolase activity to 10% of the wild-type level. This result suggested that: (i) the positive charge on the side chain is essential for function and (ii) bidentate interactions of the arginine side chain contribute an additional order of magnitude to catalysis.

The essential Tyr128 side chain was substituted by phenylalanine and leucine. The Y128F protein was fully active,
**FIG. 5. Effects of phosphate-binding loop mutations on phosphohydrolase activity and phosphoenzyme formation.** ATPase activity was assayed as described under "Experimental Procedures." Aliquots (2 µl) of serial 2-fold dilutions of each phosphocellulose BV preparation were included in the reaction mixtures. Between two and four titration experiments were performed for each protein, and the specific activity was calculated from the average of the slopes of the titration curves. The ATPase specific activities shown are normalized to the specific activity of wild type (WT) BVP (1 pmol of 32Pi released/15 min/ng of protein).

whereas Y128L was about one-third as active as wild-type BVP. Thus, neither a hydroxyl group nor an aromatic group was strictly essential at this position; rather, a bulky aliphatic side chain sufficed for BVP function.

**Mutational Effects on Phosphoenzyme Formation—**The recombinant BVP proteins containing mutations in the phosphate-binding motif were assayed for phosphoenzyme formation during a brief reaction with [γ-32P]ATP at pH 5.5. The reaction products were analyzed by SDS-PAGE (Fig. 6). There was good concordance between the mutational effect on steady-state ATP hydrolysis at pH 7.5 and the yield of phosphoenzyme at pH 5.5. For example, the conservative mutants T120V, T120S, R125K, T126S, Y128F, and Y128L that retained ATPase activity were also competent to form the phosphoenzyme. The mutant proteins that were unable to hydrolyze ATP in the steady state were also grossly defective in phosphoenzyme formation. These data provide correlative evidence that the covalent BVP thiophosphate adduct is a genuine reaction intermediate.

**Conserved Asp60 Is Dispensable for Catalysis—**Asp60, which is conserved in all of the metazoan RNA triphosphatase enzymes that include the RNA triphosphatase component of the mRNA capping apparatus. We take advantage of the fact that BVP (unlike the metazoan capping enzymes) can use nucleoside triphosphates as substrates for γ phosphate cleavage. Thus, we could study BVP and mutated versions thereof using commercially available nucleoside triphosphates instead of triphosphate-terminated RNAs, which are tedious to prepare. The experiments presented here provide insights into BVP catalysis and reveal interesting mechanistic distinctions between the RNA triphosphatases and the protein phosphatase branches of the cysteine phosphatase superfamily.

**DISCUSSION**

Here we have initiated an analysis of the catalytic mechanism of BVP, which we regard as a prototype of the metazoan RNA triphosphatase enzyme family that includes the RNA triphosphatase component of the mRNA capping apparatus. We take advantage of the fact that BVP (unlike the metazoan capping enzymes) can use nucleoside triphosphates as substrates for γ phosphate cleavage. Thus, we could study BVP and mutated versions thereof using commercially available nucleoside triphosphates instead of triphosphate-terminated RNAs, which are tedious to prepare. The experiments presented here provide insights into BVP catalysis and reveal interesting mechanistic distinctions between the RNA triphosphatases and the protein phosphatase branches of the cysteine phosphatase superfamily.

We demonstrate formation of a BVP phosphoenzyme and identify the linkage as a thiophosphate based on its chemical lability. Although we have not directly mapped the location of the phosphoamino acid, it is likely that the phosphate is linked to Cys119 because: (i) replacement of Cys119 by alanine or serine abrogates phosphoenzyme formation, (ii) of the five cysteines in BVP only Cys101 is conserved in all of the metazoan RNA triphosphatases, and (iii) there is definitive evidence that the equivalent cysteine in protein-tyrosine phosphatases and dual specificity protein phosphatases acts as the reactive nucleophile (22, 33).

The cysteine thiolate nucleophile is situated within the defining motif of the cysteine phosphatase superfamily HCXXXHXXR/T/S/G. This motif comprises a phosphate-binding loop that facilitates catalysis by protein-tyrosine phosphatases and dual specificity protein phosphatases in two ways. (i) The backbone amides of the loop interact with the cysteine side chain and stabilize it as the reactive thiolate in the ground state, and (ii) the backbone amides and the invariant arginine side chain interact with the phosphate oxygens to activate the bound substrate for nucleophilic attack and to stabilize the trigonal planar transition state. The replacement of the cata-

| Mutant | ATPase (% of WT) |
|--------|------------------|
| H18A   | ≤ 0.1            |
| H18Y   | ≤ 0.1            |
| H18N   | ≤ 0.1            |
| C119A  | ≤ 0.1            |
| C119V  | ≤ 0.1            |
| C119S  | ≤ 0.1            |
| T120A  | 7               |
| T120V  | 200             |
| T120S  | 26              |
| H121A  | ≤ 0.1            |
| H121Q  | 2               |
| H121N  | 8               |
| N124A  | ≤ 0.1            |
| N124Q  | ≤ 0.1            |
| N124D  | ≤ 0.1            |
| R125A  | ≤ 0.1            |
| R125K  | 10              |
| T126A  | ≤ 0.1            |
| T126V  | ≤ 0.1            |
| T126S  | 65              |
| Y128A  | ≤ 0.1            |
| Y128F  | 160             |
| Y128L  | 32              |

Asn, and Glu. The purity of the recombinant D60A, D60N, and D60E proteins was confirmed by SDS-PAGE (Fig. 7A). The specific ATPase activities of the D60 mutants were determined by enzyme titration. The remarkable finding was that the D60A and D60N mutations had virtually no effect on ATP hydrolysis (Fig. 7B) or the formation of the [γ32P]BVP phosphoenzyme (Fig. 7C). We surmised from this result that hydrolysis of the β-γ phosphoanhydride of ATP by BVP is not limited by the step of expelling the ADP leaving group. Replacing Asp60 by glutamate inactivated the enzyme for steady-state ATP hydrolysis and phosphoenzyme formation (Fig. 7). Thus, although the acidic moiety at position 60 is not required for catalysis, there is a strong constraint on the size of the linker arm, such that projection of the carboxylate away from the main chain by an additional methylene group is catastrophic for BVP function.

An alternative explanation for the nonessentiality of Asp60 is that another aspartate residue in BVP serves as the essential proton donor to expel ADP. Phylogenetic considerations made us skeptical of this scenario, insofar as Asp60 is the only aspartic acid in BVP that is conserved in the seven other metazoan and plant RNA triphosphatases. Nor are there any glutamate residues of BVP that are conserved in all of the other proteins. Nonetheless, we tested the effects on alanine mutation at Asp69 and Asp101, which are the only other aspartates immediately upstream of the BVP phosphate-binding motif (Fig. 1). The D69A mutant was 65% as active as wild-type BVP in ATP hydrolysis, whereas D101A was twice as active as wild-type BVP. Thus, neither Asp60 nor Asp101 is essential for catalysis.
lytic arginine in *Yersinia* protein-tyrosine phosphatase by alanine or lysine reduced $k_{\text{cat}}$ by nearly 4 orders of magnitude (34). The failure of the conservative lysine substitution to appreciably restore catalytic power is interpreted as a stringent requirement for the bidentate interaction of two guanidinium nitrogens of this arginine with the equatorial phosphate oxygens in the transition state (22, 33, 34). This model is in keeping with the 5000-fold decrement in $k_{\text{cat}}$ when the Arg residue of human protein-tyrosine phosphatase 1B was replaced by lysine (31).

We find that Ala substitution of the BVP arginine reduced phosphohydrolase specific activity by at least 3 orders of magnitude, but, unlike the case of the protein-tyrosine phosphatases, the introduction of a lysine resulted in the recovery of at least 2 orders of magnitude of catalytic power to a level of 10% of wild-type activity. Clearly, the bidentate complex of arginine with the transition state is less critical for BVP catalysis than for protein-tyrosine phosphatase. It is possible that another side chain of BVP is in contact with the phosphate and compensates in the transition state for a "second" contact made by the catalytic arginine of protein-tyrosine phosphatase. Alternatively, it may simply be the case that BVP does not fully exploit the bidentate hydrogen-bonding capacity of arginine to accelerate phosphoryl transfer and therefore a monodentate contact of lysine suffices for 10% of the full activity.

We find that the conserved histidine immediately preceding the cysteine nucleophile is essential for catalysis by BVP and that Asn did not function in lieu of His. Substitution of the equivalent histidine of *Yersinia* protein-tyrosine phosphatase by Asn and Ala reduced $k_{\text{cat}}$ by 2 and 3 orders of magnitude, respectively (35). Replacing the His side chain of human protein-tyrosine phosphatase 1B by alanine reduced $k_{\text{cat}}$ by 2 orders of magnitude (31). The conserved histidine side chain participates in a hydrogen bonding network that serves to stabilize the cysteine thiolate of protein-tyrosine phosphatase (23). Our mutational data are consistent with a similar essential function in BVP.

Thr$^{126}$ is essential for catalysis by BVP and is conserved as a threonine or serine on the protein-tyrosine phosphatases and dual specificity protein phosphatases. The hydroxyl moiety of the side chain is necessary and sufficient for BVP function, insofar as activity is restored when serine is present. Replacing the Thr or Ser residues of protein-tyrosine phosphatases and dual specificity protein phosphatases by alanine lowers $k_{\text{cat}}$ by at least 2 orders of magnitude (36–38). The crystal structures of protein-tyrosine phosphatases and dual specificity protein phosphatases reveal that the O$_\gamma$ of serine or threonine donates a hydrogen bond to the active site cysteine, an interaction that may stabilize the cysteine thiolate leaving group during hydrolysis of the phosphoenzyme. The hydrogen bond between O$_\gamma$ and the S$_\gamma$ of cysteine is visualized in the transition state and the cysteiny1-phosphate intermediate (22, 23, 33, 34). Although it has been reported for protein-tyrosine phosphatases and dual specificity protein phosphatases that elimination of O$_\gamma$ selectively impedes the hydrolysis reaction of the cysteiny1-phosphate (36, 37), we did not detect formation of the covalent phosphatase by the T126A mutant of BVP. Thus, the hydroxyl may play a relatively greater role in the first step of BVP catalysis than it does in the case of protein-tyrosine phosphatases and dual specificity protein phosphatases. BVP appears similar in its utilization of the catalytic threonine to the mammalian low molecular weight protein-tyrosine phosphatase, in which the equivalent serine is proposed to facilitate attack of the cysteine thiolate on the scissile phosphate (38).

A novel finding was that three other side chains within the conserved phosphate-binding motif of BVP (Thr$^{120}$, His$^{121}$, and Asn$^{124}$) and one side chain immediately flanking the motif (Tyr$^{126}$) are essential for phosphohydrolase activity. Structural studies have established that the backbone amide nitrogens of the phosphate-binding loop form hydrogen bonds to the phosphate oxygen atoms in the enzyme-substrate complex, the transition state and the cysteiny1-phosphate intermediate (33), but the roles of the side chains of the loop (other than those discussed above) have received little attention because they are not strictly conserved in the protein-tyrosine phosphatases and dual specificity protein phosphatases. However, Thr$^{120}$, His$^{121}$, Asn$^{124}$, and Tyr$^{126}$ of BVP are tightly conserved in the other metazoan RNA triphosphatases (Fig. 1). We presume that these essential side chains either: (i) participate directly in substrate binding or catalysis (perhaps His$^{121}$ or Asn$^{124}$) or (ii) function indirectly by ensuring the proper conformation of the
loop (Thr	extsuperscript{120} or Tyr	extsuperscript{128}) in a manner that is specific to the RNA triphosphatase subfamily of cysteinyl phosphatases.

A key finding of the present study was that the conserved aspartate of BVP is dispensable for phosphohydrolase activity and phosphoenzyme formation. This result is in stark contrast to the essential role of aspartate in catalysis by other cysteinyl phosphatases. For example, an alanine substitution of the conserved aspartic acid in mammalian protein-tyrosine phosphatase 1B decreases $k_{\text{cat}}$ by 5 orders of magnitude (31). Replacing the aspartic acid by asparagine, which had no significant effect on BVP, reduced the $k_{\text{cat}}$ of Yersinia protein-tyrosine phosphatase by 3 orders of magnitude (30) and slowed the activity of the human dual specificity protein phosphatase VHR by 2 orders of magnitude (22).

A simple explanation for the benign effects of the D60A and D60N mutations of BVP is that the enzyme is cleaving a phosphoanhydride bond in which the $pK_a$ of $\sim$6.7 for the bridging oxygen of leaving group ADP is sufficiently low that it does not require facilitated expulsion by a proton donor. This is in contrast to the phosphomonoester reactions of protein-tyrosine phosphatases and dual specificity protein phosphatases, where the serine leaving group has a $pK_a$ of 14 and the tyrosine leaving group has a $pK_a$ of 10. With protein-tyrosine phosphatases and dual specificity protein phosphatases, the leaving group $pK_a$ has little effect on catalysis because its expulsion is driven by proton donation from the aspartic acid (22, 26). However, when the Asp is replaced by Asn, the $k_{\text{cat}}$ of the mutant enzyme displays a significant dependence on leaving group $pK_a$, with reactivity being enhanced when the $pK_a$ is low and impeded when $pK_a$ is high (22). Regulated closure of the general acid loop of a wild type dual specificity protein phosphatase enzyme is critical when the $pK_a$ of the leaving group is high, but much less important when the leaving group has a low $pK_a$ (26). To our knowledge, BVP is the first case in which the presumptive “natural” phosphatase substrate (a phosphoanhydride) is cleaved efficiently without the aid of a conserved general acid.

Finally, it is instructive to compare the mutational effects on BVP to those reported by Wen et al. (6) for the RNA triphosphatase domain of mammalian mRNA capping enzyme. They found, as did we, that replacing the invariant Arg in the phosphatase domain of mammalian mRNA capping enzyme. They found, as did we, that replacing the invariant Arg in the phophatase domain of mammalian mRNA capping enzyme. They found, as did we, that replacing the invariant Arg in the "natural" phosphatase substrate (a phosphoanhydride) is cleaved efficiently without the aid of a conserved general acid.

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