Spectroscopic and Kinetic Studies of the Histidine Residues of Bovine Low-Molecular-Weight Phosphotyrosyl Protein Phosphatase[†,‡]

June P. Davis, Ming-Ming Zhou, and Robert L. Van Etten*

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907-1393

Received August 19, 1993; Revised Manuscript Received November 18, 1993[⊗]

ABSTRACT: The role of the two conserved histidine residues in the low-molecular-weight phosphotyrosyl protein phosphatases was investigated using site-directed mutagenesis of the recombinant bovine heart enzyme. His-66 and His-72 were individually mutated to alanine and to asparagine. A double mutant, containing only alanines in place of the histidines, was also created. The ^{1}H NMR spectra of the purified proteins revealed no apparent tertiary structure alterations. Microscopic pK_as for the two histidines were determined from a pH titration of the wild-type enzyme using ^{1}H NMR spectroscopy and an MLEV-17 spectral editing scheme to more readily follow shifts in the specific histidine resonance peaks. His-66 titrates with an apparent pK_a of 8.4 while for His-72 the value is 9.2. Since earlier chemical modification experiments indicated that the wild-type enzyme was inactivated by the histidine-selective modification reagent diethyl pyrocarbonate (DEP), the histidine mutants were tested for sensitivity to DEP. Both of the histidine single mutants were inactivated by DEP, and surprisingly, the double mutant containing no histidines was also readily inactivated by DEP. Thus, for this protein, modification by DEP is not specific for histidine residues. Kinetic studies of the mutant proteins reveal that neither histidine is essential in the catalytic mechanism. His-66 mutants showed virtually identical catalytic properties compared to wild-type enzyme, whereas His-72 mutants had reduced specific activity and higher phosphate K_i and lower K_m values at pH 5 and higher. It is proposed that His-72, although not essential for catalysis, may serve a significant structural role at the enzyme active site.

Protein tyrosine phosphorylation is an important regulatory mechanism involved in many diverse cellular processes such as cell growth, proliferation, and differentiation (Fischer et al., 1991) (Charbonneau & Tonks, 1992). The phosphorylation state of a protein reflects a competition between the action of protein tyrosine kinases and protein tyrosine phosphatases (PTPases).[1] Several transmembrane, receptor-like, and nonreceptor PTPases have been cloned and characterized. Although very diverse in size and structural organization, each member of this gene family possesses a conserved segment of 240 residues which has been suggested to comprise an independently folding catalytic domain (Charbonneau & Tonks, 1992). In addition to these high-molecular-weight PTPases, a group of low-molecular-weight cytoplasmic protein tyrosine phosphatases may be important in mediating intracellular protein tyrosine dephosphorylation. These small PTPases have molecular weights of approximately 18 000 and possess no apparent sequence homology with the large PTP1B or CD45 type phosphatases described above. The cDNAs for the bovine and human enzymes have been cloned and the genes overexpressed in *Escherichia coli* (Wo et al., 1992a,b). The recombinant enzymes differ only in that the N-terminal alanine is not acetylated. The kinetic and mechanistic properties of the recombinant bovine enzyme are effectively identical to those of the tissue enzyme (Wo et al., 1992b).

Although initially characterized as acid phosphatases, the low-molecular-weight cytoplasmic phosphatases have since been shown to possess strong activity toward various phosphotyrosyl (but not phosphoseryl or phosphothreonyl) peptides and proteins including angiotensin, tyrosine kinase P^{40}, erythrocyte band 3, and EGF receptor (Chernoff & Li, 1985; Boivin & Galand, 1986; Waheed et al., 1988; Ramponi et al., 1989; and Zhang & Van Etten, 1990). Low M_r PTPases have been isolated from a variety of mammalian tissues including human liver (Taga & Van Etten, 1982), placenta (Waheed et al., 1988), and red cell (Boivin et al., 1987); bovine liver (Heinrikson, 1969) and heart (Chernoff & Li, 1985; Zhang & Van Etten, 1990); and rat brain (Oakada et al., 1986) and liver (Manao et al., 1992). Amino acid sequencing of several of these enzymes has revealed a high degree of sequence homology (Camici et al., 1989; Wo et al., 1992b; Dissing et al., 1991; Manao et al., 1992).

Much is known regarding the kinetics and mechanism of this class of enzymes. An early study by Saini et al. (1981) used ^{31}P NMR as well as a chiral phosphomonoester to observe the phosphotransferase activity of bovine liver PTPase and to provide stereochemical evidence that the phosphomonoester hydrolysis proceeds via a double-displacement mechanism involving the formation of a covalent phosphoenzyme intermediate. Extensive kinetic and mechanistic studies of the bovine heart PTPase provided further evidence for the existence of a covalent phosphoenzyme intermediate, with the breakdown of this intermediate being the rate-limiting step in the catalytic mechanism (Zhang & Van Etten, 1991b).

Studies by various groups have implicated the importance of cysteine, histidine, and arginine residues at the enzyme active site. This class of low-molecular-weight PTPases contains 8 conserved sulfhydryl residues, all of which are in

† This work was supported by USDHHS Research Grant GM 27003 and by AIDS Research Center Core Support Grant AI 27713.

‡ The nucleotide sequences reported in this paper have been submitted to GenBank as updates to Accession Number M83656.

* To whom correspondence should be addressed. FAX (317) 494-0239.

⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1994.

[1] Abbreviations: PTPase, phosphotyrosyl protein phosphatase; BHPTP, bovine heart phosphotyrosyl protein phosphatase; pNPP, *p*-nitrophenyl phosphate; DEP, diethyl pyrocarbonate.

0006-2960/94/0433-1278$04.50/0

the free thiol form. The enzymes are readily inactivated by thiol modification reagents such as iodoacetate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Hg^{2+} and Ag^{+}, *p*-hydroxymercuribenzoate, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, and phenylarsine oxide (Bittencourt & Chaimovich, 1976; Lawrence & Van Etten, 1981; Laidler et al., 1982; Zhang et al., 1992). Recently, conclusive evidence in support of a cysteine residue as the enzymatic nucleophile was provided by direct observation of the stoichiometrically-trapped phosphocysteine intermediate using ^{31}P NMR (Wo et al., 1992b). Using site-directed mutagenesis, cysteine 12 has been implicated as the enzymatic nucleophile (Davis et al., 1992; Cirri et al., 1993). The use of cysteine as a nucleophile stands in contrast to the situation with the high-molecular-weight acid phosphatases, where histidine serves as the enzymatic nucleophile [Ostanin et al. (1992) and references cited therein].

Covalent modification studies using histidine-selective reagents such as Rose Bengal dye-sensitized photooxidation (Lawrence & Van Etten, 1981) and diethyl pyrocarbonate (Taga & Van Etten, 1982; Dayton, 1987; Waheed et al., 1988) could be taken to suggest that one or more histidine residues are located at or near the active site of the low-molecular-weight phosphatases. Recently, a ^{1}H NMR technique was developed in this laboratory to allow easy detection and identification of the histidine resonance peaks (Zhou et al., 1993). When the human isoenzymes or the bovine heart enzyme was incubated with the competitive inhibitor vanadate ion, a perturbation in the chemical shift of His-72 was observed. No perturbation of the His-66 resonance (or the other histidines in the human enzymes) was observed. This suggested that His-72 is located at the active site of the enzyme and may be involved in interacting with the substrate phosphate moiety.

In order to further elucidate the role of the histidines in the low-molecular-weight PTPases, histidine mutants of BHPTP have been constructed and analyzed. The kinetic results support the localization of His-72 at the enzyme active site. In addition, a somewhat surprising finding regarding the specificity of modification by diethyl pyrocarbonate is described.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotide primers were synthesized in the Purdue University Laboratory for Macromolecular Structure on an ABI Model 380A synthesizer. The pET vector and *E. coli* host strains were from Novagen. Restriction enzymes were from New England Biolabs. Diethyl pyrocarbonate (DEP) and *p*-nitrophenyl phosphate (pNPP) were from Sigma. Deuterium oxide, deuterium chloride, and sodium deuteroxide were obtained from Cambridge Isotope Laboratories.

Site-Directed Mutagenesis. Mutant proteins were obtained through the use of site-directed mutagenesis. Using a pET-11d plasmid containing wild-type BHPTP as a template (Wo et al., 1992b), a polymerase chain reaction (PCR) (Kleppe et al., 1971) was used to generate mutagenic insert DNA (Innis et al., 1990). The following oligonucleotide primers and their complements were used to generate mutagenic insert fragments: H66N: 5′-GCCTAAGAAATaATGGCAT-3′; H72N: 5′-ACACAGCCaATAAAGCAAG-3′; H66,72A: 5′-GAAATgcTGGCATTAACACAGCCgcTAAAG-3′; and H72A: 5′-GGCATTAATACAGCCgcTAAAGCAAGACAG-3′. The nucleotides which are different from those coding for wild-type BHPTP (i.e., those which create the desired mutation) are shown in lower case letters. These primers change the CAT histidine codon to the AAT asparagine codon or to the GCT alanine codon.[2] The following primers which occur at the 5′- and 3′-end of the insert DNA, respectively, were also used: 424C: 5′-GGATCCATGGCTGAGCAGGTGACCAAGTCG-3′; and 561C: 5′-TATTTCGACGGATCCACTC-3′. Amplifications were performed on an Ericomp Single Block System instrument. In a total volume of 100 μL, 0.1 μg of template DNA was mixed with 100 pmol each of forward and reverse primer, 0.2 mM dNTPs, 10 μL of the (10×) buffer supplied by the manufacturer, and 2 units of Vent polymerase (New England Biolabs). Denaturation was carried out at 94 °C for 30 s, annealing at 45 °C for 1 min, and polymerization at 72 °C for 1.5 min. Altogether, 25 cycles were performed, with the last polymerization step lasting 10 min. Two separate amplifications were required to generate the complete 743 bp mutagenic insert. For the first amplification, plasmid DNA containing the wild-type insert was used as the template DNA. In general, one tube contained the mutagenic forward primer plus 561C, which is the complement to the 3′-end of the insert BHPTP and contains a *Bam*H1 restriction site. A separate tube contained the reverse mutagenic primer plus 424C, which is identical to the 5′-end of the insert BHPTP and contains an *Nco*I restriction site. After amplification, the PCR products were purified by electroelution from a 1.2% agarose gel. For the second amplification, the two mutagenic fragments were combined along with the 424C and 561C primers to produce the 743 bp insert DNA. The insert DNA (0.5 μg) was next digested with *Nco*I and *Bam*H1 followed by ligation to pET-11d plasmid which had also been digested with *Nco*I and *Bam*H1. The ligation mix was then used for transformation to *E. coli* DH5α. Individual colonies were selected for DNA sequencing analysis to confirm the presence of the desired mutation. For protein expression, the mutagenic plasmids were transformed to *E. coli* HMS174(DE3).

DNA Sequencing. Double-stranded DNA sequencing was performed on isolated plasmids using the Sanger dideoxy chain-termination method (Sanger et al., 1977) according to the U.S. Biochemical Co. protocol for Sequenase Version 2.

Expression and Purification of Mutant BHPTPs. Prior to large-scale purification of the mutants, a small-scale expression experiment was used to confirm expression of the correct-size protein. An overnight culture of *E. coli* HMS174(DE3) carrying the mutant pET plasmid was diluted 1:10 in LB medium containing 50 μg/mL ampicillin. After 2.5 h of growth at 37 °C, BHPTP expression was induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.4 mM. After 3-h further incubation, 1.5 mL of cells was harvested by spinning for 5 min in a microcentrifuge. The cells were lysed by a lysozyme freeze/thaw procedure as described earlier (Wo et al., 1992b). Crude extracts were then subjected to SDS–PAGE analysis (Laemmli, 1970) using a 12% gel with Coomassie Brilliant Blue R-250 staining.

Large-scale expression of BHPTP and its derivatives was accomplished by growth in M9ZB medium (1% casein hydrolysate, 0.5% NaCl, 0.1% NH_4Cl, 0.3% KH_2PO_4, 0.6% NaH_2PO_4, 0.4% glucose, and 0.001 M $MgSO_4$) containing 50 μg/mL ampicillin. An overnight culture (200 mL) was diluted into 2 L of fresh medium and incubated for 2.5 h at

[2] The H66,72A reverse primer oligonucleotide was faulty and generated a mutagenic insert fragment in which the histidine 66 position was changed to alanine but the histidine 72 position remained unchanged. The histidine double mutant H66,72A was generated using the H72A mutagenic primers together with a plasmid already containing the H66A mutation as a template.

37 °C with vigorous shaking. BHPTP expression was induced by addition of 0.4 mM IPTG. After 3-h further incubation, the cells were pelleted by centrifugation at 3000*g* for 10 min. The cells were resuspended in ion-exchange buffer (25 mM sodium acetate, pH 5.0, 10 mM sodium phosphate, 1 mM EDTA, 66 mM NaCl) containing 1 mM DTT and lysed using a French press. Cell debris was pelleted by centrifugation, and the supernatant was loaded onto an SP-Sephadex C-50 cation-exchange column equilibrated in the pH 5 ion-exchange buffer. After extensive washing, enzyme was eluted from the column with 300 mM NaH_2PO_4, pH 5.1, and 1 mM EDTA. The fractions containing high phosphatase activity were concentrated and applied to a Sephadex G-75 size-exclusion column that was equilibrated with the pH 5, sodium acetate ion-exchange buffer.

Reverse-Phase High-Pressure Liquid Chromatography. Estimation of BHPTP purity was performed on an IBM LC/9533 ternary gradient liquid chromatograph equipped with an IBM LC/9523 variable-wavelength UV detector. Enzyme (10–20 μg) was loaded on a Synchropak RP-P C18 column (4.6 × 250 mm) and eluted with a linear gradient of 2–62% acetonitrile (containing 0.1% trifluoroacetic acid) for 60 min.

^1H NMR Analysis. The purified protein samples were prepared for NMR experiments by loading onto a G-25 size-exclusion column (1.5 × 4 cm) preequilibrated with 150 mM NaCl in D_2O. Fractions with enzyme activity were combined and concentrated to about 0.6 mL using a Filtron microconcentrator. Then the pH* was adjusted to the desired value with small aliquots of either 0.1 M DCl or 0.1 M NaOD. The pH measurements were done on a Corning Model 130 pH meter employing an Ingold slim-body combination electrode. The reported pH values are glass electrode readings with no correction for isotope effects (i.e., pH*). Typically protein concentrations of 1–2 mM were used for the NMR experiments. ^1H NMR experiments were conducted on a Varian VXR-500 (500-MHz ^1H) NMR spectrometer at the ambient temperature of about 20 °C. The pulse sequence designed to distinguish histidine $C^{\epsilon 1}H$ resonances from residual amide resonances is based on a ($90°_x$–spin-lock$_y$–acq) scheme, in which the spin-lock is generated using an MLEV-17 composite-pulse sequence (Bax & Davis, 1985). The MLEV-17 spin-lock time can be optimized experimentally depending on protein molecular weight. The spin-lock time used for this family of enzymes was about 30 ms.

Suppression of the residual HDO signal was achieved by presaturating the water resonance during a relaxation delay of 2.5 s. All chemical shifts were referenced externally to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0 ppm. With protein concentrations of 1–1.5 mM, excellent spectra were obtained in 64–128 transients. The free induction decay was zero-filled to 16K points and multiplied by a Gaussian weighting function (GB = 0.10) prior to Fourier transformation.

Enzymatic Activity Assay. During enzyme purification, phosphatase activity was measured as described previously (Zhang & Van Etten, 1990). The assay was initiated by mixing 5–10 μL of enzyme solution with 400 μL of 10 mM *p*-nitrophenyl phosphate (pNPP) in pH 5 assay buffer (100 mM sodium acetate, 1 mM EDTA, I = 0.15 M adjusted with NaCl). After 3–6 min, the reaction was quenched by adding 1 mL of 1 N NaOH. The amount of *p*-nitrophenol produced was calculated using a molar extinction coefficient of 18 000 M^{-1} cm^{-1} at 405 nm. The protein concentration was determined colorimetrically (Lowry et al., 1951) or from the UV absorbance using an extinction coefficient $E^{0.1\%}_{280nm}$ of 0.992 that was computed using PCGENE software and the program PHYSCHEM.

Steady-State Kinetics. V_{max} and K_m values for pNPP were determined for the WT, H66N, H66A, H72N, H72A, and H66,72A enzymes at pH 5 (100 mM sodium acetate, 86 mM sodium chloride, and 1 mM EDTA) and pH 7 (50 mM 3,3-dimethylglutarate, 16 mM sodium chloride, and 1 mM EDTA). The hydrolysis of pNPP was followed as described above except the reaction time was 6 min. Eight different pNPP concentrations ranging from $0.1K_m$ to $10K_m$ were used, and triplicate measurements were taken. The V_{max} and K_m values were obtained by fitting the data directly to the Michaelis–Menten equation using the computer program KINFIT (Knack & Röhm, 1981).

A more detailed analysis of the pH dependence of the Michaelis–Menten parameters for pNPP was performed on the WT, H66A, H72A, and H66,72A enzymes. The following buffers were used: 100 mM 3,3-dimethylglutarate, pH 3.5; 100 mM sodium acetate, pH 4.0–5.0; 100 mM Bis-Tris, pH 6.0–7.0; 10 mM glycinamide, pH 7.5–8.0. Each buffer contained 1 mM EDTA, and the ionic strength was adjusted to 0.15 M using NaCl. Eight different pNPP concentrations from $0.2K_m$ to $(3–10)K_m$ were used. (In some cases at the higher pH values, the maximum pNPP concentration was only $3K_m$ in order to avoid the sharp increases in ionic strength caused by the pNPP dianion.) The assay was performed at 37 °C for 4 min.

Inhibition constants for inorganic phosphate were determined for H66A, H72A, and WT enzymes at pH 5 (100 mM sodium acetate, I = 0.15 M) and 37 °C. Eight different concentrations of pNPP ranging from $0.1K_m$ to $10K_m$ were used. The K_m and V_{max} values were determined for three different inhibitor concentrations and in the absence of inhibitor. Inhibition constants were evaluated using the program ENZYME (Lutz & Rodbard, 1985).

Hydrolysis of Aryl and Alkyl Phosphomonoesters. V_{max} and K_m values for some representative aryl and alkyl phosphomonoesters were determined for the H72A mutant and WT BHPTP. The following phosphate monoester substrates were tested in the pH 5 assay buffer: 4-nitrophenyl, 4-(trifluoromethyl)phenyl, 4-ethylphenyl, 3-phenylpropyl, and 5-phenylpentyl. These substrates were synthesized as described earlier (Zhang & Van Etten, 1991b). The assay was carried out at 37 °C for 6 min. Triplicate measurements were taken. Phosphate hydrolysis was monitored by determination of the amount of inorganic phosphate released as described earlier.

pH Titration of Histidine Residues by NMR. In pH titration experiments, the pH* was measured with an Ingold slim-body combination electrode and adjusted in an Eppendorf microcentrifuge tube after removal of the sample from the NMR tube. The protein samples were titrated between pH* 5 and 10 with 2–2.5-μL aliquots of either 0.1 M DCl or 0.1 M NaOD, and NMR spectra were recorded every 0.1–0.2 pH unit. In order to minimize protein denaturation, very small aliquots of either DCl or NaOD were added to the protein sample solution while it was gently stirred. The pH* was measured before and after each NMR spectrum was acquired, and the values usually agreed to within ±0.03 pH unit. The variation with pH of the histidine resonance chemical shift (δ_{obs}) was analyzed using the following equation,

$$\delta_{obs} = \delta_A + (\delta_{AH} - \delta_A)\frac{[H^+]}{K_a + [H^+]}$$

where K_a is the dissociation constant, δ_A is the chemical shift

of the resonance of the unprotonated histidine, δ_{AH} is the chemical shift of the resonance of the protonated histidine, and [H+] is calculated from pH*. Experimental data were fitted to this equation using ENZFITTER, a nonlinear least-squares data analysis program (Leatherbarrow, 1987).

Modification by Diethyl Pyrocarbonate. Catalytic amounts of H66A, H72A, and H66,72A were tested for susceptibility to inactivation by diethyl pyrocarbonate (DEP). Simultaneous inactivation of WT BHPTP was performed for comparison. The modification was conducted in 50 mM 3,3-dimethylglutarate, 16 mM NaCl, and 1 mM EDTA, pH 6.0, buffer at room temperature (23 °C). A fresh stock solution of DEP in absolute ethanol was prepared immediately before use. The reaction was initiated by addition of 10 μL of 0.2 M DEP to 190 μL of enzyme solution. For each enzyme, a control sample was prepared containing 190 μL of enzyme solution plus 10 μL of absolute ethanol. At various times, 10-μL aliquots were removed from the reaction mixture and assayed for remaining enzyme activity. The assay was performed for 3 min at 37 °C in pH 5 sodium acetate buffer containing 10 mM pNPP. The percentage of residual activity of the modified sample was calculated relative to the control sample, and a pseudo-first-order rate constant for inactivation was determined using a linear regression program.

The inactivation of the double histidine mutant H66,72A was also tested using a lower concentration of DEP. The effect of an added competitive inhibitor, inorganic phosphate, was also tested. One enzyme sample contained 0.5 mM DEP, and the other sample contained 0.5 mM DEP plus 5 mM inorganic phosphate. Control samples contained enzyme plus ethanol or enzyme plus ethanol plus 5 mM inorganic phosphate. At various times, aliquots were removed from the reaction mixture and analyzed as described above.

Larger-scale modification experiments were employed to allow spectrophotometric observation of the DEP-modified enzyme. In this case, 0.7–1.1 mg/mL enzyme in 5 mM 3,3-dimethylglutarate was reacted with a 200-fold equivalent excess of DEP. UV spectra in the 200–300-nm region were recorded on a Beckman DU-68 spectrophotometer.

RESULTS

Overexpression and Purification of BHPTP Mutants. A T7 polymerase-dependent expression system was used to overexpress WT and mutant BHPTPs in *E. coli* HMS174-(DE3). Site-directed mutagenesis of the conserved histidine residues was achieved using PCR methodology as described in Experimental Procedures. Five histidine mutants were made: H66N, H66A, H72N, H72A, and H66,72A. DNA sequencing of the complete BHPTP coding region in the plasmid allowed confirmation of both the presence of the desired mutation and the absence of undesired mutations. Small-scale expression experiments followed by SDS–PAGE analysis of the crude extracts confirmed overexpression of the proper-size protein (data not shown).

Large-scale expression of the mutants produced 25–50 mg of enzyme/L of culture. Two chromatographic steps, a cation-exchange column followed by a size-exclusion column, were sufficient to purify the protein to homogeneity. The predominant impurity remaining after the ion-exchange step was identified as the 29-kDa β-lactamase protein produced by the pET plasmid. Size-exclusion chromatography permitted removal of this impurity. In each case, protein purity was estimated to be >95% by reverse-phase high-pressure liquid chromatography (data not shown).

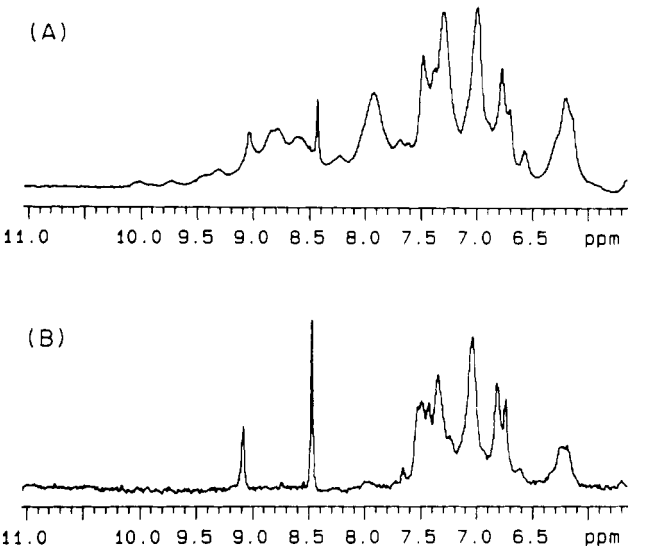

FIGURE 1: ^{1}H NMR spectra of 1.5 mM BHPTP in 150 mM NaCl/D_2O at pH 5.5, obtained (A) using a single 90° pulse sequence; or (B) using the MLEV-17 pulse sequence with a spin-lock time of 30 ms.

^{1}H NMR Analysis. Figure 1 shows ^{1}H spectra of BHPTP. Two histidine resonances were readily detected using the spin-lock pulse sequence employed here, whereas in conventional 1D spectra, one histidine peak at about 9.07 ppm was severely overlapped by the unexchanged amide proton peaks. Without the use of the present spin-lock pulse sequence, this histidine peak was effectively buried under the NH peaks during pH titration experiments even after several exchanges of deuterium oxide solvent.

Assignment of the histidine resonances of BHPTP was accomplished by comparison with the spectra of the corresponding histidine to asparagine mutants, as shown in Figure 2. Comparison of the aromatic region of WT and mutant enzymes also revealed that the histidine to asparagine mutation at both positions was conservative and did not result in detectable changes in the global or local conformation of the enzyme. In the H66N spectrum (Figure 2A) the peak at 8.45 ppm disappeared, while the peak at 9.07 ppm remained effectively unchanged, indicating that the peak at 8.45 ppm in the WT spectrum (Figure 2C) is due to His-66 and the peak at 9.07 ppm is due to the His-72 residue. This assignment was confirmed using an H72N mutant. The NMR spectrum of this protein (Figure 2B) revealed that the peak at 9.07 ppm disappeared as a consequence of the His-72 to Asn mutation. These histidine assignments were further confirmed using the corresponding two histidine to alanine mutants of BHPTP. The ^{1}H NMR spectra (data not shown) were very similar to those of the H66N and H72N mutants. Moreover, in a H66,-72A mutant in which both histidines were changed to alanines, both of the relevant peaks at 8.45 and 9.07 ppm of the BHPTP spectrum were absent from the spectrum (data not shown).

Steady-State Kinetics of BHPTP Mutants. V_{max} and K_m values for the substrate *p*-nitrophenyl phosphate were determined at pH 5 and 7, 37 °C, for the histidine mutants and for WT enzyme, measured simultaneously. These data are summarized in Table 1. Mutant enzymes in which the histidine at position 66 is converted to asparagine or alanine have essentially unaltered kinetic parameters for pNPP. However, compared to WT enzyme, the mutant H72A has 5.9- and 4.4-fold lower activity at pH 5 and 7, respectively. Interestingly, the K_m values for this mutant are also lower. The activity of H72N is higher than the corresponding alanine

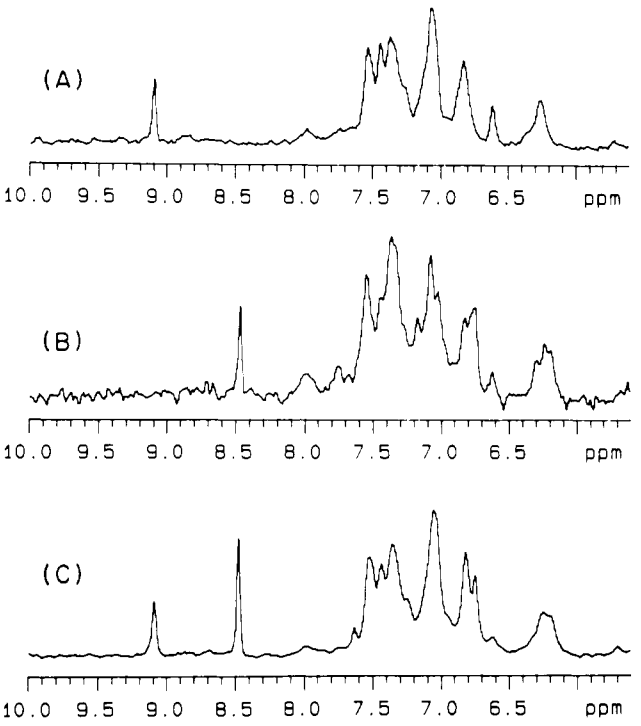

FIGURE 2: ^{1}H NMR spectra of (A) 1 mM H66N BHPTP; and (B) 1 mM H72N BHPTP; and (C) 1.5 mM BHPTP wild-type enzyme, in 150 mM NaCl/D_2O at pH 5.5. The spin-lock time used was 30 ms.

Table 1: Kinetic Parameters of WT and Mutant BHPTP Using pNPP as Substrate

	pH 5[a]		pH 7[b]	
mutant	K_m (mM)	V_{max} (units/mg)	K_m (mM)	V_{max} (units/mg)
WT	0.31	89	2.45	61
H66N	0.31	64	3.92	58
H66A	0.28	86	2.20	63
H72N	0.32	26	1.59	25
H72A	0.28	15	0.95	14
H66,72A	3.1	11	2.51	12

[a] Measurements performed in pH 5.0, 100 mM sodium acetate buffer, 1 mM EDTA, and NaCl added to make I = 0.15 M. [b] Measurements performed in pH 7.0, 50 mM 3,3-dimethylglutarate buffer, 1 mM EDTA, and NaCl added to make I = 0.15 M.

mutant, but is still 2.4–3.4-fold lower than WT. As expected, the H66,72A double mutant has a specific activity no greater than that of the single mutant, H72A. However, the substrate K_m is 10-fold higher than for WT enzyme at pH 5.

A characterization of the pH dependence of WT, H66A, H72A, and H66,72A under conditions where ionic strength was carefully controlled (see Experimental Procedures) revealed further differences in K_m values (Table 2). Although

Table 2: pH Dependence of Michaelis–Menten Parameters[a]

		pH:						
mutant		3.5	4.0	5.0	6.0	6.5	7.0	7.5	8.0
WT	V_{max}	57	81	110	98	102	82	116	121
	K_m	1.5	0.33	0.41	2.0	6.9	22	46	(196)
H66A	V_{max}		70	100	84	98			
	K_m		0.51	0.33	1.8	5.8			
H72A	V_{max}		11	15	13	15	18	18	19
	K_m		1.2	0.19	0.41	1.1	3.6	6.6	18
H66,72A	V_{max}			14	8.2	10			
	K_m			4.3	2.2	3.1			

[a] V_{max} is in units/mg and K_m is in mM. Buffers: pH 3.5, 100 mM 3,3-dimethylglutarate; pH 4.0, 100 mM sodium acetate; pH 5.0, 100 mM sodium acetate; pH 6.0, 100 mM Bis-Tris; pH 6.5, 100 mM Bis-Tris; pH 7.0, 100 mM Bis-Tris; pH 7.5, 10 mM glycinamide; pH 8.0, 10 mM glycinamide. All buffers contained 1 mM EDTA and had an ionic strength of 0.15 M.

valuable and convenient for comparative purposes, the use of 50 mM 3,3-dimethylglutarate buffer presents a complication to the determination of precise K_m values. Because the ionic strength varies significantly from 0.15 M at the higher substrate concentrations, the observed K_m values were found to reflect a significant dependence on ionic strength as well as pH. The higher K_m values observed for WT enzyme in the higher pH range are consistent with the enzyme specificity for the monoanion form of *p*-nitrophenyl phosphate. Because it is impossible to maintain a constant ionic strength of 0.15 M at the higher substrate concentrations, the K_m and V_{max} values reported for pH 7.5 and 8.0 for WT enzyme are only rough estimates. However, because H72A exhibited much lower K_m values, an optimal range of substrate concentrations [$0.3K_m$ to $(4–8)K_m$] could be used to obtain precise kinetic data for that protein. The lower K_m values that are observed for H72A indicate that the substrate binds to this mutant with a higher apparent affinity compared to wild-type. All of the histidine mutants had increased K_m values at the lower pH of 4.

Inhibition constants for inorganic phosphate were measured for H66A, H72A, and WT BHPTP at pH 5, since substrate K_m values are not true equilibrium binding constants. The wild-type and H66A enzymes had effectively identical K_i values of 1.87 ± 0.06 mM and 1.77 ± 0.10 mM, respectively, whereas H72A had a higher K_i of 2.92 ± 0.13 mM. The higher K_i observed for H72A reflects a reduced affinity for phosphate ion.

Hydrolysis of Aryl and Alkyl Phosphomonoesters. To assess if the lower activity of the H72A mutant reflects a change in the rate-determining step of the catalytic mechanism from dephosphorylation of the covalent phosphoenzyme intermediate to phosphorylation of the enzyme, several phosphate monoesters with significantly different leaving group pK_a values were tested (Table 3). The results for WT enzyme

Table 3: Michaelis–Menten Parameters for Hydrolysis of Aryl and Alkyl Phosphate Monoesters[a]

	wild-type BHPTP				H72A		
phosphate ester	leaving group pK_a	k_{cat} (s^{-1})	K_m (mM)	$10^{-3} \times k_{cat}/K_m$ (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	$10^{-3} \times k_{cat}/K_m$ (M^{-1} s^{-1})
aryl phosphomonoester:							
4-nitrophenyl	7.14	22.8	0.39	58	4.3	0.27	16
4-(trifluoromethyl)phenyl	8.68	22.8	1.03	22	4.2	0.55	7.6
4-ethylphenyl	10.0	20.9	2.0	10	3.7	1.02	3.6
alkyl phosphomonoester:							
$Ar(CH_2)_3OPO_3H_2$	15.79	5.3	23.8	0.22	1.2	10.7	0.11
$Ar(CH_2)_5OPO_3H_2$	15.9	7.2	15.2	0.47	1.8	9.9	0.18

[a] Measurements made at pH 5, 37 °C.

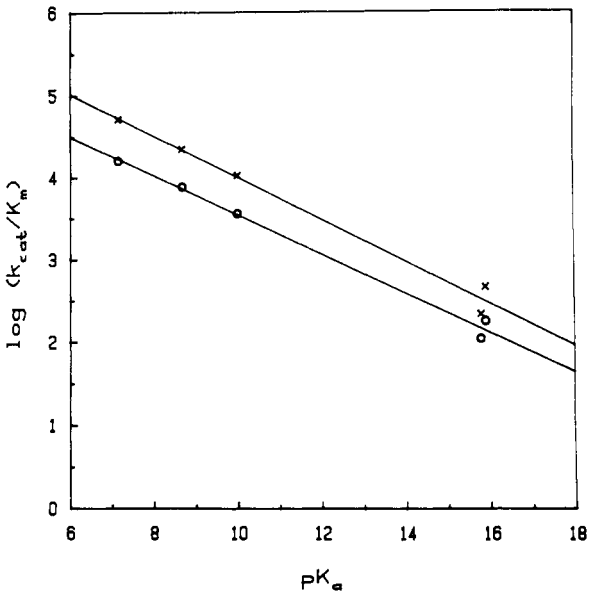

FIGURE 3: Modified Bronsted plot for k_{cat}/K_m at pH 5.0, 37 °C. The data points are from Table 3, and lines are generated by a linear regression method. Legend: (×), WT BHPTP; and (○), H72A BHPTP. The slope corresponding to WT data is –0.25, and the slope corresponding to H72A data is –0.24.

agree well with those reported earlier for the tissue enzyme (Zhang & Van Etten, 1991a). The k_{cat} values of the aryl substrates are effectively constant for both enzymes, consistent with the formation of a covalent phosphoenzyme intermediate, and with dephosphorylation of this intermediate being the rate-limiting step in the catalytic mechanism. As has previously been reported for WT enzyme (Zhang & Van Etten, 1991a), the lower k_{cat} values observed for alkyl phosphomonoesters are due to rate-limiting phosphorylation. The linear correlation of $\log(k_{cat}/K_m)$ versus leaving group pK_a for the two classes of substrates indicates a uniform catalytic mechanism for the phosphorylation event. There is also a good correlation between $\log k_{cat}/K_m$ vs leaving group pK_a for the H72A mutant using the aryl and alkyl phosphomonoesters (see Figure 3).

$$\text{WT:} \quad \log(k_{cat}/K_m) = -0.25pK_a + 6.55$$

$$\text{H72A:} \quad \log(k_{cat}/K_m) = -0.24pK_a + 5.93$$

Since k_{cat}/K_m monitors the phosphorylation step, the effectively identical slopes of the lines indicate that the H72A mutation has not interfered with this mechanistic step under the condition tested. The β_{lg} value of –0.25 is typical for reactions involving protonation of the leaving group (Kirby & Varvoglis, 1967; Bunton et al., 1967). Since the value measured for H72A is the same as the value measured for WT enzyme, it is unlikely that His-72 is acting as a general acid to donate a proton to the leaving phenol/alcohol at pH 5.

Titration of the Histidine Residues by NMR. Recently, a new technique has been developed in this laboratory to permit the facile detection of histidine resonance peaks (Zhou et al., 1993). NMR spectra were recorded for each of the histidine mutants. The absence of one of the histidine peaks in each single mutant allowed conclusive assignment of the two histidine resonances (Figure 2). A pH titration of WT enzyme was then used to follow chemical shifts of the histidine resonances. As shown in a series of ^{1}H NMR spectra of WT BHPTP at different pH conditions (Figure 4), the two histidine peaks undergo a pH-dependent chemical shift change. Typical

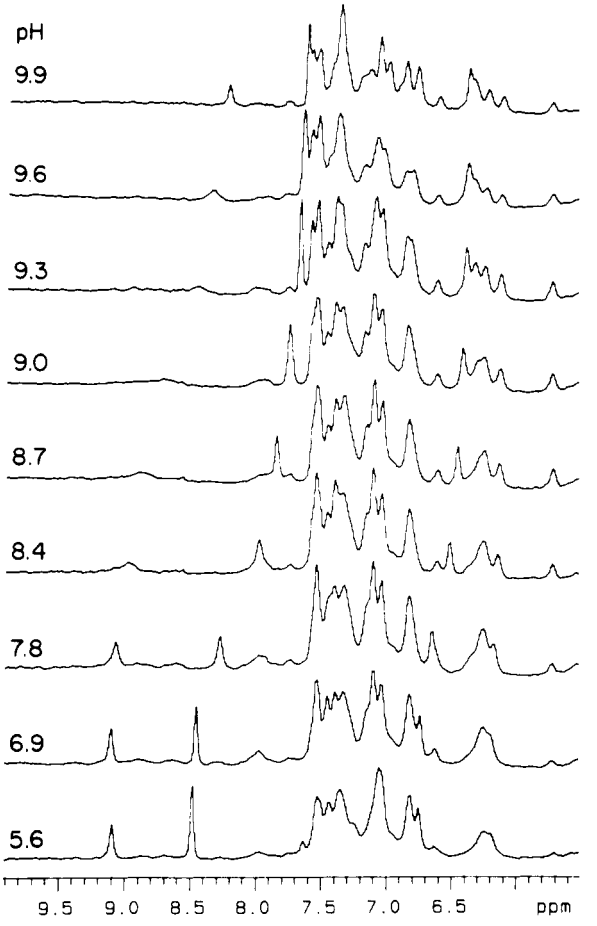

FIGURE 4: ^{1}H NMR spectra illustrating the pH titration of WT BHPTP. 2 mM BHPTP in 150 mM NaCl/D_2O at different pH values. The spin-lock time was 30 ms.

for such titration curves, the histidine resonances exhibit peak broadening in the transition region (around the pK_a) of the pH titration. Apparently as a consequence of some particular feature of the active site region, the His-72 resonance is significantly weaker than that of His-66. It would be extremely difficult to follow its chemical shift change without the use of the current spin-lock pulse sequence approach. Except for the chemical shift change of the two histidine $C^{\delta 2}H$ peaks as a function of pH, there are no significant changes apparent elsewhere in the aromatic region. This result indicates that the enzyme structure is not significantly disturbed throughout the pH titration. Chemical shifts, δ_{HA} and δ_A, for the protonated and deprotonated forms of the histidine residue, respectively, were determined for the His-66 and His-72 $C^{\epsilon 1}H$'s. For His-66, δ_{HA} and δ_A were 8.43 and 7.54 ppm, respectively, and for His-72, δ_{HA} and δ_A were 9.06 and 7.78 ppm, respectively. A plot of the titration data showing the chemical shifts as a function of pH is displayed in Figure 5. The pK_a values obtained by fitting the titration data for the two histidine peaks were 8.36 ppm for His-66 and 9.19 ppm for His-72.

Modification by Diethyl Pyrocarbonate. Catalytic amounts of WT, H66A, H72A, H66,72A, and C17A were reacted with 10 mM DEP at pH 6, 23 °C, in 50 mM 3,3-dimethylglutarate and 1 mM EDTA, I = 0.15 M (Figure 6). First-order rate constants for inactivation were estimated using the first four data points (at longer times there was increased deviation from linearity, presumably due to the decomposition of DEP). The rate constants were 0.032, 0.039, 0.092, and 0.013 min^{-1} for WT, H66A, H72A, and C17A, respectively.

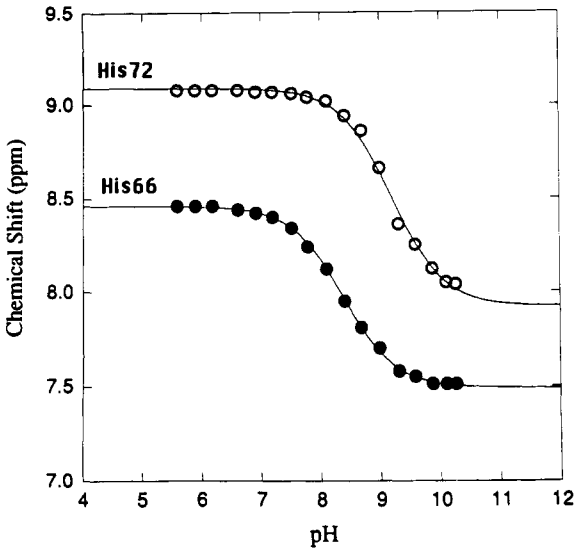

FIGURE 5: ^{1}H NMR titration curves of the histidine C$^{\epsilon 1}$H's of BHPTP. The solid lines represent the curves calculated using the chemical shift data reported in Results. (●) His 66, pK_a = 9.19; (○) His 72, pK_a = 8.36.

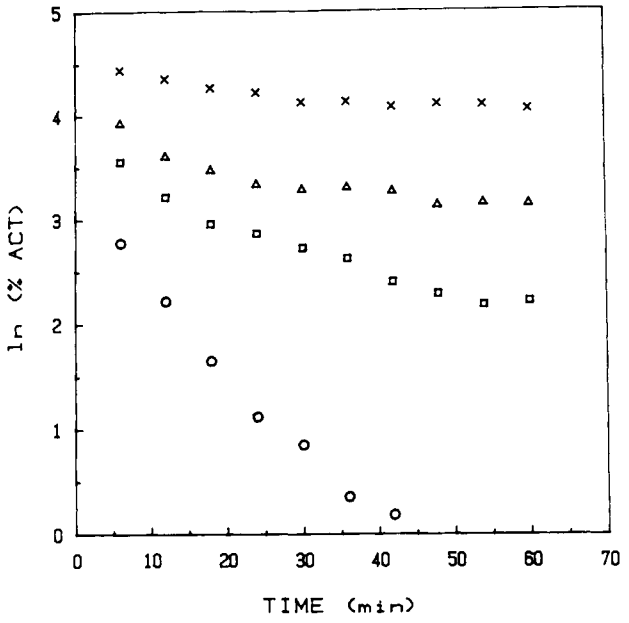

FIGURE 6: Diethyl pyrocarbonate inactivation of BHPTP mutants. 10 mM DEP was added to catalytic amounts of the following BHPTP enzymes: (△) wild type; (□) H66A; (○) H72A; and (×) C17A in pH 6, 50 mM 3,3-dimethylglutarate buffer at room temperature.

Interestingly, the rate constant for inactivation of H66,72A could not be determined under these conditions because, at this DEP concentration, the enzyme was completely inactive before the first data point could be measured ($t_{1/2}$ ~22 min for WT and $t_{1/2}$ < 3 min for H66,72A). To test if a competitive inhibitor could protect H66,72A from inactivation, a lower concentration of DEP (0.5 mM) was used. The inactivation rate in the presence of 5 mM inorganic phosphate was 3.5-fold slower than in its absence, 0.018 min^{-1} and 0.064 min^{-1}, respectively (see Figure 7). The rate of inactivation of this mutant at 23 °C is still faster than the rate constant calculated for inactivation of WT at 37 °C using 0.5 mM DEP (0.024 min^{-1}) (Zhang et al., 1992).

Larger-scale inactivation experiments were conducted in 5 mM 3,3-dimethylglutarate, pH 7. UV spectra were recorded in the region 200–300 nm. A control experiment using

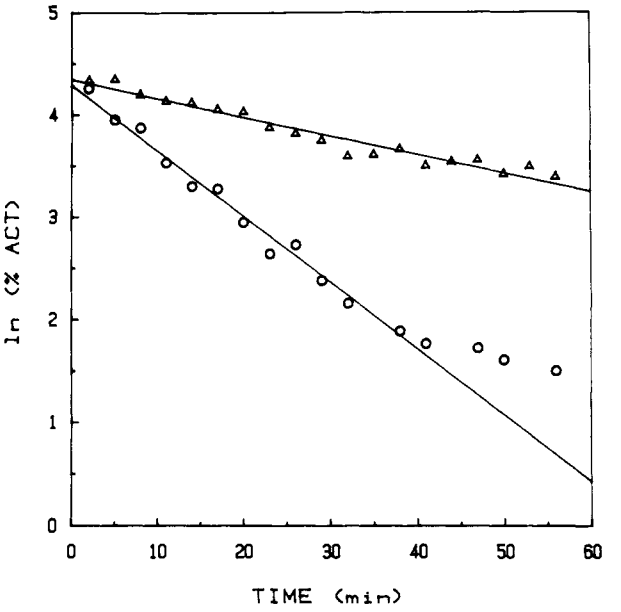

FIGURE 7: Diethyl pyrocarbonate inactivation of the H66,72A mutant in pH 6, 50 mM 3,3-dimethylglutarate buffer at room temperature. Legend: (○) H66,72A plus 0.5 mM DEP; (△) H66,72A plus 0.5 mM DEP plus 5 mM inorganic phosphate.

lysozyme under these conditions reproduced the spectrum reported by Li et al. (1993) (data not shown). Using the carbethoxyhistidine extinction coefficient of ϵ_{242} = 3200 M^{-1} cm^{-1}, a value of 1.24 for moles of carbethoxyhistidine present was calculated. This agrees reasonably well with the actual presence of one histidine in lysozyme. A similar experiment was performed on wild-type BHPTP. After 1 h 37 min, no further change in the spectrum was observed and the residual enzyme activity remained constant. A calculation of moles of carbethoxyhistidine present gave a value of 2.9. It should be noted that this enzyme contains only two histidines. NH_2-OH is known to decompose the carbethoxyhistidine moiety and regenerate native histidine (Melchior & Fahrney, 1970; Lundblad, 1991). However, NH_2OH reportedly does not regenerate modified cysteines[3] or histidines that have been modified by reaction with two molecules of DEP (Miles, 1977). The addition of 0.24 M NH_2OH to modified WT enzyme did not restore enzyme activity, consistent with earlier observations for the tissue enzyme (Zhang et al., 1992).[4]

A large-scale modification of this histidine double mutant also resulted in an increase in absorbance at 242 nm. Using an extinction coefficient of 3200 M^{-1} cm^{-1}, 1.13 mol of carbethoxyhistidine would be predicted to be present in this mutant (which of course contains no histidine residues). Garrison and Himes (1975) have suggested that DEP can react with carboxylate buffers to form a mixed anhydride, which then reacts with cysteine in a protein to form thiol esters which absorb in the 240-nm region. However, the histidine double mutant is also readily inactivated by DEP in

[3] Some discrepancy exists in the literature regarding the regeneration of DEP-modified cysteine residues. Miles (1977) states that NH_2OH does not regenerate modified sulfhydryl residues. However, the reference cited in support of that statement does not include any mention of such studies of sulfhydryl modification reactions. Others have reported that NH_2OH can regenerate the cysteine from thiol esters formed by reaction of cysteine with DEP in carboxylate buffers (Garrison & Himes, 1975).

[4] In an earlier study, the NH_2OH that was used not only failed to reactivate the enzyme, but also caused inactivation of residual enzyme. However, reevaluation of this experiment with a fresh sample of NH_2OH showed no enzyme inactivation.

pH 6 Bis-Tris buffer. Therefore, although DEP may be reacting with a cysteine residue, prior reaction with a carboxylic acid buffer is not a requirement.

DISCUSSION

Among the low-molecular-weight phosphotyrosyl protein phosphatases sequenced to date, His-66 and His-72 are conserved. In order to assess the role of histidines in the low-molecular-weight PTPases, several histidine mutants of the bovine heart enzyme were constructed and analyzed. Both of the histidines, His-66 and His-72, were individually replaced by asparagine and by alanine; a double mutant containing alanines in place of histidines was also made.

All of the mutants retained phosphatase activity. The His-66 mutants had kinetic parameters very similar to those of the wild-type enzyme, confirming that this residue is neither mechanistically nor structurally important to the catalytic function of the enzyme. On the other hand, His-72 mutants had significantly reduced catalytic activity. In order to confirm that the reduced activity of H72A was not due to a change in the catalytic mechanism of the enzyme from rate-limiting dephosphorylation to phosphorylation, several aryl and alkyl phosphomonoesters were tested. The dependence of k_{cat}/K_m versus leaving group pK_a was the same for H72A as for WT enzyme. Therefore, His-72 is not required in the phosphorylation step at pH 5, and no significant change in the catalytic mechanism has occurred. To the extent that competitive inhibition constants K_i for phosphate ion reflect alterations in the true substrate binding constants K_s, it is clear that substrate binding to H66A would be expected to be unaffected, while a small but experimentally significant decrease in binding affinity may be expected with the H72A mutants. Decreased K_m values are probably the result of lowered k_3 values (Zhang & Van Etten, 1991b).

The His-72 to asparagine mutant had a higher activity than the corresponding alanine mutant. This may suggest a structural role for the histidine. Although the asparagine nitrogen would be unlikely to participate in electrostatic interactions, it is accepted that the nitrogen of the asparagine conserves the position occupied by the N-1 atom in the imidazole ring (Lowe et al., 1985) and therefore may preserve some important hydrogen bonding interaction. If such an interaction is important to the local active site structure, then its loss may account for the reduced activity of the mutant.

At pH 5, though not at pH 7, the H66,72A double mutant had a 10-fold higher K_m compared to WT enzyme (Tables 1 and 2). This may be related to structural alterations and in turn to a decreased structural stability of this mutant at pH 5 since, unlike the other mutants, this enzyme was observed to precipitate upon storage at 4 °C when concentrated to a protein concentration >5 mg/mL. These effects may be due to a lower isoelectric point for this mutant compared to wild-type or the single histidine mutants (pI_{WT} = 6.87, pI_{H72A} = 6.73, p$I_{H66,72A}$ = 6.37). (Isoelectric points were computed using the PCGENE software program PHYSCHEM.)

The histidines were found to have relatively high apparent pK_a values, which may be the result of ionic interactions that in turn can affect protein structural stability. The current finding of a pK_a of 8.4 for His-66 agrees well with the pK_a assignment of 8.3 made earlier by Dayton (1987) using ^{1}H NMR and enzyme isolated from bovine liver. An identification of the other histidine resonance and determination of its pK_a were not possible at that time due to the severe overlap of unexchanged amide protons in the spectrum. However, by using the present MLEV spin-lock pulse sequence which effectively suppresses signals due to amide proton peaks, in conjunction with the histidine single mutants in which one peak was absent, it was possible to positively identify the His-72 resonance peak. Compared to typical histidine pK_a values (Lundblad, 1991), His-72 titrates with an unusually high apparent pK_a of 9.2. Interestingly, a recent survey of the literature revealed no other protein histidine residues with such a high pK_a (Fasman, 1989).[5] The high pK_a values observed for the BHPTP histidines may be due to a direct interaction of the protonated histidines with anionic side chains such as those of aspartate or glutamate. Such an interaction would serve to elevate the histidine pK_a and reduce the pK_a of the acidic residue.

Chemical modification experiments are often quite helpful in implicating the presence of certain amino acid residues at the enzyme active site. The reagent may directly modify catalytic residues or merely modify a residue near the enzyme active site and thereby sterically hinder or block substrate binding. The low molecular-weight PTPases were previously shown to be sensitive to various modification reagents selective for cysteine, arginine, and histidine residues. Modification by diethyl pyrocarbonate was further explored in this work. Earlier studies of the pH dependence of DEP modification showed that the residue responsible for loss of enzyme activity has an apparent pK_a of 7.2 (Zhang et al., 1992). Since this value is consistent with a normal histidine pK_a and because the actual His-72 pK_a was unknown at that time, the conclusion was made that the DEP-modified residue was most likely a histidine with a pK_a of 7.2. However, the present site-directed mutagenesis study and direct pK_a determination for His-72 have shown this not to be the case. The modification by DEP was performed at pH 6, and while normally this would be optimal for selective histidine modification, the histidines in BHPTP would not be very reactive at this pH because they would still be in the predominantly protonated, nonnucleophilic form.

Assessment of the modification by DEP of the histidine to alanine mutants indicated that the single histidine mutants and the double mutant containing no histidines are each readily inactivated by DEP. The rate of inactivation of H66A was 1.2-fold faster than WT, H72A was 2.9-fold faster, and the inactivation of H66,72A was at least 10-fold faster than WT enzyme. A similar trend was observed for the histidine to asparagine mutants. The faster inactivation of H72A is consistent with kinetic results suggesting that replacement of this histidine with alanine creates a more open or accessible active site pocket. It was surprising to find that the double mutant, H66,72A, was even more rapidly inactivated by DEP and that the increase in the rate of inactivation was even greater than the sum of the increases in the rates of inactivation of the single mutants. As noted earlier, this mutant may have a reduced structural stability, and this may account for the increased inactivation rate. Addition of the competitive inhibitor inorganic phosphate significantly reduced, but did not eliminate, enzyme inactivation. The phosphate may serve to protect the sensitive active site residue(s) responsible for loss of catalytic activity, or it may just act to stabilize the enzyme structure.

The large-scale inactivation experiments using direct detection of the putative carbethoxyhistidine moiety by monitoring increases in the 242-nm absorbance indicate that this method alone is not reliable to determine moles of histidine modified by DEP. Calculations of the moles of carbethox-

[5] In fact, the highest histidine pK_a cited in Fasman (1989) is only 8.2.

yhistidine present suggested that wild-type BHPTP contained 2.9 mol of histidine and H66,72A contained 1.1 mol of histidine. Since in actuality the wild-type enzyme contains only two histidines and the H66,72A mutant contains no histidines, caution clearly is advised in the interpretation of such DEP spectrophotometric measurements.

In work in progress, preliminary DTNB titrations of the modified WT and H66,72A enzymes appear to indicate that sulfhydryl residues in BHPTP are modified by DEP under the conditions used. Consistent with this, modification of a C17A mutant by DEP showed that this mutant has a 2.5-fold slower rate of inactivation compared to that of wild-type enzyme. However, because the WT enzyme possesses eight free cysteines, it is difficult to obtain sufficiently precise quantitative data on the number of cysteines that are modified. Such experiments may be possible using radiolabeled iodoacetate or other sulfhydryl-reactive reagents.

Thus, in contrast to high-molecular-weight acid phosphatases, which have two highly important or absolutely essential histidine residues, the present type of phosphomonesterase has none. This result is perhaps all the more striking when one considers the similarity in the pH dependence of, for example, the (high-molecular-weight) human prostatic and the (low-molecular-weight) bovine enzymes (Taga & Van Etten, 1982; Van Etten, 1982). The molecular basis of these differences may not be revealed until detailed structural information is available for the two types of phosphatases.

ACKNOWLEDGMENT

We thank Christine Pokalsky for synthesis of alkyl phosphomonoesters.

REFERENCES

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355–360.
Bittencourt, H. M. S., & Chaimovich, H. (1976) *Biochim. Biophys. Acta 438*, 153–158.
Boivin, P., & Galand, C. (1986) *Biochem. Biophys. Res. Commun. 134*, 557–564.
Boivin, P., Galand, C., & Bertrand, O. (1987) *Int. J. Biochem. 19*, 613–618.
Bunton, C. A., Fendler, E. J., Humeres, E., & Yang, K.-U. (1967) *J. Org. Chem. 32*, 2806–2811.
Camici, G., Manao, G., Cappugi, G., Modesti, A., Stefani, M., & Ramponi, G. (1989) *J. Biol. Chem. 264*, 2560–2567.
Charbonneau, H., & Tonks, N. K. (1992) *Annu. Rev. Cell Biol. 8*, 469–493.
Chernoff, J., & Li, H. C. (1985) *Arch. Biochem. Biophys. 240*, 135–145.
Cirri, P., Chiarugi, P., Camici, G., Manao, G., Raugei, G., Cappugi, G., & Ramponi, G. (1993) *FEBS Lett. 214*, 647–657.
Davis, J. P., Zhou, M.-M., Wo, Y.-Y. P., Zhang, Z.-Y., & Van Etten, R. L. (1992) *ASBMB Fall Symposium—Structure and Function of Protein Kinases and Phosphatases*, Keystone, CO, Oct 23–26, 1992, Abstract, p 99.
Dayton, B. D. (1987) Ph.D. Thesis, Purdue University.
Dissing, J., Johnsen, A. H., & Sensabaugh, G. F. (1991) *J. Biol. Chem. 266*, 20619–20625.
Fasman, G. D. (1989) in *Practical Handbook of Biochemistry and Molecular Biology*, pp 359–362, CRC Press, Boca Raton, FL.
Fischer, E. H., Charbonneau, H., & Tonks, N. K. (1991) *Science 253*, 401–406.
Garrison, C. K., & Himes, R. H. (1975) *Biochem. Biophys. Res. Commun. 67*, 1251–1255.
Heinrikson, R. L. (1969) *J. Biol. Chem. 244*, 299–307.
Innis, M. A., Gelfand, D. H., Sninsky, J. J., & White, T. J. (1990) *PCR Protocols: A Guide To Methods and Applications*, Academic Press, Inc., San Diego, CA.
Kirby, A. J., & Varvoglis, A. G. (1967) *J. Am. Chem. Soc. 89*, 415–423.
Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I., & Khorana, H. G. (1971) *J. Mol. Biol. 56*, 341–361.
Knack, I., & Röhm, K. H. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 1119–1130.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Laidler, P. M., Taga, E. M., & Van Etten, R. L. (1982) *Arch. Biochem. Biophys. 216*, 512–521.
Lawrence, G. L., & Van Etten, R. L. (1981) *Arch. Biochem. Biophys. 206*, 122–131.
Leatherbarrow, R. J. (1987) *Enzfitter A Non-Linear Regression Data Analysis Program for the IBMPC*, Elsevier BIOSOFT, Cambridge.
Li, C., Moore, D. S., & Rosenberg, R. C. (1993) *J. Biol. Chem. 268*, 11090–11096.
Lowe, D. M., Fersht, A. R., & Wilkinson, A. J. (1985) *Biochemistry 24*, 5106–5109.
Lowry, O. H., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.
Lundblad, R. L. (1991) in *Chemical Reagents for Protein Modification*, 2nd ed., p 8, CRC Press, Boca Raton, FL.
Lutz, R. A., & Rodbard, D. (1985) *Clin. Chem. 31*, 656.
Manao, G., Pazzagli, L., Cirri, P., Caselli, A., Camici, G., Cappugi, G., Saeed, A., & Ramponi, G. (1992) *J. Protein Chem. 11*, 333–345.
Melchior, W. B., Jr., & Fahrney, D. (1970) *Biochemistry 9*, 251.
Miles, E. W. (1977) *Methods Enzymol. 47*, 431–442.
Oakada, M., Owada, K., & Nakagawa, H. (1986) *Biochem. J. 239*, 155–162.
Ostanin, K., Harms, E., Stevis, P., Kuciel, R., Zhou, M.-M., & Van Etten, R. L. (1992) *J. Biol. Chem. 267*, 22830–22836.
Ramponi, G., Manao, G., Camici, G., Cappugi, G., Ruggiero, M., & Bottaro, D. P. (1989) *FEBS Lett. 250*, 469–473.
Saini, M. S., Buchwald, S. C., Van Etten, R. L., & Knowles, J. R. (1981) *J. Biol. Chem. 256*, 10453–10455.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Taga, E. M, & Van Etten, R. L. (1982) *Arch. Biochem. Biophys. 214*, 505–515.
Van Etten, R. L. (1982) *Ann. N.Y. Acad. Sci. 390*, 26–53.
Waheed, A., Laider, P. M., Wo, Y.-Y. P., & Van Etten, R. L. (1988) *Biochemistry 27*, 4265–4273.
Wo, Y.-Y. P., McCormack, A. L., Shabanowitz, J., Hunt, D. F., Davis, J. P., Mitchell, G. L., & Van Etten, R. L. (1992a) *J. Biol. Chem. 267*, 10856–10865.
Wo, Y.-Y. P., Zhou, M.-M., Stevis, P., Davis, J. P., Zhang, Z.-Y., & Van Etten, R. L. (1992b) *Biochemistry 31*, 1712–1721.
Zhang, Z.-Y., & Van Etten, R. L. (1990) *Arch. Biochem. Biophys. 228*, 39–49.
Zhang, Z.-Y., & Van Etten, R. L. (1991a) *Biochemistry 30*, 8954–8959.
Zhang, Z.-Y., & Van Etten, R. L. (1991b) *J. Biol. Chem. 266*, 1516–1525.
Zhang, Z.-Y., Davis, J. P., & Van Etten, R. L. (1992) *Biochemistry 31*, 1701–1711.
Zhou, M.-M., Davis, J. P., & Van Etten, R. L. (1993) *Biochemistry 32*, 8479–8486.