Asp_{129} of Low Molecular Weight Protein Tyrosine Phosphatase Is Involved in Leaving Group Protonation*

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Zhongtao Zhang, Etti Harms, and Robert L. Van Etten

From the Departments of Chemistry and Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Site-directed mutagenesis was used to explore the functions of a number of acidic residues of bovine low molecular weight protein tyrosine phosphatase. Residues Asp-129, Asp-56, and Asp-92 were mutated to Ala or Asn. The mutant enzymes D56A, D56N, and D92A showed no significant changes in \( V_{\text{max}} \) values, although they did exhibit significantly altered \( K_m \) values. In contrast, the D129A mutant enzyme exhibited a greater than 2000-fold reduction in \( V_{\text{max}} \), using p-nitrophenyl phosphate as a substrate. The \( V_{\text{max}} \) values of D129A also exhibited a leaving group dependence, an altered solvent isotope effect limiting step of the catalytic reaction. Asp-129 is considered to be the proton donor to the leaving group pNPP, p-nitrophenyl phosphate; HPLC, high pressure liquid chromatography.

Protein tyrosine phosphatases employ a cysteine residue as a nucleophile in the catalysis of phosphomonoester hydrolysis (1-4). The enzymatic reaction proceeds through the formation of a covalent cysteinylphosphate intermediate, and the step leading to its formation is presumably accompanied by the protonation of the phenolic or other leaving group, since this would be expected to be necessary to enforce a favorable partitioning of the trigonal bipyramidal S_{2a}(P) transition state (6) toward loss of oxygen rather than sulfur. For bovine heart low molecular weight protein tyrosine-phosphatase (BPTP), the decomposition of the cysteinylphosphate enzyme intermediate is the rate-limiting step (6). The minimal kinetic scheme for the catalysis is given in Scheme I.

For wild type BPTP, \( k_1 \) is the rate-limiting step, and as a result, BPTP exhibits no dependence on the leaving group \( pK_a \) values, no isotope effect on \( V_{\text{max}} \) when aryl phosphates are used as substrates, but it does exhibit phosphotransferase activity when alternative phosphate acceptors are present. The identification of Cys-12 as the nucleophile was established by site-directed mutagenesis, kinetic studies, and spectroscopic studies on the non-fusion, recombinant protein, while the necessary role of Arg-18 and comparable uniformity of histidine in the protein have been established (7, 8).

However, the possible requirement of a proton donating group remained uncertain and this topic required further study. An Escherichia coli phosphomonoesterase that utilizes histidine as an active site nucleophile has an absolute requirement for an active site carboxylic acid that serves as a proton donor to the leaving alcohol or phenol (9). A mechanistic requirement for leaving group protonation seemed equally critical in the case of cysteine nucleophiles, consistent with related results for a bacterial phosphatase (10). The availability of high resolution solution and crystal structures of BPTP (11, 12), a prototypical mammalian phosphotyrosyl protein phosphatase, has now made it possible to answer this important question with precision. In the present study, acidic residues near the active site, namely Asp-92, Asp-56, and Asp-129, have been mutated to either Ala or Asn. The kinetic properties of these mutant proteins are characterized, and the proposed function of Asp-129 as a proton donor is considered in detail.

**EXPERIMENTAL PROCEDURES**

**BPTP Mutagenesis**—Site-directed mutations within the BPTP gene (2) were generated by the method of Vanderkam et al. (13). A 765-base pair XbaI-BamHI fragment containing the BPTP gene was transferred from plasmid pVEBH4 (2) into the corresponding sites of bacteriophage M13mp18. The following primers were used to initiate second strand synthesis: 5'-GTCCACCCCAACCCGACCTG-3', 5'-GTCCACCGACCAAGACCTG-3', 5'-CTATGCCATGGCCAGGAC-3', and 5'-CATTGAGCTTCCTTATGCGAAC-3'. The corresponding mutants were D66N, D56A, D92A, and D129A, respectively. Mutagenesis was performed using the T3-Gen in vitro mutagenesis kit from U. S. Biochemical Corp. Initial screening for the presence of the desired mutation was performed using single base nucleotide sequence reactions. The complete nucleotide sequence of the mutant BPTP genes in the final plasmid constructs was determined in order to verify the mutation as well as the integrity of the remainder of the gene.

**Expression, Purification, and Assay of Mutant Proteins**—The mutant enzymes were expressed and purified in the same way as the wild type bovine and human enzymes (2, 14). The homogeneity of each mutant protein was confirmed by SDS-polyacrylamide gel electrophoresis in 10% gels (15). The BPTP concentration was determined from the UV absorbance using an extinction coefficient (\( \varepsilon_{280} \)) value of 1.00 that has been previously established (16). The kinetic parameters toward p-nitrophenyl phosphate (pNPP) and other substrates (6) were measured in 0.1 M sodium acetate, pH 5.0 buffer, containing 1 mM EDTA with an ionic strength of 0.15 M adjusted by sodium chloride. Assays were conducted at 37 °C with an incubation time of 4 min except in the case of D129A, where the incubation time was increased to 30 min. Linearity was established by preliminary measurements over these time periods. For \( V_{\text{max}} \) and \( K_m \) measurements, eight different substrate concentrations were used, duplicate or triplicate measurements were made, and \( V_{\text{max}} \) and \( K_m \) values were obtained by fitting the data directly to the Michaelis-Menten equation using
for kinetic assays. Stock solutions of 100 mM acetate, 1 mM trifluoroacetic acid and acetonitrile (containing 0.1% trifluoroacetic acid) were made and mixed to prepare H$_2$O-D$_2$O buffers. The pellets were dissolved in a pH 5.0 buffer (lower) and dried using a Speed Vac. The pellet was dissolved in a pH 5.0 buffer (upper) and then dried using a Speed Vac. The pellet was dissolved in a pH 5.0 buffer containing 0.1% aqueous pNPP ranging from 0.1 to 10 mM. The protein was eluted by using a gradient of 0.1% aqueous solvent. The detector was set to monitor UV absorbance at 214 nm and to maximal sensitivity in order to detect any contaminant proteins. The major fraction, which accounted for 96% of the injected protein, was then dried using a Speed Vac. The pelleted samples were dissolved in a pH 5.0 buffer (upper) consisting of 100 mM sodium acetate containing 1 mM EDTA, 2 mM dithiothreitol, and 7 mM urea. The resulting solution was dialyzed for 24 h against 4 liters of pH 5.0 assay buffer. The resulting enzyme was used for kinetic assays.

**Solvent Isotope Effect Studies—Proton inventory studies** (18) of wild type and D129A mutant enzyme-catalyzed hydrolysis of pNPP were conducted as follows. Stock solutions of 100 mM acetate, 1 mM EDTA, 1 mM NaCl, pH 5.5, D$_2$O buffer. SigmaPlot software. Inhibition constants for inorganic phosphate were determined at pH 5.0 and 37 °C, using eight different concentrations of pNPP ranging from 0.1 to 10 mM. The data obtained in the absence of inhibitor and with four different inhibitor concentrations were evaluated using the program ENZYME (17).

**RESULTS AND DISCUSSION**

**Expression and Purification of Mutant Enzymes**—The D129A, D56A, D56N, and D92A mutant enzymes were expressed in E. coli and purified to homogeneity as described previously for the wild type BPTP (2). No significant differences were detected in the behavior of mutant and wild type enzymes during this procedure. All the mutant enzymes were efficiently overproduced to levels of 50–100 mg/liter of cell culture. The expression level of the D56N mutant was effectively identical to that of wild type enzyme, in contrast to the conclusion in a previous report (20), where the D56N mutant had been inadvertently produced due to incorporation of a sequencing error (discussed in Ref. 2). Relative molecular mass of each of these enzymes were determined by SDS-polyacrylamide gel electrophoresis and were equal to that of wild type enzyme. Comparison of the NMR spectrum of the aromatic region of the D129A mutant protein with that of the wild type enzyme (Fig. 1) shows the expected sharp and generally coincident $^1$H resonances characteristic of the properly folded protein, including the distinct C$^\alpha$H resonances of His-66 and His-72 (7). The latter residues serve an important role in forming the substrate binding loop of the active site (12). Minor spectral differences, especially in the 7.6–8.2 ppm region, are due in part to slight variations in NH exchange. The changes at 6.1 and 6.9 are consistent with $^1$H resonance shifts of Tyr-131, which is near the active site (11, 12.) From these spectral results, the D129A mutant appears to be correctly folded.

**Kinetic Parameters of Mutant Enzymes**—The effects of mutations D129A, D56N, D56A, and D92A on the kinetic parameters of BPTP were examined using pNPP as substrate at pH 5.0 (Table I). The most striking result is obtained with the mutant D129A, which exhibits a more than 2,000-fold reduction in $V_{max}$ values. This is consistent with the conclusion that Asp-129 serves a highly critical role in catalysis by BPTP. The effects of the mutations D65A, D56N, and D92N on $V_{max}$ were minimal, indicating that Asp-56 and Asp-92 are not directly involved in catalysis. However, these mutations do result in experimentally significant effects on $K_m$. D56A exhibits 2.5- and 3-fold increases in $K_m$ values for the substrates pNPP and phenyl phosphate, respectively. The mutation D56N resulted in a 5-fold increase in the $K_m$ value for pNPP. This is also in contrast to a previous report in which it was claimed that the D56N enzyme exhibited no change in $K_m$ and a 3-fold reduction in $V_{max}$ values in comparison to the wild type enzyme (20). The most striking increase in $K_m$ is observed for D92A, where a 16-fold increase is observed. These results indicate that Asp-92 and to a lesser extent Asp-56 serve in part to maintain the substrate binding affinity. From Scheme I, $K_m = (k_d k_p)/(k_+ k_*)$ and $K_{app} = (k_d/k_+ + k_p) 	imes K_m$, where $K_+ = (k_d + k_p)/k_d$. Therefore, the similar $K_m$ values of wild type, Asp-56, and Asp-92 mutant enzymes indicate that the increased $K_m$ values for the Asp-92 and Asp-56 mutant enzymes are probably due to reduced true affinities for substrate (i.e. increased $K_m$ values). This is consistent with the structure of the protein (12), in which Asp-92 is observed to form a salt bridge with Arg-18 (Fig. 2). Arg-18 is known to bind the phosphate moiety of the substrate. Although it is not shown in the figure, Asp-56 is about 7 Å from the active site nucleophile Cys-12 and forms a salt bridge with Arg-58 (12). The affinities for inorganic phosphate are changed as well, from a $K_m$ value of 2.0 mM at pH 5.0 for wild type BPTP to 6.2 mM for the D129A mutant enzyme. This may be due to the loss of a hydrogen bond between the phosphate and the carboxylic acid.

**D$_2$O Isotope Effect**—Earlier studies (16) showed that substrates such as pNPP with a good leaving group exhibited no isotope effect, while substrates such as 4-phenylbutyl phosphate with a poor leaving group resulted in an isotope effect $k_d^{H_2O}/k_d^{D_2O}$ of 2.38. This was attributed to a change in rate-limiting step, from dephosphorylation ($k_d$) with pNPP to phosphorylation ($k_+$) with 4-phenylbutyl phosphate. Further proton inventory experiments indicated that only one proton was involved in the phosphorylation step (16). This readily suggests the hypothesis that a proton donor must be present to protonate the leaving group in the phosphorylation step, and that proton transfer to the leaving group is at least partially rate-
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FIG. 2. Stereo diagram of the active site structure of BPTP. The figure was drawn with MolScript. Arg-18 forms a salt bridge with Asp-92. The nucleophile (Cys-12) and proton donor (Asp-129) are situated on opposite sides of the phosphate ion shown at the active site.

...limiting. In the present case, the D₂O solvent isotope effect that is measured for the D129A mutant enzyme at pH 5.0 using pNFP as a substrate is substantially altered; a small inverse isotopic effect \( V_{\text{D}_2\text{O}}/V_{\text{H}_2\text{O}} \) of 0.78 is observed for the low residual activity. \( K_c \) is unaffected.) In order to further exclude possible contamination by the wild type enzyme, portions of the D129A protein obtained after the G-75 column step were additionally purified by reverse phase HPLC. The repurified, renatured D129A enzyme exhibited identical kinetic properties and isotope effects before and after these procedures. The solvent isotope effect of the D129A mutant enzyme is in contrast to that of the wild type enzyme, which exhibits no solvent isotope effect when pNPP is used as a substrate. This difference is also evident in proton inventory studies performed simultaneously on the wild type and D129A mutant enzymes at pH 5.0 (Fig. 3). Although the data indicate a linear relationship between \( V_{\text{D}_2\text{O}}/V_{\text{H}_2\text{O}} \) and deuterium fraction \( n \), the isotope effect is too small to effectively distinguish linearity from a polynomial function (18).

The inverse solvent isotope effect indicates that either there is no proton transfer or that the proton transfer is not rate-limiting in the phosphorylation step of the D129A mutant enzyme, in contrast to the solvent isotope effect observed for the wild type enzyme (16). As illustrated by the example of papain (21), the small inverse isotope effect exhibited by D129A could result from the fact that protonation of the leaving group by the solvent is essential, and the fractionation factor favors D₂O (18). It is reasonable to conclude that the mutation of Asp-129 to Ala eliminates facile proton transfer to the leaving group.

BPTP catalyzes the hydrolysis of pNPP through the minimal mechanism presented in Scheme I, in which the rate-limiting step is normally dephosphorylation (6). This typically results in constant \( V_{\text{max}} \) values regardless of the pKₐ values of the aryl leaving groups of the substrates (6). In contrast, when the substrates 4-nitro-, 4-cyano-, 4-acetyl-, and 2,6-dichloro-4-nitrophenyl phosphates were used as substrates with the D129A mutant enzyme, \( V_{\text{max}} \) was highly dependent on leaving group pKₐ (data not shown). Although some substrates with leaving group pKₐ values over 8.5 are available, measurement of their kinetic parameters are effectively precluded due to their extremely low reactivity with the mutant enzyme.) This is consistent with a mechanism in which the phosphorylation step is rate-limiting (\( k_2 \)). Again, the differing behavior of wild type and D129A BPTP implicates the participation of Asp-129 in leaving group protonation during the phosphorylation step of BPTP catalysis, and leads to the conclusion that this step is severely retarded in the mutant D129A.

Partitioning experiments also showed a marked difference between the D129A mutant and the wild type enzyme. \( V_{\text{max}} \) values of the wild type enzyme increase substantially in the presence of a number of slightly nucleophilic alcohols such as methanol, ethanol, ethylene glycol, or propanediol, which act as phosphate acceptors (5, 16). Thus, in the presence of 2 M methanol, the \( V_{\text{max}} \) value of wild type enzyme increases by 100% at pH 5.0 (assayed by p-nitrophenol release). However, the \( V_{\text{max}} \) value of the D129A mutant enzyme is unchanged by the presence of 1–3 M methanol (data not shown). This is also consistent with a shift in mechanism from rate-limiting dephosphorylation for the wild type enzyme to rate-limiting phosphorylation for the D129A mutant enzyme.

Thus, the mutation of Asp-129 to Ala eliminates the proton donor that normally (16) facilitates loss of the leaving group in...
the phosphorylation step. This evidently results in leaving group protonation directly from the solvent, consistent with the inverse D,O solvent isotope effect. Attempts were made to substitute an external buffer general acid and thereby aid the catalysis by D129A. Provided the ionic strength is maintained constant by addition of sodium chloride, the kinetic parameters catalysis by D129A. Provided the ionic strength is maintained constant by addition of sodium chloride, the kinetic parameters of the D129A mutant did not change with acetate buffer concentration (0.1–1.5 M) at pH 5.0 and 7.0, respectively. This suggests that an external buffer general acid and thereby aid the leaving group in BPTP catalysis further clarifies the detailed catalytic mechanism and proton-donating group cannot readily transfer a proton to the leaving group.

The identification of Asp-129 as the proton donor for the leaving group in BPTP catalysis further clarifies the detailed catalytic mechanism of protein tyrosine phosphatases. Our investigation should aid in the search for related residues in other protein tyrosine phosphatases such as human PTP1B (21, 22), CD45 (23), and related enzymes. In the recently published crystal structure of human PTP1B (22), a potential proton donor residue was not evident. It is reasonable to suggest that further structural and mutagenic studies would be expected to lead to such an identification.

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