Altering the Nucleophile Specificity of a Protein-tyrosine Phosphatase-catalyzed Reaction

PROBING THE FUNCTION OF THE INARIANT GLUTAMINE RESIDUES

Yu Zhao‡, Li Wu‡, Seong J. Noh§, Kun-Liang Guan§, and Zhong-Yin Zhang‡

From the ‡Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461, and §Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, Michigan 48109

Protein-tyrosine phosphatases (PTPases) catalyze a cysteinyl phosphate intermediate, in which the phosphoryl group cannot be transferred to nucleophiles other than water. The dual specificity phosphatases and the low molecular weight phosphatases utilize the same chemical mechanism for catalysis and contain the same (H/V)(C)(X)R(S/T) signature motif present in PTPases. Interestingly, the latter two groups of phosphatases do catalyze phosphoryl transfers to alcohols in addition to water. Unique to the PTPase family are two invariant Gln residues which are located at the active site. Mutations at Gln-446 (and to a much smaller extent Gln-450) to Ala, Asn, or Met (but not Glu) residues disrupt a bifurcated hydrogen bond between the side chain of Gln-446 and the nucleophilic water and confer phosphotransferase activity to the Yersinia PTPase. Thus, the conserved Gln-446 residue is responsible for maintaining PTPases' strict hydrolytic activity and for preventing the PTPases from acting as kinases to phosphorylate undesirable substrates. This explains why phosphoryl transfer from the phosphoenzyme intermediate in PTPases can only occur to water and not to other nucleophilic acceptors. Detailed kinetic analyses also suggest roles for Gln-446 and Gln-450 in PTPase catalysis. Although Gln-446 is not essential for the phosphoenzyme formation step, it plays an important role during the hydrolysis of the intermediate by sequestering and positioning the nucleophilic water in the active site for an in-line attack on the phosphorus atom of the cysteinyl phosphate intermediate. Gln-450 interacts through a bound water molecule with the phosphoryl moiety and may play a role for the precise alignment of active site residues, which are important for substrate binding and transition state stabilization for both of the chemical steps.

Protein-tyrosine phosphatases (PTPase) consist of a family of enzymes that catalyze the removal of phosphoryl groups on tyrosine residues in proteins that are introduced by protein-tyrosine kinases. So far, approximately 100 PTPases have been identified, and the predicted total number of human PTPases may reach 500 based on the available genome sequencing data (1, 2). Amino acid sequence alignment of PTPases from bacteria, yeast, and mammalian organisms indicates that the only structural element that has amino acid sequence identity among all PTPases corresponds to the catalytic domain that spans over 250 residues (3). The hallmark feature that defines the PTPases is the active site sequence (H/V)(C)(X)R(S/T) in the catalytic domain also known as the PTPase signature motif (4). Work from a number of laboratories has led to the conclusion that PTPase catalysis proceeds through a double displacement mechanism involving a covalent phosphoenzyme intermediate. The side chain of the active site Cys residue serves as a nucleophile to accept the phosphoryl group from the phosphorytrosine in a substrate and form a kinetically competent cysteinyl phosphorolate. Detailed kinetic analyses also suggest roles for Gln-446 and Gln-450 in PTPase catalysis. Although Gln-446 is not essential for the phosphoenzyme formation step, it plays an important role during the hydrolysis of the intermediate by sequestering and positioning the nucleophilic water in the active site for an in-line attack on the phosphorus atom of the cysteinyl phosphate intermediate. Gln-450 interacts through a bound water molecule with the phosphoryl moiety and may play a role for the precise alignment of active site residues, which are important for substrate binding and transition state stabilization for both of the chemical steps.

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Roles of the Conserved Gln Residues in PTPase Catalysis

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FIG. 1. Sequence alignment of the PTPase catalytic domains starting from the active site signature motif to the C terminus including the invariant glutamine residues. The sequences shown here were retrieved from the GenBank™ using GCG program: Yos51 (the Yersinia PTPase) (387–468, accession no. M30457), hPTP1B (207–297, accession no. M137124), mSHPTP (445–529, accession no. M90389), and Ref. 18 and 19). Both Gln-446 and Gln-450 form hydrogen bonds with a conserved structural water, WAT1 (WAT505 in the PTPase complexed with oxyanion (22)). The oligonucleotide primers used were as follows: Q446A, GGTATTATGGTAGCAAAAGATGAGC; Q450A, Q450E, AAAA-

EXPERIMENTAL PROCEDURES

Materials—p-Nitrophenyl phosphate (pNPP) was obtained from Fluka. Ethylene glycol, β-naphthyl phosphate, 4-methyl umbelliferyl phosphate, and phenyl phosphate were purchased from Sigma. Aryl phosphate monoesters, 4-cyanophenyl, 4-ethoxycarbonylphenyl, 4-fluorophenyl, 4-bromophenyl, 4-chlorophenyl, 4-methylphenyl phosphate monoesters, 4-acetylphenyl, and phenyl phosphate were purchased from Sigma. Aryl phosphate monoesters by the Bio-Rad, and DNA sequencing kit from U. S. Biochemical Corp.

Expression and Purification of the Recombinant Phosphatases—The wild type Yersinia PTPase and the mutants Q446A, Q446E, Q446M, Q446N, Q450A, Q450E, Q450M were expressed under the control of T7 promoter in Esherichia coli B1.L2 (DE3) grown at 23–25 °C after induction with 0.4 mM isopropyl-1-thio-β-D-galactopryanoside, and recombinant proteins were purified to homogeneity as described previously (23). The recombinant dual specificity phosphatase VHR was expressed in E. coli and purified to homogeneity as described previously (24). The yeast low molecular weight phosphatase Sp1 was expressed in E. coli and purified to homogeneity as described previously (25).

Urea Denaturation—The urea-induced Yersinia PTPase denaturation was studied by monitoring the decrease in the intrinsic tryptophan fluorescence at 340 nm (slit width, 5 nm) with an excitation wavelength of 295 nm (slit width, 3.5 nm). The experimental details were described in Zhang et al. (23).

Steady-state Kinetics—Initial rates for the hydrolysis of pNPP and other aryl phosphate monooesters by the Yersinia PTPases was measured as described previously (8). Buffers used were as follow: pH 3.8–5.7, 100 mM acetate; pH 5.8–6.5, 50 mM succinate; pH 6.6–7.3, 50 mM 3,3-dimethylglutarate; and pH 7.5–9.0, 100 mM Tris. All of the buffer systems contained 1 mM EDTA and the ionic strength of the solutions was kept at 0.15 M using NaCl. The enzyme active site concentration was treated as the protein concentration determined from the absorption at 280 nm using the coefficient A1 mmol/l of 0.352 (8). The Michaelis-Menten kinetic parameters were determined from a direct fit of the v versus [S] data to the Michaelis-Menten equation using the nonlinear regression program KinetAsyst (IntelliKinetics, State College, PA). Inhibition constants for the Yersinia PTPases by arsenate were determined as described previously (4).

Pre-steady-state Kinetics—Pre-steady-state kinetic measurements of the Gln-446 and Gln-450 mutant Yersinia PTPases catalyzed hydrolysis of pNPP were conducted at pH 6.0 and 4.5 °C. The reaction was monitored by the increase in absorbance at 410 nm of the p-nitrophenolate product. The enzyme concentrations were 42 μM for Q446A and 49 μM for Q450A, and the pNPP concentration was 20 μM. The details for data collection and analysis were as described (8).

FIG. 2. Active site features of the Yersinia PTPase detailing the interactions of Gln-446 and Gln-450. The numbers along the dashed lines are distances (Å) taken from the crystal structure of the Yersinia PTPase complexed with tungstate (Brookhaven Protein Data Bank accession code 1YTW).
Detection of Reaction Products by 32P NMR—Dephosphorylation of pNPP in the presence of phosphohistidine (1 M ethylene glycol) was monitored on a Varian VXR 500 MHz spectrometer operating at 202.3 MHz using the following parameters: acquisition time, 3.0 s; pulse width, 37 ms; delay time, 1.0 s; spectral width, 8,000 Hz. All spectra were recorded with a 25-ppm broad band. The pH of the reaction was locked on D2O resonance line. The buffer used contained 50 mM succinate (pH 6.0), 1 mM EDTA, 1 mM ethylene glycol, and 20% D2O. The chemical shift of inorganic phosphate in the same buffer was set to zero. The concentration of pNPP was 20 mM, and the reaction was initiated with the addition of a catalytic amount of enzyme (1.5 - 2.0 × 10−3 M) in 3 mM buffer mentioned above. NMR data were collected at two time points of the reaction (at which the sample was incubated for 5 min in boiling water to inactivate the enzyme): one at 1 min to 1.5 h (wild type enzyme at 1 min, Q446A at 30 min, Q450A at 1.5 h, etc.), the other at 12 h. Multiple scans were taken to reduce the noise.

Protein Phosphatase Assays—The phosphorylated substrate (myelin basic protein (MBP), GST-Elk, Kemptide, or GST-Rb), diluted in phosphatase buffer (20 mM HEPES, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol), was added to an equal volume of phosphatase (wild type or mutant Yersinia PTPase, also in phosphatase buffer, to a final volume of 20 μL. This phosphatase reaction mixture was incubated at 37 °C for one hour. For MBP, GST-Elk, and GST-Rb, 20 μL of SDS sample buffer was added to each reaction mixture to quench the phosphatase reaction. Half of this final mixture was resolved on 15% SDS gel and subjected to autoradiography. For Kemptide, all of the reaction mixture at the end of 1 h of incubation was blotted onto P81 filter paper (Whatman). The filters were then washed four times with 80 mM phosphoric acid for 10 min each, washed with 95% ethanol, dried, and counted in the scintillation counter.

The amount of wild type or mutant Yersinia PTPase used was normalized to an equal amount of phosphatase activity in each of the reactions. The following amounts of each phosphatase was used for all experiments except for the second round of Rh dephosphorylation, where different amounts of the same phosphatases were incubated for experiments except for the second round of Rb dephosphorylation, normalized to an equal amount of phosphatase activity in each of the experiments. The concentrations of phosphatase (in μg) was 0.84, wild type; 1.08, Q446A; 0.64, Q446E; 3.35, Q446M; 3.08, Q450E; 5.72, Q450A; 57.0, Q450E; and 5.5, Q450M.

Preparation of Phosphorylated Protein Substrates—1) MBP and GST-Elk (a transcription factor phosphorylated by extracellular signal-regulated kinase-1), GST-Erk (1.5 μg), GST-super mitogen-activated protein or extracellular signal-regulated kinase (0.5 μg), 10 μCi of γ-32P ATP and either 10 μg of MBP or 10 μg GST-Elk were mixed together in the kinase buffer (18 mM HEPES, pH 7.4, 10 mM magnesium acetate, 50 μM ATP) to a final volume of 20 μL, and was incubated at 30 °C for 30 min. The incubation mixture was chached by the addition of 80 μL of phosphatase buffer. Ten μL of this mixture was used for phosphatase assay. 2) Kemptide (synthetic peptide as substrate of protein kinase A, Leu-Arg-Arg-Ala-Ser-Leu-Gly). Protein kinase A (catalytic subunit) was mixed with 10 μCi of the synthetic peptide, Kemptide, and 20 μCi of γ-32P ATP in 50 μL of the protein kinase A kinase buffer (20 mM Tris, 10 mM dithiothreitol, 5 mM NaF, 10 mM MgOAc, 200 μM ATP). This mixture was incubated at 30 °C for 1 h to allow phosphorylation of the Kemptide. The kinase reaction was chached by the addition of 200 μL of the phosphatase buffer. Ten μL of this mixture was then used for phosphatase assay. 3) GST-Rb (C-terminal domain of retinoblastoma tumor suppressor protein). GST-CDK4 and GST-cyclin D2 (1 μg each) were activated by incubation in 40 μL of proliferating Jurkat cell lysate (supplemented with 1 μM ATP) at room temperature for 1 h. The activated kinase was affinity purified on glutathione-agarose and eluted in 25 mM HEPES, pH 7.0, containing 10 μM glutathione. Half of the eluent (10 μL) was mixed with 10 μL of the cycldenpendent kinase kinase buffer (50 mM HEPES, pH 7.0, 10 mM MgCl2, 5 mM MgOAc, 1 mM dithiothreitol, 20 μM ATP) containing 10 μCi of γ-32P ATP. This reaction mixture was incubated at 37 °C for 1 h. The kinase reaction was then quenched by the addition of 80 μL of the phosphatase buffer, and 10 μL of this mixture was used for phosphatase assay.

RESULTS

Physicochemical Properties of the Gln-446 and Gln-450 Mutant Yersinia PTPases—To evaluate the role of the conserved Gln residues in PTPase catalysis, these Gln residues were altered by site-directed mutagenesis. The carboxyamide side chain does not ionize at physiological pHs but is relatively polar, being capable of both donating and accepting hydrogen bonds. The Gln residue was changed to (a) Glu, which is isosteric with Gln but can ionize as the pH is raised from the acidic range, (b) Asn, which is the lower homolog (one methylene group shortened) with the carboxyamide group retained, (c) Met, which is similar in size to Gln but lacks the carboxyamide group, and (d) Ala, which completely eliminates the Gln side chain. All of the mutations were verified by DNA sequencing. Initially, the mutant Yersinia PTPases were expressed in E. coli as described for the wild type enzyme, i.e., 6 h growth at 37 °C after 0.4 mM isopropyl-1-thio-β-d-galactopyranoside induction. Like the wild type, Q446E remained soluble in the cell lysate. However, more than half of the total recombinant Q446A, Q446N, and Q450E protein was found in the pellet, and the majority of the expressed protein for mutant Q446M, Q450M, and Q450A became insoluble at the same expression conditions. Subsequently, when the cells were grown at room temperature overnight after isopropyl-1-thio-β-d-galactopyranoside induction, the amount of the soluble protein obtained increased markedly. All of the mutant PTPases were purified to near homogeneity as judged by SDS-polyacrylamide gel electrophoresis, using procedures identical to those described for the wild type enzyme (23).

The Gln-446 and Gln-450 mutants had chromatographic and ultraviolet absorption spectral characteristics similar to those of the wild type. The λmax values of the fluorescence emission spectra of the mutants were the same as the wild type. To assess the conformational stability of the mutants, urea-induced denaturation was studied (see “Experimental Procedures”). As can be seen in Table I, the free energies of unfolding for Q446E and Q450E are similar to the wild type, whereas the competitive inhibition constants for the binding of arsenate denaturant were determined by fitting the data to \( \Delta G'_{m} = \Delta G'_{m} + m \cdot [\text{Urea}] \), where \( \Delta G'_{m} \) is the free energy change for protein unfolding at zero urea concentration, [Urea]m is the concentration of urea at which all of the protein is denatured. Errors were standard errors derived from direct fitting of the data to the equation.

### Table I

| Yersinia PTPase | \( \Delta G'_{m} \) kcal/mol | \( m \) | [Urea]m | kcal/(mol · m) |
|----------------|----------------------------|------|---------|----------------|
| Wild type      | 3.15 ± 0.27                | 1.0  | 1.38    |
| Q446A          | 3.08 ± 0.19                | 0.96 | 1.28    |
| Q446E          | 3.08 ± 0.19                | 0.95 | 1.28    |
| Q450A          | 3.08 ± 0.19                | 0.97 | 1.36    |
| Q450E          | 3.08 ± 0.19                | 0.95 | 1.36    |
Gln-446 and Gln-450 generally had rather modest effects on the kinetic parameters, although the effects were greater for the Gln-450 mutants than the Gln-446 mutants. For example, substitutions at Gln-446 generally led to less than 10- and 5-fold reduction in \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) values, respectively. In contrast, substitutions at Gln-450 resulted in a decrease in \( k_{\text{cat}} \) by 10-fold and \( k_{\text{cat}}/K_m \) by 10–100-fold. Third, with the exception of Q446E and Q450E, the affinity of the native and mutant PTPases toward arsenate did not change appreciably with pH, and alterations at positions 446 and 450 did not lead to significant changes in the \( K_v \) values.

**Leaving Group Dependence**—The leaving group dependence of the wild type *Yersinia* PTPase as well as the Q446A and Q450A mutants was investigated using a series of aryl phosphates. To minimize nonspecific steric effects, only para-substituted phenyl phosphates were used. Fig. 3 shows the Brønsted plots which relate the logarithms of \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \), respectively, to the \( pK_a \) values of the leaving groups for 4-nitrophenyl, 4-acetylphenyl, 4-cyanophenyl, 4-ethoxyacarbonylphenyl, 4-fluorophenyl, 4-bromophenyl, 4-chlorophenyl, 4-methylphenyl, 4-ethyl phosphate, and 4-methoxymethyl phosphate. Linear least-squares fitting of the Brønsted plots yielded the slopes, which correspond to the \( pK_a \) values. The \( pK_a \) values describe the leaving group dependence of the reaction and can often be regarded as an approximate measure of the extent of bond cleavage between the reaction center and the leaving group. For the wild type enzyme, the \( pK_a \) for \( k_{\text{cat}} \) was 0.036 ± 0.032, whereas the \( pK_a \) for \( k_{\text{cat}}/K_m \) was −0.19 ± 0.051. Similarly, Q446A exhibited a \( pK_a \) for \( k_{\text{cat}} \) of −0.011 ± 0.011, and a \( pK_a \) for \( k_{\text{cat}}/K_m \) of −0.12 ± 0.050, whereas Q450A exhibited a \( pK_a \) for \( k_{\text{cat}} \) of −0.011 ± 0.037, and a \( pK_a \) for \( k_{\text{cat}}/K_m \) of −0.13 ± 0.053. Thus, elimination of the carboxamide functional group from residues 446 or 450 had negligible effect on the \( pK_a \) values.

**Pre-steady-state Analysis**—The overall mechanism of hydrolysis of aryl phosphates catalyzed by the *Yersinia* PTPase involves a number of steps that are represented schematically in Scheme 1. The reaction proceeds through a sequence involving binding of substrate which is then cleaved with phosphoryl group transfer (\( k_b \)) to the nucleophilic Cys residue. Subsequent back-catalyzed reaction with water cleaves the phosphoenzyme intermediate (E-P) (\( k_b \)) and release of phosphate completes the catalytic cycle. Burst kinetics was observed with the *Yersinia* PTPase at pH 6.0 and 3.5 °C using pNPP as a substrate (8). It was concluded that under these conditions the rate-limiting step corresponds to E-P hydrolysis. Burst kinetics was also observed at pH 6.0 and 4.5 °C with all of the Gln mutants characterized. The stopped flow traces for the Q446A and Q450A mutant reactions are shown in Fig. 4. The observed rate constant for E-P formation (\( k_b \)) in the Q446A and Q450A reactions was slightly higher (1.5–2-fold), whereas the rate constant for E-P hydrolysis (\( k_b \)) was lowered by 4–5-fold in comparison with those of the wild type reaction reported in a previous study (8). It is not clear whether the slightly higher burst rate is significant since rate constants in the range of several hundreds per second are barely within the detection limit of a typical stopped flow spectrophotometer. Collectively, these results suggest that alteration of either Gln-446 or Gln-450 does not change the rate-limiting step of the dephosphorylation of pNPP by the enzyme.

**Substitutions at Gln-446 Confer Phosphotransferase Activity to the *Yersinia* PTPase**—PTPase catalysis involves a cysteinyl phosphate intermediate that can only be hydrolyzed by water. Alcohols, such as methanol, ethanol, ethylene glycol, and glycerol, are not phosphoryl acceptors in the PTPase-catalyzed...

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**TABLE II**

Kinetic parameters of the *Yersinia* PTPase and its conserved Gln mutants

| pH  | *Yersinia* PTPase | \( k_{\text{cat}} \) | \( K_m \) | \( k_{\text{cat}}/K_m \) | Arsenate \( K_v \) |
|-----|------------------|--------------------|--------|-----------------|----------------|
| Wild type | 816 ± 29 | 2.50 ± 0.26 | 326 ± 35 | 0.32 ± 0.05 |
| Q446A | 85.6 ± 1.4 | 0.19 ± 0.01 | 450 ± 24 | 0.66 ± 0.09 |
| Q446E | 487 ± 18 | 2.53 ± 0.31 | 192 ± 24 | 0.38 ± 0.07 |
| Q446M | 63.7 ± 2.7 | 0.63 ± 0.11 | 101 ± 18 | 1.10 ± 0.19 |
| Q446N | 43.0 ± 0.4 | 0.40 ± 0.01 | 108.6 ± 0.3 | 2.06 ± 0.17 |
| Q450A | 66.4 ± 2.1 | 5.88 ± 0.65 | 113 ± 1.3 | 1.06 ± 0.10 |
| Q450E | 27.2 ± 0.3 | 9.49 ± 0.66 | 2.9 ± 0.2 | 1.16 ± 0.49 |
| Q450M | 21.9 ± 0.5 | 6.22 ± 0.35 | 3.5 ± 0.2 | 2.42 ± 0.46 |
| Wild type | 285 ± 8 | 3.23 ± 0.24 | 88.2 ± 6.9 | 1.45 ± 0.11 |
| Q446A | 61.8 ± 0.7 | 0.30 ± 0.02 | 206 ± 15 | 1.18 ± 0.06 |
| Q446E | 233 ± 9 | 6.64 ± 0.59 | 351 ± 3.4 | 0.68 ± 0.06 |
| Q446M | 42.5 ± 3.1 | 0.89 ± 0.05 | 61.6 ± 0.6 | 1.67 ± 0.17 |
| Q450A | 38.1 ± 0.5 | 3.26 ± 0.13 | 11.7 ± 0.5 | 2.46 ± 0.11 |
| Q450E | 7.44 ± 0.53 | 24.0 ± 3.9 | 0.31 ± 0.05 | 9.00 ± 1.09 |
| Q450M | 33.0 ± 0.5 | 4.40 ± 0.16 | 7.5 ± 0.3 | 2.85 ± 0.23 |

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reaction. For example, when the Yersinia PTPase was incubated with \( p_{\text{NPP}} \) in the presence of 1 M ethylene glycol at pH 6.0, no phosphoryl group transfer to the alcohol was observed and only the hydrolysis product, inorganic phosphate, was detected using \(^{31}\text{P NMR} \) (Fig. 5A). In contrast, when the same experiment was performed with the Q446A, Q446N, or Q446M mutant, in addition to hydrolysis, ethylene glycol phosphate was generated during the process of dephosphorylation of \( p_{\text{NPP}} \) (data for Q446A is shown in Fig. 5B). Phosphoryl transfer to alcohol also occurred with the Q450A and Q450M mutants, although only a minute amount of ethylene glycol phosphate could be detected even after prolonged incubation with the mutant phosphatases (data for Q4450A is shown in Fig. 5C). Interestingly, like the wild type enzyme, neither Q446E nor Q450E was able to produce the alkyl phosphate product (data not shown).

FIG. 3. Effect of aryl phosphate leaving group \( p_{\text{K}} \) on \( k_{\text{cat}} \) (A) and \( k_{\text{cat}}/K_{\text{m}} \) (B) for the wild type (●), Q446A (■), and Q450A (○) Yersinia PTPase. The lines were drawn by a linear regression method (Kleidagraph, Abelbeck Software).

FIG. 4. Burst kinetics observed with Q446A and Q450A using \( p_{\text{NPP}} \) as a substrate at pH 6 and 4.5 °C. The \( p_{\text{NPP}} \) concentration was 20 mM. Each stopped flow trace was an average of six to eight individual experiments. The specific rate constants were analyzed by fitting the experimental data directly to the theoretical equation (the solid line): \( [p_{\text{nitrophenolate}}] = A t + B(1 - e^{-kt}) + C \) through the use of the nonlinear least squares algorithm in KISS (Kinetic Instruments, Inc.). For Q446A, \( k_3 \) and \( k_4 \) were 538 s\(^{-1}\) and 13.5 s\(^{-1}\), respectively, and for Q450A, \( k_3 \) and \( k_4 \) were 721 s\(^{-1}\) and 10.1 s\(^{-1}\), respectively.

FIG. 5. Phosphoryl transfer reaction catalyzed by the Yersinia PTPase monitored by \(^{31}\text{P NMR spectroscopy} \). Panels A, B, and C are the NMR spectra of reactions catalyzed by the native, Q446A, and Q450A Yersinia PTPase, respectively. The chemical shift of inorganic phosphate is set to zero. The chemical shift of \( p_{\text{NPP}} \) is −1.4 ppm and that of ethylene glycol phosphate is 1.3 ppm.
Roles of the Conserved Gln Residues in PTPase Catalysis

When the rate-limiting step is the hydrolysis of E-P, Equation 1 can be reduced to Equation 2. Thus, the presence of ethylene glycol will increase the rate of E-P breakdown and thus accelerate the overall reaction rate (Scheme 2 and Equation 2), provided ethylene glycol can serve as a phosphoryl acceptor.

\[ k_{\text{cat}} = \frac{k_3 k_4 + k_5 [\text{ROH}]}{k_2 + k_3 + k_5 [\text{ROH}]} \]  

(Eq. 1)

\[ k_{\text{cat}} = k_3 + k_5 [\text{ROH}] \]  

(Eq. 2)

It turned out that the presence of ethylene glycol did not have any effect on the rate of catalysis by the wild type and the mutants Q446E and Q450E, since they were unable to catalyze the transfer of a phosphoryl group to an alcohol. However, as shown in Table III, mutant PTPases that could catalyze phosphoryl transfer to alcohols as judged by the \(^{31P}\) NMR data did exhibit increased overall reaction rate in the presence of 1 M ethylene glycol. This was especially so for the Q446A mutant for which the \(k_{\text{cat}}\) for overall reaction was accelerated 1.5-fold in the presence of 1 M ethylene glycol, consistent with the relative amount of products generated as measured by \(^{31P}\) NMR (Fig. 5B). These results support that E-P decomposition is the rate-limiting step for the hydrolysis of pNPP catalyzed by Q446A, Q446N and Q446M.

To assess the significance of Gln-446 in controlling the nucleophile specificity (\(\text{H}_2\text{O} vs.\) alcohol), we have compared the rates of phosphotransfer to ethylene glycol for Q446A, the dual specificity phosphatase VHR, and the low molecular weight phosphatase Stp1. The second-order rate constant (\(k_3\)) for phosphotransfer can be determined from Equation 2 by measuring the dependence of \(k_{\text{cat}}\) on the alcohol concentration (29). As shown in Fig. 6, \(k_3\) for Q446A is 21.6 m\(^{-1}\) s\(^{-1}\) which is 3 times faster than that for Stp1 (6.6 m\(^{-1}\) s\(^{-1}\)) and 45 times faster than that for VHR (0.48 m\(^{-1}\) s\(^{-1}\)). Since the \(Yersinia\) PTPase, VHR, and Stp1 exhibit different intrinsic catalytic activities, we also compare the ratio of the rate of phosphotransfer (\(k_3\)) to the rate of hydrolysis (\(k_3\) = \(k_3 [\text{H}_2\text{O}]\), Scheme 2 and Ref. 29). The ratio (\(k_3/k_3\)) is 0 for the \(Yersinia\) PTPase, 19.4 for the Q446A mutant, 122 for Stp1 and 4.4 for VHR, indicating that the tendency to catalyze phosphoryl transfer to ethylene glycol is zero for the \(Yersinia\) PTPase, moderate for VHR, and great for Stp1. Furthermore, replacement of Gln-446 with an Ala has rendered the \(Yersinia\) PTPase with considerable phosphotransferase potential (\(k_3/k_3\)) that is 4.4-fold higher than VHR and 6.3-fold lower than Stp1.

### Table III

**Effect of ethylene glycol**

All measurements were performed at pH 6.0 and 30°C. The reported errors were the standard deviation of the mean. For each experiment, eight substrate concentrations covering the range of 0.2–5 \(K_m\) were used.

| \(Yersinia\) PTPase (1 M) Ethylene glycol | Phosphotransfer | \(p\text{-Nitrophenol}\) | Phosphate |
| --- | --- | --- | --- |
| | | \(k_{\text{cat}}\) \(s^{-1}\) | \(K_m\) \(mM\) | \(k_{\text{cat}}\) \(s^{-1}\) | \(K_m\) \(mM\) |
| Wild type | – | 285 ± 8 | 3.23 ± 0.24 | 278 ± 9 | 3.58 ± 0.27 |
| – | + | 256 ± 5 | 2.26 ± 0.10 | 257 ± 10 | 2.40 ± 0.12 |
| Q446A | – | 61.8 ± 1.4 | 0.30 ± 0.02 | 61.2 ± 1.3 | 0.31 ± 0.02 |
| – | + | 93.4 ± 1.4 | 0.32 ± 0.01 | 96.9 ± 1.3 | 0.36 ± 0.02 |
| Q446E | – | 223 ± 9 | 11.0 ± 1.1 | 222 ± 12 | 11.1 ± 1.5 |
| – | + | 223 ± 10 | 9.8 ± 1.4 | 232 ± 19 | 10.7 ± 1.8 |
| Q446M | – | 35.3 ± 1.4 | 0.54 ± 0.05 | 35.9 ± 0.8 | 0.50 ± 0.05 |
| – | + | 49.7 ± 0.9 | 0.39 ± 0.02 | 50.5 ± 0.5 | 0.41 ± 0.03 |
| Q446N | – | 30.5 ± 0.4 | 0.78 ± 0.04 | 31.7 ± 0.4 | 0.76 ± 0.03 |
| – | + | 39.3 ± 0.8 | 0.91 ± 0.06 | 39.6 ± 0.5 | 1.05 ± 0.06 |
| Q450A | – | 35.0 ± 1.3 | 0.39 ± 0.06 | 35.3 ± 0.6 | 0.59 ± 0.24 |
| – | + | 45.9 ± 0.7 | 3.25 ± 0.18 | 48.4 ± 0.6 | 2.44 ± 0.19 |
| Q450E | – | 6.63 ± 0.36 | 26.6 ± 3.1 | 5.27 ± 0.23 | 18.7 ± 1.6 |
| – | + | 7.06 ± 0.12 | 24.5 ± 0.9 | 4.66 ± 0.15 | 3.73 ± 0.3 |
| Q450M | – | 33.0 ± 0.6 | 4.53 ± 0.10 | 30.1 ± 0.6 | 3.76 ± 0.12 |
| – | + | 36.4 ± 0.6 | 3.98 ± 0.21 | 32.2 ± 0.6 | 3.42 ± 0.15 |

* Determined by the production of \(p\text{-nitrophenol}.

* Determined by the production of inorganic phosphate.
Do Mutations at the Two Gln Residues Alter the Yersinia PTPase Substrate Specificity?—The Yersinia PTPase displays strict specificity toward Tyr(P)-containing peptides/proteins (30). This specificity for Tyr(P) has been proposed to be due to the depth of the amphipathic Tyr(P) binding pocket that exactly matches the length of Tyr(P) (20). Since the side chain of Gln-262 (equivalent to Gln-446 in the Yersinia PTPase) interacts with the phenyl ring of the phosphotyrosine (Tyr(P)), and defines a portion of the rim for the Tyr(P) binding pocket (20). A similar interaction between Gln-446 and Tyr(P) is observed in an energy minimized model of the Yersinia PTPase with Tyr(P) bound (19). In the structures of PTPases bound with substrates or oxyanions, a trapped structural water molecule, WAT1 in the Yersinia PTPase and WAT505 in PTP1B, is observed (19, 20). WAT1 forms hydrogen bonds with the side chains of Gln-450 and Gln-450, O-2 of the oxyanion, and the carboxylate of the general acid/base Asp-356 in the PTPase-oxyanion complexes (Fig. 2). In the PTPase-substrate complexes, this water molecule makes hydrogen bonds with the side chain of Gln-450, the tyrosine leaving group oxygen (the scissile oxygen), O-2 of the phosphate, and the carboxylate of the general acid/base Asp-356 (Fig. 7A, and Ref. 20). Interestingly, a new water molecule, WAT3, is identified in the Yersinia PTPase-nitrate complex as well as in an energy minimized model of the phosphocysteine intermediate (19). Nitrate is coplanar and lacks the apical oxygen present in tetrahedral oxyanion such as tungstate. Instead, WAT3 sits directly above the nitrate and is perfectly in line with the sulfur atom of the active site Cys residue. WAT3 is proposed to be the nucleophilic attacking molecule for hydrolysis of the cysteiny1 phosphate intermediate and is coordinated primarily by the side chains of Gln-446, Gln-357, Asp-356, and WAT1 through hydrogen bonds (Fig. 7B, Ref. 19).

Based on the reported structural data, Gln-446 and Gln-450 likely participate in both of the chemical steps, i.e., phosphoenzyme formation and decay. Gln-446 may function to help to align the phenyl ring of Tyr(P) in the first step and to position the attacking water in the second step. Gln-450 makes a hydrogen bond with WAT1, which in turn is in hydrogen bonding distance with the apical oxygen (O-1) and one of the phosphoryl oxygen (O-2). Thus, Gln-450 may play a role in proper stabilization of the metaphosphate-like transition state (31) as well as in leaving group stabilization and nucleophile activation. For the Yersinia PTPase, the kinetic parameter $k_{cat}/K_m$ is primarily limited by the phosphoenzyme intermediate formation step (31), whereas the $k_{cat}$ term is mostly determined by intermediate breakdown. Results from kinetic analysis of the site-directed mutants of Gln-446 and Gln-450 confirm some of the structural data and provide additional insights into the mechanisms by which these two residues effect catalysis.

Replacements of Gln-446 by Ala, Asn, or Met have minimal effect on $k_{cat}/K_m$ and only less than 10-fold reduction in $k_{cat}$. In the case of PTP1B/Q262A, $k_{cat}/K_m$ and $k_{cat}$ are reduced by 7- and 83-fold, respectively, using the tyrosine-phosphorylated lysozyme as a substrate (7). It is not clear why a larger reduction in $k_{cat}$ is observed for the PTP1B/Q262A mutant. It appears that Gln-446 does not play a significant role in the phosphoenzyme formation step. As shown in Fig. 7B, the putative nucleophilic water molecule is coordinated by a bidentate hydrogen bond with the side chain of Gln-446. Such an interaction does not exist in the Q446A, Q446N, and Q446M mutants so that the positioning of the attacking water could be affected leading to a reduced rate for the phosphoenzyme hydrolysis step. Indeed, within the PTP1B/Q262A phosphoenzyme intermediate, the position of the nucleophilic water is...
displaced by 1.5 Å from being colinear with the active site Cys-215 S-P bond.2 The moderate decrease in rate for phosphoenzyme hydrolysis observed in the Yersinia Gln-446 mutants is consistent with a dissociative transition state for which minimal activation of the nucleophilic water is required (29). In addition, a water molecule may take the place of the Gln-446 side chain and partially rescue its function in the second step. A new water is found at the position of the side chain of Gln-262 in the structure of PTP1B/Q262A phosphoenzyme complex.2 This may also explain the fact that Gln-446 mutants possess similar oxyanion binding affinity as the wild type.

Replacements of Gln-450 by Ala or Met have larger effects on both the $k_{cat}/K_m$ and $k_{cat}$, consistent with the notion that it interacts through WAT1 with a phosphoryl oxygen, and the leaving group oxygen in the first step and a phosphoryl oxygen, and the attacking water in the second step. The results obtained with Q446E and Q450E are rather interesting. Although glutamic acid and glutamine are isosteric, the Gln to Glu substitution at residue 450 manifests the most deleterious effects with up to 1200-fold decrease in $k_{cat}/K_m$ and 66-fold decrease in $k_{cat}$, 20-fold increase in $K_m$ for pNPP and 30-fold increase in $K_i$ for arsenate. Furthermore, these deleterious effects increase as the pH is raised. These results strongly suggest that in the native Yersinia PTPase, the carboxyamide side chain must donate a hydrogen bond to WAT1 through its amide group (Fig. 7). WAT1 may receive another hydrogen bond from Asp-356 or the backbone amide from Gln-357, and donate one hydrogen bond to a phosphoryl oxygen and another hydrogen bond to the leaving group oxygen or the attacking water (Fig. 7). Thus, deprotonation of the carboxylic acid at residue 450 in Q450E will cause detrimental effects due to charge repulsion. In contrast, it appears that Gln-446 can be largely replaced by a Glu residue with little change in $k_{cat}$ and only moderate increase in $K_m$ for pNPP and $K_i$ for arsenate.

Gln-446 Is the Residue Escorting the Nucleophilic Water—

The ability to catalyze phosphoryl transfer to nucleophilic acceptors in addition to water is a common feature for phosphate ester hydrolases, such as nonspecific alkaline and acid phosphatases, that involve a covalent phosphoenzyme intermediate (32). The PTPase-catalyzed reaction proceeds through a covalent phosphocysteine intermediate that is subsequently hydrolyzed by water. However, careful investigations from this laboratory have shown that the Yersinia PTPase and the human PTP1B can only catalyze the transfer of the phosphoryl group from the intermediate to water, yielding inorganic phosphate. Similar observations have also been made with other PTPases. For example, LAR-D1 PTPase does not show a discernible tendency to transfer the phosphoryl group to ethylene glycol or glycerol, and the CD45 catalytic fragment displayed no phosphotransferase activity to alcohols as cosubstrates (33). Glycerol or propane-1,2-diols, at concentrations of 4–6 M, accelerated the $k_{cat}$ of the full-length SHP-1 by 47-fold and of the PTPase domain by 8-fold. However, $31^P$ NMR spectroscopy indicates no formation of glycerol phosphate during hydrolysis of pNPP by SHP-1 in 50% glycerol (34). The increase in rate is likely caused by a glycerol induced conformational change which alleviates the autoinhibited state.

Alcohols are better nucleophiles than water for accepting a phosphoryl group (29). The fact that alcohols such as methanol, ethanol, ethylene glycol and glycerol are much better nucleophilic acceptors of phosphoryl group than water and yet they fail to serve as phosphate acceptors in the PTPase reaction indicates that the phosphoryl group in the intermediate is only accessible by water. As shown in the PTPase-oxyanion complexes, the side chain of Gln-446 interacts with the apical oxygen of the oxyanion, which effectively shields the oxyanion from the aqueous environment (18). In the PTPase-nitrate complex or the energy-minimized model of the phosphoenzyme intermediate (19) the side chain of Gln-446 coordinates the attacking nucleophilic water via a bidentate hydrogen bond. Thus, nucleophiles larger than water such as alcohols cannot gain access into the active site due to steric hindrance. Alter-

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2 D. Barford, personal communication.
natively, it is also possible that both protons of the nucleophilic water are required for proper positioning for the nucleophilic attack. For example, the nucleophilic water can donate one H-bond to Oe1 of Gln-446 and the other to the side chain of Asp-556, which is consistent with the carbonylate functioning as a general base in the phosphoenzyme hydrolysis step (21). The nucleophilic water can also receive a hydrogen bond either from Ne2 of Gln-446 or from WAT1 (Fig. 7). Such interactions would position the water in an optimal position for nucleophilic attack on the phosphorus atom (Fig. 7). Since alcohols are incapable of making the same interactions and they can only donate one H-bond, they cannot serve as phosphoryl acceptors.

Substitutions at Gln-446 by an Ala, an Asn, or a Met abolishes the specific bidentate hydrogen bonding interactions with the nucleophilic water and results in either smaller side chains (Q446A and Q446N) or a side chain with an increased mobility (Q446M), such that alcohols can replace the nucleophilic water and gain access to the phosphoenzyme intermediate. This notion is supported by the observations that Q446A, Q446N, and Q446M all displayed phosphoryl transfer activities. Q450A and Q450M also exhibited minor phosphoryl transfer activities. This implies that the Gln-450 to Glu substitution, whereas Gln-446 is important for the optimal positioning of the nucleophilic water molecule in the phosphoenzyme hydrolysis step.

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REFERENCES

1. Hunter, T. (1995) Cell 80, 225–236
2. Tonks, N. K., and Neel, B. G. (1996) Cell 87, 365–368
3. Zhang, Z.-Y., Wang, Y., and Dixon, J. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1624–1627
4. Zhang, Z.-Y., Wang, Y., Wu, L., Fauman, E., Stuecky, J. A., Schubert, H. L., Saper, M. A., and Dixon, J. E. (1994) Biochemistry 33, 15266–15270
5. Guan, K. L., and Dixon, J. E. (1991) J. Biol. Chem. 266, 17026–17030
6. Cho, H., Krishnaraj, R., Kitas, E., Bannwarth, W., Walsh, C. T., and Anderson, K. S. (1992) J. Am. Chem. Soc. 114, 7286–7290
7. Flint, A. J., Taganis, T., Barford, D., and Tonks, N. K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1680–1685
8. Zhang, Z.-Y., Palety, B. A., Wu, L., and Zhao, Y. (1995) Biochemistry 34, 16389–16396
9. Lohse, D. L., Denu, J. M., Santoro, N., and Dixon, J. E. (1997) Biochemistry 36, 4588–4575
10. Denu, J. M., Stuecky, J. A., Saper, M. A., and Dixon, J. E. (1996) Cell 87, 361–364
11. Zhang, Z.-Y. (1997) Curr. Top. Cell. Regul. 35, 21–68
12. Barford, D., Jia, Z., and Tonks, N. K. (1995) Nat. Struct. Biol. 2, 1043–1053
13. Fauman, E. B., and Saper, M. A. (1996) Trends Biochem. Sci. 21, 413–417
14. Hengge, A. C., Zhao, Y., Wu, L., and Zhang, Z.-Y. (1997) Biochemistry 36, 7926–7936
15. Bliiska, J. B., Guan, K. L., Dixon, J. E., and Falkow, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1187–1191
16. Barford, D., Flint, A. J., and Tonks, N. K. (1994) Science 263, 1397–1404
17. Stuecky, J. A., Schubert, H. L., Fauman, E., Zhang, Z.-Y., Dixon, J. E., and Saper, M. A. (1994) Nature 370, 571–575
18. Schubert, H. L., Fauman, E. B., Stuecky, J. A., Dixon, J. E., and Saper, M. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2711–2715
19. Zhang, Z.-Y., Wang, Y., Wu, L., and Zhao, Y. (1995) Biochemistry 34, 16379–16386
20. Jia, Z., Barford, D., Flint, A. J., and Tonks, N. K. (1995) Science 268, 1744–1748
21. Wu, L., and Zhang, Z.-Y. (1996) Biochemistry 35, 5426–5434
22. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
23. Zhang, Z.-Y., Clemens, J. C., Schubert, H. L., Stuecky, J. A., Fischer, M. W. F., Hume, D. M., Saper, M. A., and Dixon, J. E. (1992) J. Biol. Chem. 267, 23759–23766
24. Zhang, Z.-Y., Wu, L., and Chen, L. (1995) Biochemistry 34, 16886–16896
25. Zhang, Z.-Y., Zhou, G., Denu, J. M., Wu, L., Tang, X., Mondesert, O., Russell, P., Butch, E., and Guan, K.-L. (1995) Biochemistry 34, 10560–10568
26. Yuvaniyama, J., Denu, J. M., Dixon, J. E., and Saper, M. A. (1998) Science 272, 1328–1331
27. Su, X. D., Taddei, N., Stefani, M., Ramponi, G., and Nordlund, P. (1994) Nature 370, 575–578
28. Zhang, Z., Van Etten, R. L., and Staudacher, C. V. (1994) Biochemistry 33, 11097–11105
29. Zhao, Y., and Zhang, Z.-Y. (1996) Biochemistry 35, 11797–11804
30. Guan, K. L., and Dixon, J. E. (1990) Science 240, 553–556
31. Hengge, A. C., Sowa, G., Wu, L., and Zhang, Z.-Y. (1995) Biochemistry 34, 13962–13967
32. Perahia, A. (1985) Enzyme Structure and Mechanism, pp. 206–209, W. H. Freeman & Co., New York
33. Cho, H., Ramer, S. E., Itoh, M., Kitas, E., Bannwarth, W., Burn, P., Saito, H., and Walsh, C. T. (1992) Biochemistry 31, 123–138
34. Wang, J., and Walsh, C. T. (1997) Biochemistry 36, 2993–2999
35. Zhang, Z.-Y., and Van Etten, R. L. (1991) J. Biol. Chem. 266, 1516–1525
36. Santoro, M. M., and Bolen, D. W. (1988) Biochemistry 27, 8063–8068