Cloning, Expression, and Catalytic Mechanism of the Low Molecular Weight Phosphotyrosyl Protein Phosphatase from Bovine Heart[†,‡]

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ABSTRACT: The first representative of a group of mammalian, low molecular weight phosphotyrosyl protein phosphatases was cloned, sequenced and expressed in *Escherichia coli*. Using a 61-mer oligonucleotide probe based on the amino acid sequence of the purified enzyme, several overlapping cDNA clones were isolated from a bovine heart cDNA library. A full-length clone was obtained consisting of a 27-bp 5′ noncoding region, an open reading frame encoding the expected 157 amino acid protein, and an extensive 3′ nontranslated sequence. The identification of the clone as full length was consistent with results obtained in mRNA blotting experiments using poly(A)$^+$ mRNA from bovine heart. The coding sequence was placed downstream of a bacteriophage T7 promoter, and protein was expressed in *E. coli*. The expressed enzyme was soluble, and catalytically active and was readily isolated and purified. The recombinant protein had the expected M_r of 18 000 (estimated by SDS–PAGE), and it showed cross-reactivity with antisera that had been raised against both the bovine heart and the human placenta enzymes. The amino acid sequence of the N-terminal region of the expressed protein showed that methionine had been removed, resulting in a sequence identical to that of the enzyme isolated from the bovine tissue, with the exception that the N-terminal alanine of the protein from tissue is acetylated. A kinetically competent phosphoenzyme intermediate was trapped from a phosphatase-catalyzed reaction. Using ^{31}P NMR, the covalent intermediate was identified as a cysteinyl phosphate. By analogy with the nomenclature used for serine esterases, these enzymes may be called cysteine phosphatases.

Recently, we described the purification and properties of a homogeneous low molecular weight phosphotyrosyl protein phosphatase from bovine heart (Zhang & Van Etten, 1990). This enzyme had previously been identified as a member of a group of "acid" phosphatases (Chernoff & Li, 1985), although in point of fact V_{max} is almost unchanged over the pH range 5–8 for this enzyme (Taga & Van Etten, 1982). Acid phosphatases [orthophosphoric monoester phosphohydrolase (acid optimum), EC 3.1.3.2] are ubiquitous in nature and have been isolated from various mammalian tissues and characterized to varying extents (Hollander, 1971). Based on their molecular weight, they can be categorized into several groups, i.e., 18 000, 35 000, approximately 100 000, and larger than 200 000 (Dipietro & Zengerle, 1967; Rehkop & Van Etten, 1975). Although the very large M_r forms are membrane bound, and the 100-kDa forms are known to be either secretory or lysosomal enzymes, the 18-kDa enzymes are soluble cytoplasmic proteins (Heinrikson, 1969; Aranjo et al., 1976; Taga & Van Etten, 1982). These nonlysosomal phosphatases, including the low molecular weight phosphatases isolated from human red cell (Boivin et al., 1987), human placenta (Waheed et al., 1988), rat brain (Okada et al., 1986), and bovine liver and heart (Zhang & Van Etten, 1990; Chernoff & Li, 1985), all of which function efficiently at neutral pH, have been shown to be active phosphotyrosyl protein phosphatases. Although their exact physiological functions and substrates remain uncertain, these enzymes may be expected to share some degree of importance with other, structurally unrelated, protein tyrosine phosphatases (Hunter, 1989; Streuli et al., 1988; Charbonneau et al., 1988) in regulating the levels of phosphotyrosine and thus affecting diverse cellular processes.

The low molecular weight, bovine heart phosphotyrosyl protein phosphatase (BHPTP)[1] has been isolated and purified to homogeneity from tissue. The enzyme is not abundant; a yield of less than 2 mg/kg of tissue was obtained (Zhang & Van Etten, 1990). Despite the difficulty of purifying this enzyme, many features of its steady-state and pre-steady-state kinetics have been examined in detail, and a detailed energy profile for a phosphate ester hydrolysis reaction has just been described (Zhang & Van Etten, 1991).

No representative from this class of proteins has yet been cloned. Although the amino acid sequence of the bovine heart enzyme has not been reported, the related sequence of the enzyme from bovine liver was reported during the course of these studies (Camici et al., 1989). In order to facilitate a detailed analysis of the structure and function of this small but active[2] phosphotyrosyl protein phosphatase, we have utilized cloning methodologies in order to establish the cDNA sequence and to produce substantial quantities of the enzyme through the use of heterologous expression. An efficient expression system has been developed, and conditions for the convenient expression and isolation of the protein are presented.

The availability of reasonable quantities of enzyme made it possible to explore a mechanistic hypothesis that had been developed during the course of preceding work on this phosphatase, namely that a covalent intermediate is formed by attack of an enzymic nucleophile (Zhang et al., 1992). The

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[‡] The nucleotide sequence reported in this paper has been submitted to GenBank and the EMBL data bank under accession number M83656.

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[1] Abbreviations: BHPTP, bovine heart phosphotyrosyl protein phosphatase; PCR, polymerase chain reaction; *p*NPP, *p*-nitrophenyl phosphate; DTT, dithiothreitol.

[2] Interestingly, when the specific activity of this protein is compared to that of the structurally unrelated cdc25 phosphatase using *p*-nitrophenyl phosphate as a substrate (Dunphy & Kumagai, 1991), the present enzyme is several orders of magnitude more active.

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detection of a covalent intermediate occurring in the normal catalytic pathway of an enzyme-catalyzed reaction provides direct evidence about the mechanism of the reaction. For alkaline phosphatase, a phosphoserine could be isolated from incubation mixtures of inorganic phosphate and that metalloenzyme (Schwartz & Lipmann, 1961). For acid phosphatases, a covalent phosphoenzyme intermediate has been reported for the (nonhomologous) rat liver, wheat germ, and human prostate enzymes (McTigue & Van Etten, 1978, and references cited therein). In each of these cases, a phosphohistidine intermediate was identified after base hydrolysis of the labeled acid phosphatase. No such experiments have been performed on the low molecular weight phosphotyrosyl protein phosphatases, although earlier studies strongly implicated the existence of a covalent phosphoenzyme intermediate in BHPTP-catalyzed reactions (Zhang & Van Etten, 1990, 1991). Trapping of the postulated intermediate would provide conclusive evidence in support of the proposed kinetic mechanism, and it could provide information about the nature of the nucleophile. We describe such experiments here.

Materials and Methods

Materials. Bovine heart poly(A)$^+$ mRNA and a λgt11 bovine heart cDNA library were purchased from Clontech Laboratories, Inc. Nylon transfer membrane for mRNA blotting was from Micron Separations, Inc. The pET expression vectors were purchased from Novagen, Inc. (Madison, WI). Oligonucleotides that were used as probes for screening of the library or as primers for sequencing and polymerase chain amplification reactions (PCR) (Kleppe et al., 1971) were synthesized on an Applied Biosystems Model 380A DNA synthesizer in the Laboratory for Macromolecular Structure, Purdue University. A phosphotyrosyl-containing peptide related to the cytoplasmic domain of erythrocyte band 3 was obtained from Prof. Roger Roeske. BHPTP was purified to homogeneity from bovine heart by procedures that have been recently described (Zhang & Van Etten, 1990).

Protein Sequence. The majority of the sequence was determined by secondary ion mass spectrometric procedures (A. L. McCormack, D. Hunt, R. L. Van Etten, and J. P. Davis, unpublished results), but in the course of that work, the conventionally-obtained sequence of the bovine liver enzyme was described (Camici et al., 1989) and proved to be nearly identical to that of the bovine heart enzyme. Consequently, we will refer to the published sequence except where points of unique significance to the bovine heart enzyme are involved.

Isolation of cDNA Clones. Bacteriophage λgt11 containing bovine heart cDNA was grown in *Escherichia coli* strain Y1090. A total of 120 000 plaques were screened using standard methods (Maniatis et al., 1982). The hybridization was performed with a 5′-end ^{32}P-labeled 16-mer oligonucleotide probe derived from the sequence of residues 127–146 of the published amino acid sequence of the low molecular weight bovine liver acid phosphatase (Camici et al., 1989), i.e., 5′-ATCATTGAGGACCCATACTATGGCA-ATGATGCTGACTTTGAGACAGTCTACCAGCAGT-GTG-3′. Duplicate nitrocellulose filter plaque lifts were subjected to prehybridization and hybridization for 4 and 16 h, respectively, in 5× Denhardt's solution containing 5× SSPE (1× SSPE is 10 mM sodium phosphate, pH 7.4, 0.18 M NaCl, and 1 mM EDTA), 0.1% SDS, and 0.1 mg/mL denatured salmon sperm DNA. Following two preliminary washes in 6× SSC/0.1% SDS at room temperature for 30 min, the filters were washed with the same solution at 37 °C for 5 min and exposed to X-ray film (Kodak, X-Omat) for 48–72 h with an intensifying screen. In order to isolate longer clones, an *Eco*RI/*Sal*I insert of a positive λgt11 clone obtained in the first screen (i.e., BHPTP1) was labeled by the random-priming method (Feinberg & Vogelstein, 1983) using [α-^{32}P]dCTP, and this probe was used to rescreen the library. Stringent washing was performed in 2× SSC/0.1% SDS at 70 °C for 5 min.

DNA Sequencing. The *Eco*RI inserts from isolated clones were subcloned into vector pUC18 for restriction analysis and double-stranded DNA sequencing. Plasmids were prepared by established procedures (Holmes & Quigley, 1981). The plasmid DNA was denatured for 5 min in 2 M NaOH/2 mM EDTA, the DNA was precipitated with ethanol, and the nucleotide sequence was determined by the dideoxynucleotide chain termination method (Sanger et al., 1977). In order to obtain extended sequence information but still use universal primers, partial deletion subclones were constructed by digesting isolated DNA with *Pst*I or *Sal*I and religating with T4 DNA ligase.

mRNA Blotting. Bovine heart poly(A)$^+$ RNA (3 μg) was fractionated on a formaldehyde/agarose gel (1% agarose) and transferred to a nylon membrane. The coding region of the cDNA clone, BHPTP4, was labeled by the random primer method and used as a probe (Mahmoudi & Lin, 1989). The size of the resulting band was estimated by comparison with RNA standards (0.24–9.5-kb RNA ladder; BRL).

Expression of BHPTP in E. coli. Initial expression experiments involved the formation of a β-galactosidase fusion protein using pUC18 and *E. coli* strain JM107. The resulting fusion protein contained seven amino acids from the *lacZ* gene fused to the initial alanine of the phosphatase. For subsequent experiments, a bacteriophage T7 polymerase dependent bacterial expression system (Rosenberg et al., 1987) was used to express high levels of BHPTP. Using PCR methodology (Saiki et al., 1988), the beginning of the coding region of BHPTP DNA was reconstructed to include an *Nco*I site at the start codon. In addition, a *Bam*HI site located 247 bp downstream of the termination codon was introduced. The primers used in the reaction were 5′-GGATCCATGGCTGAGCAGG-TGACCAAGTCG-3′ and 5′-TATTTCGACGGATCCA-CTC-3′, and the amplification was performed for 27 cycles, with denaturation at 94 °C for 1 min, annealing at 45 °C for 2 min, and the polymerization at 72 °C for 4 min. This PCR-amplified fragment was cut with the restriction enzymes *Nco*I and *Bam*HI and ligated into the unique *Nco*I and *Bam*HI sites of the expression vector pET-11d and subsequently used to transform *E. coli* strain DH5α. Plasmid DNA, isolated from ampicillin-resistant colonies by the boiling method (Holmes & Quigley, 1981), was subjected to restriction analysis. A pET-11d derivative carrying the desired insert was designated pVEBH4. In order to express recombinant bovine heart phosphatase, pVEBH4 was transformed into *E. coli* strain HMS174(DE3).

The overnight cultures of *E. coli* HMS174(DE3) carrying pVEBH4 were diluted 1:10 in LB medium containing 50 μg/mL ampicillin and grown at 37 °C for 3 h. Foreign protein production was induced by adding isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 0.4 mM, and the cultures were incubated for 3 h more at the same temperature. The bacteria were harvested by centrifugation at 3000*g* for 10 min and lysed by sonication in 100 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 1 mM DTT. In larger scale preparations, *E. coli* cells were disrupted using a French press.

BHPTP Activity Assay. The BHPTP enzyme activity was determined by a procedure similar to that described previously

(Waheed et al., 1988). The phosphatase assay was initiated by mixing 5 μL of enzyme solution with 200–400 μL of a reaction mixture containing 20 mM *p*-nitrophenyl phosphate as a substrate in 100 mM sodium acetate, pH 5.0, 1 mM EDTA. After 3–5 min at 37 °C, the reaction was quenched by adding 1 mL of 1 N NaOH. The amount of reaction product, *p*-nitrophenoxide, was quantitated by measuring the absorbance at 405 nm and using a molar extinction coefficient of 18 000 M^{-1} cm^{-1}. The protein content of the cell extract was determined by a dye binding assay (Bradford, 1976).

V_{max} and K_m Determinations. Kinetic parameters were measured at 37 °C in 50 mM 3,3-dimethylglutarate buffer at pH 7.0 or in 100 mM sodium acetate buffer at pH 5.0, both containing 1 mM EDTA and at an ionic strength of 0.15 M, adjusting using NaCl. The hydrolysis of *p*NPP was followed as described above. The rates of dephosphorylation of a phosphotyrosyl-containing peptide and β-naphthyl phosphate were measured by determining the release of inorganic phosphate, as described previously (Zhang & Van Etten, 1991). The V_{max} and K_m values were obtained by fitting the data directly to the Michaelis–Menten expression using the computer program KINFIT (Knack & Röhm, 1981).

Purification and Partial Amino Acid Sequence Determination of Recombinant BHPTP. The initial stages of the purification of recombinant BHPTP followed a procedure that was somewhat related to the one developed for purification of the enzyme from fresh tissue (Zhang & Van Etten, 1990). Three hours after induction with IPTG, the bacteria were pelleted by centrifugation and then resuspended in 100 mM sodium acetate, pH 5.0, containing 1 mM EDTA and 1 mM DTT. The bacterial cells were disrupted using a French press, cell debris was pelleted by centrifugation, and the supernatant was loaded onto a SP-Sephadex C-50 cation-exchange column that was equilibrated with 10 mM sodium acetate, pH 4.8, containing 30 mM inorganic phosphate, 1 mM EDTA, and 60 mM NaCl. The enzyme was eluted with 300 mM Na-H_2PO_4, pH 5.1, 1 mM EDTA. The fractions with high phosphatase activity were pooled, concentrated, and applied to a Sephadex G-50 size-exclusion column that was equilibrated with the same buffer as that of the ion-exchange column. The further purification of recombinant BHPTP for protein sequence analysis or other uses where high purity was necessary was achieved by reverse-phase HPLC. It was carried out on an IBM LC/9533 ternary gradient liquid chromatograph equipped with an IBM LC/9523 variable-wavelength UV detector. The sample was run on a 4.6 × 250 mm Synchropak RP-P C18 column using solvents A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile). A linear gradient from 12 to 62% B in 50 min was employed. The flow rate was 1 mL/min, and the column effluent was monitored at 210 nm. The amino acid sequence of the N-terminal region of the purified protein was then determined by using an Applied Biosystems 470A gas-phase sequenator. Amino acid analyses were performed as previously described (Zhang & Van Etten, 1990).

Immunoblotting. The cell extracts were directly analyzed by SDS–PAGE with a 15% gel, and one-third of the gel was stained with Coomassie Blue. The remainder of the gel was subjected to immunoblot analysis. The electrophoretic transfer was performed at 4 °C, 240 mA for 3 h in an electrotransfer solution containing 20 mM Tris base, 0.15 M glycine, and 25% methanol. The blots were incubated overnight at room temperature in a blocking solution consisting of 3% BSA in 20 mM Tris-HCl and 500 mM NaCl, pH 7.4. Rabbit antisera to the low molecular weight human placenta phosphatase (Waheed et al., 1988) and to the bovine heart phosphatase (Zhang & Van Etten, 1990) were separately used as primary antibodies. Rabbit immunoglobin was detected with peroxidase-conjugated goat anti-rabbit IgG and 125 mL of a solution containing 100 μL of hydrogen peroxide (30%) plus 60 mg of 4-chloro-1-naphthol.

Synthesis of ^{32}P-Labeled Substrate. ^{32}P-labeled *p*NPP was synthesized as follows: 5 mCi of ^{32}P-labeled inorganic phosphate (supplied in 0.5 mL of solution) was mixed with 5 mL of acetonitrile in a 50-mL round flask, and water in the mixture was removed by rotary evaporation. Crystalline phosphoric acid (50 mg, 0.5 mmol) was added to the flask containing a solution of acetonitrile (7.5 mL), *p*-nitrophenol (0.5 g, 3.6 mmol), triethylamine (0.21 mL, 1.5 mmol), and 5 mCi of [^{32}P]phosphate. After all the solids dissolved, trichloroacetonitrile (0.4 mL, 4 mmol) was added to start the reaction, and the reaction was allowed to proceed at 35–40 °C for 2.5 h. Solvent and excess trichloroacetonitrile were removed by rotary evaporation at 50 °C. The remaining residue was dissolved in 7.5 mL of H_2O. The unreacted *p*-nitrophenol was removed by extraction three times with ether at pH 5. Cyclohexylamine (0.3 mL, 2.6 mmol) was introduced to the aqueous phase, and water was removed by rotary evaporation at 50 °C. After recrystallization in 95% ethanol, 0.14 g of ^{32}P-labeled *p*NPP dicyclohexylammonium salt was obtained. The radioactive substrate was dissolved in 8 mL of 0.1 M acetate, 1 mM EDTA, pH 5.0 buffer, to give 42 mM *p*NPP. The ^{32}P specific radioactivity of the substrate stock was 2900 CPM/nmol.

Preparation and Properties of ^{32}P-Labeled Enzyme. In a typical trapping experiment to obtain ^{32}P-labeled enzyme, we used 0.4–0.6 mg of BHPTP (specific enzyme activity 90–113 units/mg). First, the enzyme (in 0.5 mL of pH 5.0, 0.1 M acetate buffer with 1 mM EDTA, I = 0.15 M) was reacted for 10 s at room temperature with 0.5 mL of a solution of 42 mM ^{32}P-labeled substrate in the same buffer. Then, the mixture was quickly transferred to a syringe which was connected to a Ballou mixer (Ballou & Palmer, 1974), and the reaction mixture was quenched by rapidly mixing it with an equal volume of 2 N NaOH solution. The ^{32}P-labeled enzyme was separated by passing the quenched reaction mixture through a 1.6 × 35 cm G-25 column preequilibrated with 0.15 M ammonium bicarbonate, pH 8.4 buffer containing 4 M urea. The protein content was followed by absorption at 280 nm, and the radioactivity was quantitated by counting 30-μL aliquots from each fraction (3 mL/fraction) using a Packard 1600CA Tri-Carb liquid scintillation analyzer.

The chemical stability of the phosphoenzyme intermediate was examined as follows. Briefly, 100 μL of the post-G-25 sample was incubated at 37 °C under different chemical conditions in 4 M urea. The reaction was stopped by quickly passing the reaction mixture through a PD-10 column equilibrated with 4 M urea in pH 8.4, 0.15 M ammonium bicarbonate buffer. The protein and inorganic phosphate eluted in different fractions, so the amount of phosphate hydrolyzed could be determined by counting the radioactivity associated with protein and phosphate.

Tryptic digestion of the ^{32}P-labeled enzyme was performed in the presence of 4 M urea. The peptides were separated using reverse-phase HPLC under two different conditions. In the first case, solvent A was 0.1 M ammonium bicarbonate, pH 8.5, and solvent C was 0.1 M ammonium bicarbonate, pH 8.5, in 70% acetonitrile. The reasons for choosing this condition were based primarily on the stability of the postulated phosphoenzyme intermediate, which was expected to be rel-

atively stable at alkaline pH, if the intermediate was a thiol phosphate or a phosphoramidate. In addition, a pH of 8.5 was at the edge of the manufacturer's recommended conditions for the reverse-phase column. In the second case, normal reverse-phase HPLC conditions were used, i.e., solvent A contained 0.1% TFA in water and solvent C contained 0.1% TFA in acetonitrile. The flow rate was 1 mL/min and the gradient was a 1% increment in solvent C per minute.

Identification of the Phosphorylated Residue by ^{31}P NMR Spectroscopy. The enzyme used in NMR experiments was recombinant BHPTP that was expressed in *E. coli* and purified as described. Phosphorylation of recombinant BHPTP with unlabeled *p*NPP was similar to that described for the preparation of ^{32}P-labeled enzyme, except that the reaction temperature was 4 °C, and an additional Plexiglass tee mixer was used to first mix enzyme (1 mL, 3 mg) and *p*NPP substrate (1 mL, 150 mM) solutions. The phosphorylated enzyme was isolated by passing the quenched reaction mixture through a G-25 size-exclusion column (2.5 × 30 cm) preequilibrated with 50 mM ammonium bicarbonate, pH 10 buffer containing 0.25% SDS. Phosphoenzyme from three such experiments was combined and concentrated using an Amicon membrane filtration system with a YM3 membrane.

All ^{31}P NMR spectra were recorded on a 200-MHz Varian XL-200A spectrometer operating at 81 MHz (^{31}P frequency). Unless otherwise stated, the acquisition conditions were 65° pulse, 4.1-kHz sweep width, 1.5-s acquisition time, and 1-s relaxation delay. Protons were broad-band decoupled. Samples (each approximately 9 mg in 0.6–0.7 mL) were contained in 5-mm NMR tubes. All chemical shifts that are described here are externally referenced to 85% phosphoric acid. Samples contained 10% D_2O for the field-frequency lock. All NMR spectra were obtained with samples at 20 °C.

Results

Isolation and Characterization of the cDNA for BHPTP. A bovine heart cDNA library was first screened with a synthetic 61-mer oligonucleotide probe. The probe sequence was chosen to make use of the established advantage of a single, long, nondegenerate probe (Lathe, 1985). The screening of only 120 000 recombinants revealed three hybridizing clones. Among these, only one clone (designated BHPTP1) contained an extensive coding sequence, including an open reading frame of 89 amino acids and an entire 3′ untranslated region. Compared to the known size of the isolated enzyme and to the amino acid sequence of the liver enzyme, it was clear that BHPTP1 was not a full-length clone. Therefore, an additional screening of the λ library was carried out using a ^{32}P-labeled *Eco*RI/*Sal*I fragment of BHPTP1. Eight additional clones were isolated and characterized. Sequencing results revealed that one cDNA clone, BHPTP4, contained an open reading frame of 471 nucleotides that would be sufficient to code for a protein of 157 amino acid residues, which is the number of amino acids present in the protein that is isolated from bovine heart. In fact, the inferred protein sequence differed at only one residue (i.e., D56 instead of N56) from that reported for the bovine liver enzyme so the difference probably represents a protein sequencing error in that study (Camici et al., 1989).[3] Furthermore, another clone, BHPTP10, was found to be the longest clone, and it comprised 1381 nucleotides, including a 27-bp 5′ noncoding sequence, the entire translated region, and an 880-bp 3′ nontranslated region. A schematic diagram of the sequencing strategy and the restriction map are shown in Figure 1. The cDNA sequence and derived protein sequence are presented in Figure 2. An unexpected finding was that BHPTP10 contained two in-frame AUG codons, and the

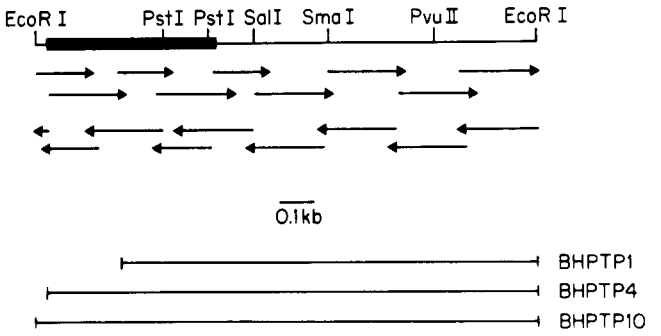

FIGURE 1: Restriction map, nucleotide sequencing strategy, and the structures of three independent cDNA clones coding for the low molecular weight bovine heart phosphotyrosyl protein phosphatase. The locations of several restriction sites on the cDNA are indicated. The limits of the map correspond to the size of the longest clone with the coding region indicated by the heavy line.

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                                                Met Ala Glu Gln Val Thr Lys
  1  GGA TCT ATG AAG AGT TGG GTG GGC GAG ATG GCT GAG CAG GTG ACC AAG

  8  Ser Val Leu Phe Val Cys Leu Gly Asn Ile Cys Arg Ser Pro Ile Ala
 49  TCG GTG CTG TTC GTG TGT CTG GGT AAC ATC TGT CGA TCG CCC ATC GCA

 24  Glu Ala Val Phe Arg Lys Leu Val Thr Asp Gln Asn Ile Ser Asp Asn
 97  GAA GCG GTT TTC AGG AAA CTT GTA ACT GAT CAA AAC ATT TCA GAT AAT

 40  Trp Val Ile Asp Ser Gly Ala Val Ser Asp Trp Asn Val Gly Arg Ser
145  TGG GTC ATT GAC AGT GGC GCT GTT TCT GAC TGG AAC GTG GGC CGG TCA

 56  Pro Asp Pro Arg Ala Val Ser Cys Leu Arg Asn His Gly Ile Asn Thr
193  CCA GAT CCA AGA GCT GTG AGC TGC CTA AGA AAT CAT GGC ATT AAC ACA

 72  Ala His Lys Ala Arg Gln Val Thr Lys Glu Asp Phe Val Thr Phe Asp
241  GCC CAT AAA GCA AGA CAG GTT ACC AAA GAA GAC TTT GTC ACT TTT GAT

 88  Tyr Ile Leu Cys Met Asp Glu Ser Asn Leu Arg Asp Leu Asn Arg Lys
289  TAT ATA CTA TGT ATG GAT GAG AGC AAT CTG AGA GAT TTG AAT AGA AAA

104  Ser Asn Gln Val Lys Asn Cys Arg Ala Lys Ile Glu Leu Leu Gly Ser
337  AGT AAT CAA GTT AAA AAC TGC AGA GCG AAA ATC GAA CTG CTC GGG AGC

120  Tyr Asp Pro Gln Lys Gln Leu Ile Ile Glu Asp Pro Tyr Tyr Gly Asn
385  TAT GAT CCA CAA AAA CAA CTT ATC ATT GAA GAT CCC TAT TAT GGC AAC

136  Asp Ala Asp Phe Glu Thr Val Tyr Gln Gln Cys Val Arg Cys Cys Arg
433  GAC GCC GAC TTT GAG ACC GTC TAC CAG CAG TGC GTG CGG TGC TGC AGG

152  Ala Phe Leu Glu Lys Val Arg  *
481  GCC TTC CTG GAG AAG GTG CGC TGA CCG GCC GTC CCG CTG TGG CCC AGG
          538        548        558        568        578        588
 5'  CTCGTCCCCCCAGGCCGTGTGTCCAGTGTCAACAGTGTCACCGTTCCGAGCCAAGGTCGC
          598        608        618        628        638        648
     AGCCTCTCTTCAGTCGACTCACTGTTTCCTACCTGAAAAAATAATTGTAGATGGAAATCA
          658        668        678        688        698        708
     GTCTTTGTGTTTGGCGAAAGAGTAAATAAAAAATCTTTATGTGGGGTAAGAGTTCCTTAG
          718        728        738        748        758        768
     ACTAGCTAAACTTCCCACTATGCCCCAGTTACAAAAAGAGTGGAACCGTCGAAATAGTGG
          778        788        798        808        818        828
     AGCAGCACAGTAGACCATCGTGAAGGGAAGTGAGACGCCCCCGGGGCCTGCGCTGCCCCC
          838        848        858        868        878        888
     GCTGTGGACGGGCCCTGCCGCACCGCCTCTCCACGGGCACCGCCTCTCCACGGCCGGCCC
          898        908        918        928        938        948
     AGCGGCGGTGAGCTGCCAGGCGCGCACTCTCAGGACAGGACGGAGCGAGGGTCGTGGGAC
          958        968        978        988        998       1008
     TCCTCGTCAGCACCTTCACTCCTTCTTAGTTTGTGTCTGTGTGGTTCGTTTCATGAGGAA
         1018       1028       1038       1048       1058       1068
     GTCACGTTATGTGTTCCCATTGGAACCCCTTTGCCTCTGTGGAAATGTAGTTATGTCCCGGA
         1078       1088       1098       1108       1118       1128
     GAAGGACGTGTGTGCTGGTTTGTATTATGGTCAGCTGGGGACTGTGTCTGCTTCTACATG
         1138       1148       1158       1168       1178       1188
     CGTACAATTGCCTGAATGTCAATTACAGATCACCTTGTGTGGGAAAAGTATTAAGTTGGA
         1198       1208       1218       1228       1238       1248
     AAAAGCCCTTTTATGTTGGAATGCTTAAATATCAATATATTAAGTATTGTTTTGTCAGTG
         1258       1268       1278       1288       1298       1308
     CCTGGTTATAGGGAAATATACATAATTTGTGCACACTTTGAAATAACTAATCTAAAATTC
         1318       1328       1338       1348       1358       1368
     ATATGAATATATGTCAGAAAAAATTGTTCTCAAATAAACTGGGGAAATTACAAAAATAAA
         1378
     AAAAAAAAAAAA 3'
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FIGURE 2: Combined nucleotide sequence and deduced amino acid sequence of BHPTP. The initiating methionine is not present on the enzyme isolated from tissue, which begins with *N*-acetylalanine. The asterisk indicates the termination site.

[3] Aspartic acid rather than asparagine is also found in this position in the homologous human isoenzymes (Wo et al., 1992).

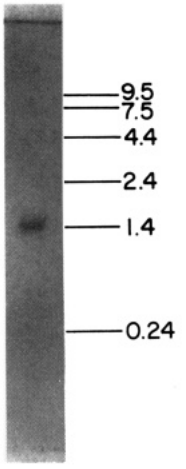

FIGURE 3: Northern blot analysis of poly(A)$^+$ mRNA from bovine heart, using the coding region of BHPTP4 as a probe. The molecular size of the mRNA was determined by comparison with the mobility of RNA standards electrophoresed in the same gel.

second of these AUG triplets immediately preceded the confirmed first amino acid of the isolated protein (i.e., Ala). In each of these isolated BHPTP clones, a putative polyadenylation signal, AATAAA, was located 25 bp upstream from the polyadenine tail.

Although the longest clone that was isolated contained only a 27-bp 5′ untranslated sequence, the size of the isolated cDNA clone BHPTP10 (1381 bp) closely matched the maximal size of mRNA in tissue (1400 bp). Moreover, only one size of transcript was observed (Figure 3).

Expression and Purification of Recombinant BHPTP. To confirm that the cDNA coded for functional BHPTP enzyme, the coding region of the cDNA cloned in pUC18 was initially expressed as a fusion protein using the *lac* promoter supplied by the vector. The expressed protein was isolated, it was confirmed by protein sequencing that it contained seven additional amino acids (i.e., TMITNSG) at the N-terminal end of the phosphatase protein, and it was also found that the calculated mass of a related fusion protein agreed exactly with that determined by electrospray mass spectrometry. Interestingly, the expressed fusion protein was catalytically active. However, since further experimentation provided a route to a high-level expression system yielding a nonfusion protein, only those experiments will now be described in detail.

The protein coding region was inserted downstream of the bacteriophage T7 promoter of the expression vector, pET-11d, to yield plasmid pVEBH4. Bacterial extracts were analyzed both by SDS–PAGE with Coomassie Blue staining (Figure 4A) and by immunoblotting using polyclonal antibodies against the low molecular weight bovine heart phosphatase (Figure 4B). Bacteria transformed with pVEBH4 produced a prominent polypeptide, M_r 18 000 (Figure 4A, lane 1), which was consistent with the molecular weight of BHPTP as isolated from tissue. This band was absent in preparations of bacteria containing the expression vector without an insert (Figure 4A, lane 2). Consistent with previous observations on enzyme isolated from tissue, the recombinant BHPTP also cross-reacted with polyclonal antibodies obtained against the human placental enzyme (Figure 4C, lane 1), thus suggesting one or more regions of highly conserved structure. The BHPTP was exclusively expressed as a soluble protein. This conclusion is based on results obtained using a phosphatase activity assay as well as SDS–PAGE analyses of supernatant, possible inclusion bodies, and insoluble cell debris separated from the crude extract.

For purposes of further characterization, including protein sequencing, amino acid analysis, and kinetic studies, we carried out large-scale expression experiments (2 L of culture) to isolate recombinant BHPTP. Three purification steps were sufficient to purify the protein. The gel filtration elution profile on a Sephadex G-50 column revealed that a contaminating, bacterial protein impurity that was present following the Sephadex C-50 ion-exchange chromatography step (Figure 5A) could be efficiently removed using experimental conditions that were developed in preliminary experiments. HPLC results showed that the BHPTP preparation was greater than 90%

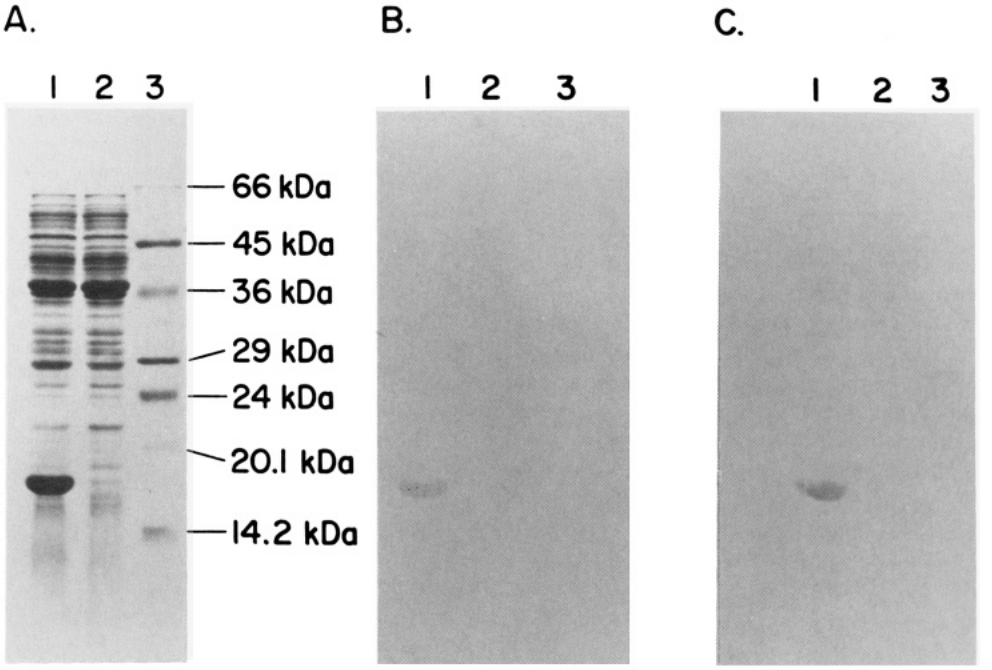

FIGURE 4: Expression of recombinant BHPTP in *E. coli*. Bacteria (HMS174(DE3)) were transformed with plasmids, grown and induced with IPTG as described under Materials and Methods. The lysed cell extracts were analyzed by SDS–PAGE and Coomassie Blue staining (A) or immunoblotting using antisera specific to bovine heart (B) and human placenta (C) enzymes. Lane 1: bacteria containing the BHPTP expression vector. Lane 2: bacteria containing pET-11d vector but no insert. Lane 3: SDS molecular weight markers.

Table I: Michaelis Parameters for Native and Recombinant Forms of Bovine Heart Phosphotyrosyl Protein Phosphatase

		BHPTP		rBHPTP	
substrate	pH	K_m (mM)	V_{max} (μmol/min mg^{-1})	K_m (mM)	V_{max} (μmol/min mg^{-1})
β-naphthyl phosphate	7	4.7 ± 0.3	78 ± 4	4.6 ± 0.2	78 ± 4
p-nitrophenyl phosphate	7	3.5 ± 0.2	103 ± 5	3.2 ● 0.2	100 ± 5
p-nitrophenyl phosphate	5	0.41 ± 0.3	114 ± 9	0.38 ± 0.3	100 ± 10
band 3-related peptide[a]	5	1.0 ± 0.2	1.2 ● 0.1		
band 3-related peptide[a]	7			2.1 ± 0.6	0.3 ± 0.1

[a] AcLQDDY(OPO_3H_2)EDDM(*S*-benzyl)EENL.

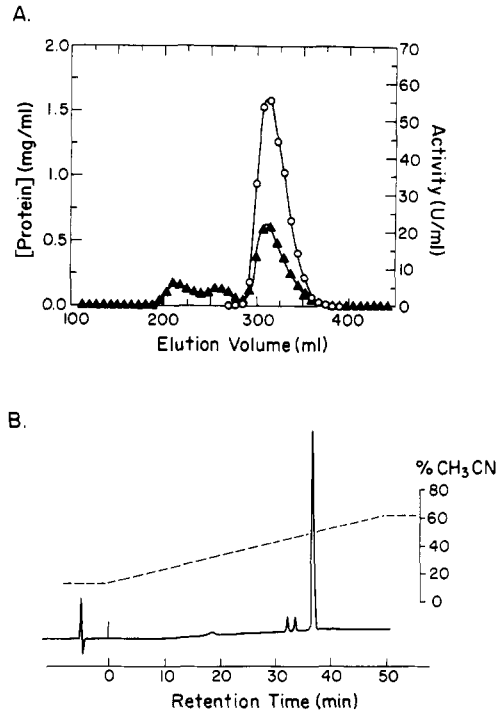

FIGURE 5: Purification of expressed BHPTP. (A) Sephadex G-50 gel filtration column elution profile. Protein content was monitored at 280 nm (▲). The enzymatic activity of phosphatase was measured at 405 nm using *p*-nitrophenyl phosphate as substrate (○). (B) Reverse-phase HPLC chromatogram showing the purification of BHPTP. The major peak represents the recombinant BHPTP. Refer to Materials and Methods for details.

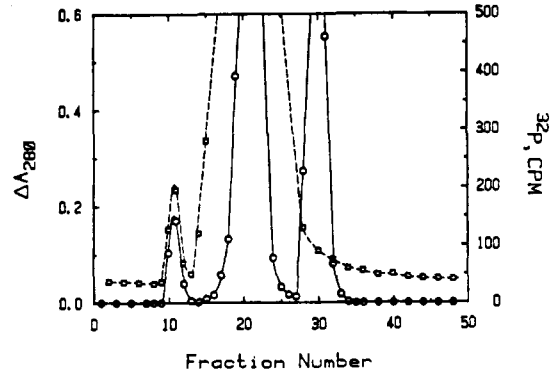

FIGURE 6: Isolation of ^{32}P-labeled BHPTP by Sephadex G-25 chromatography. The separation conditions were specified in the text: (○), ΔA_{280}, left scale; (□), radioactivity, right scale.

Table II: Chemical Stability of the ^{32}P-Labeled BHPTP Phosphoenzyme Intermediate

assay conditions[a]	percent hydrolyzed
pH < 1, 4 M HCl, 37 °C, 2 h	57
pH 1, 1 M HAc, 37 °C, 1.5 h	54
pH 4, 100 mM acetate, 37 °C, 10 min	37
pH 7, 100 mM Tris, 37 °C, 15 min	0
pH 7, 100 mM Tris, 0.1 mM I_2, 37 °C, 5 min	41
pH 8.5, 0.15 M NH_4HCO_3, 37 °C, 1 h	0
pH 8.5, 0.15 M NH_4HCO_3, 0.4 mM I_2, 37 °C, 10 min	50
pH 13, 1.5 M NaOH, 37 °C, 2 h	54

[a] In all of the assay mixtures, 4 M urea was included.

pure after size fractionation using the G-50 column (Figure 5B). Approximately 19 mg of BHPTP was obtained from 2 L of culture; this corresponded to about 20% of total protein content.

The pure protein as obtained from reverse-phase HPLC was analyzed by gas-phase amino acid sequencing. The expressed BHPTP did not have an N-terminal methionine, but it did have the expected initial sequence, Ala-Glu-Gln-Val-Thr-Lys, corresponding to the enzyme isolated from bovine tissue. However, in contrast to the case with the tissue enzyme, the N-terminal residue of the expressed protein was not acetylated. The catalytic activity of the enzyme was also characterized. Michaelis kinetic constants for the native and the recombinant protein are given in Table I. Within the limits of experimental error, the kinetic properties are the same.

Trapping experiments were designed that allowed us to obtain phosphorylated enzyme. In experiments with ^{32}P-labeled *p*NPP, the phosphorylated enzyme eluted in the breakthrough of the G-25 column and the protein peak was coincident with a radioactivity peak. A typical G-25 chromatography separation of the phosphorylated enzyme is shown in Figure 6. Initially, some of the experiments gave very low labeling stoichiometries. Furthermore, when either the intact, labeled protein or the trypsin-digested peptide mixture was loaded onto the HPLC, all of the radioactivity was found to be associated with the fractions in the breakthrough. Eventually, it was found that the major reason for the initially low yield in trapping and for the ready loss of ^{32}P label during sample manipulations was the refolding of the NaOH-quenched enzyme during and after the G-25 size-exclusion chromatography in the pH 8.4 ammonium bicarbonate buffer. The "trapped" enzyme was in fact found to be fully active when aliquots of the post-G-25 sample were subsequently assayed at both pH 5.0 and pH 8.4. However, the enzyme was not active in the presence of 4 M urea at pH 8.4. That is, 4 M urea was needed to keep the trapped protein in the denatured state. Consequently, in subsequent experiments, 4 M urea was included in the G-25 buffer to prevent the refolding of the quenched enzyme. When this was done, labeled protein was obtained in trapping experiments conducted with 0.4 mg of enzyme in an amount that corresponded to a labeling stoichiometry of at least 74%.

The results from chemical stability studies of the phosphoenzyme intermediate are summarized in Table II. The properties of the covalent phosphoenzyme intermediate suggested that it was most likely a phosphocysteinyl enzyme (see Discussion), but the possibility of phosphohistidine could not be ruled out completely. An attempt to isolate the peptide

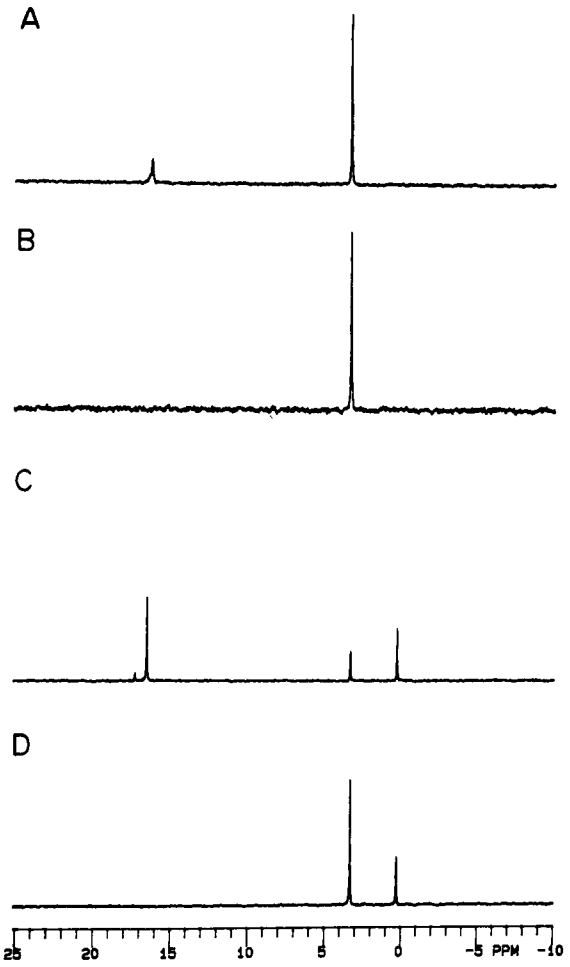

FIGURE 7: ^{31}P NMR spectra of the trapped phosphoenzyme intermediate and cysteamine *S*-phosphate. (A) The trapped phosphoenzyme intermediate sample contained 50 mM NH_4HCO_3, pH 10, 0.25%, SDS, 10% D_2O, approximately 0.6 mM enzyme, and 2 mM P_i. NMR line broadening was 8 Hz, and 25 000 scans were collected. (B) The sample from (A) after the addition of I_2; 8000 scans were collected. (C) The ^{31}P NMR spectrum of a sample containing 10 mM cysteamine *S*-phosphate, 2 mM P_i, and 5 mM *p*NPP in 50 mM NH_4HCO_3, pH 10, 0.25% SDS, 10% D_2O. (D) Spectrum obtained from the preceding sample immediately after adding I_2. The chemical shifts are reported relative to external 85% H_3PO_4. In each spectrum, the P_i resonance appears at 3.3 ppm.

that contained the ^{32}P label using the normal reverse-phase HPLC conditions (0.1% TFA in water and acetonitrile) did not yield a significantly labeled radioactive peak, suggesting that the protein–phosphate linkage was not stable under these conditions (pH = 1, 70 min), which was consistent with the results from the chemical stability studies.

Definitive proof of the structural nature of the phosphorylated residue was sought through the use of ^{31}P NMR spectroscopy. As shown in Figure 7A, the trapped, phosphorylated intermediate that was obtained by reaction of (nonradioactive) *p*NPP and recombinant BHPTP gave a single ^{31}P NMR resonance with a chemical shift of +16.2 ppm. The peak had a width at half-height that was approximately a factor of 3 larger than that of the inorganic phosphate peak. This is consistent with shorter T_2 as expected for a protein-bound phospho group. Under the same conditions, the substrate *p*NPP exhibited a ^{31}P resonance at +0.3 ppm (Figure 7C).

The chemical shift of the peak, +16.2 ppm, is not consistent with the phosphorylation of a histidine residue. Model ^{31}P NMR studies on τ- and π-phosphohistidine (also termed 3′- and 1′-phosphohistidine) at pH > 8 showed chemical shifts of −3.9 and −4.9 ppm (Gassner et al., 1977). In fact, phosphorylated amino acid residues including the phosphomonoesters phosphoserine, phosphothreonine, and phosphotyrosine, the acyl phosphate phosphoaspartate, and the phosphoramidates phospholysine, phosphoarginine, and phosphohistidine all appear at less than 5 ppm, relative to 85% phosphoric acid (Vogel et al., 1989). A recent study by Pas et al. demonstrated that phosphocysteine and phosphohistidine in *E. coli* phosphotransferase system EII^{Mtl} protein at pH 8 showed peaks at +15.0 and −3.8 ppm, respectively, relative to external 85% phosphoric acid (Pas et al., 1991). The present signal at 16.2 ppm is consistent with the presence of a phosphorylated cysteine residue in BHPTP, but not a phosphohistidine. The slight difference in chemical shift between the phosphocysteines of BHPTP and the *E. coli* phosphotransferase system EII^{Mtl} is probably due to local environmental effects around the phospho groups in the proteins. Since the present ^{31}P NMR spectrum was collected under conditions in which BHPTP was in a denatured state, its chemical shift would be expected to be close to that of model compounds such as cysteamine *S*-phosphate. To ascertain that this signal is due to phosphocysteine, a ^{31}P NMR spectrum of cysteamine *S*-phosphate was obtained under the same conditions (Figure 7C). The peak observed for this model compound at 16.4 ppm clearly confirms that the peak of 16.2 ppm results from phosphorylated cysteine at the active site of the enzyme. The substrate, *p*NPP, appears at +0.3 ppm in the spectrum. Finally, the phosphoenzyme intermediate was rapidly decomposed upon addition of iodine to the solution, in a manner that was effectively identical to the destruction of cysteamine *S*-phosphate (compare parts B and D of Figure 7).

Discussion

The present work describes the isolation and primary structure determination of cDNA clones encoding a representative low molecular weight phosphotyrosyl protein phosphatase. We also provide evidence establishing the effective identity of the enzyme isolated from tissue with the recombinant BHPTP. The isolated clones contain two nearby, in-frame initiation codons. In comparison with the proposed consensus sequence (CCA/GCCAUGG) for eukaryotic initiation sites (Kozak, 1984), it appears that the sequence around the 5′-proximal AUG triplet (GCGAGAUGG) represents a better match than that around the upstream initiation codon (GAUCUAUGA). In studies of the tissue enzyme, no slightly larger forms of the protein appear to exist which would result from use of the alternative initiation site. The amino acid composition of the protein isolated from tissue (Zhang & Van Etten, 1990) and that of the present expressed protein (data not shown) are identical to the composition that is inferred from the cDNA sequence starting at the second AUG codon.

Results obtained upon amino acid sequencing of the expressed BHPTP revealed the removal of the N-terminal methionine, presumably by an aminopeptidase of *E. coli*. In general, methionine is deformylated during protein synthesis in *E. coli*, but not necessarily cleaved (Adams, 1968). However, small residues like alanine near the N-terminus are thought to favor the efficiency of methionine removal (Liang et al., 1985), and carefully supported examples of such processing are known (Yang & Wells, 1990). While N-terminal acetylation is often important for the biological activity of proteins (Driessen et al., 1985), this may often be due to the fact that N-terminal acetylated proteins are more resistant to proteolysis. Nothing is known of the biological half-life of the present protein, although the availability of N-acetylated and unacetylated forms should now make such a study possible. In any event, the catalytic activity of the recombinant protein,

at least when measured against synthetic or peptide substrates, is not significantly changed (Table I). This is also consistent with the findings made by Yang and Wells (1990) for the case of glutaredoxin.

Protein overexpression in *E. coli*, using a T7 polymerase dependent system, confirms that the cDNA encodes the low molecular weight, enzymatically active, bovine heart phosphotyrosyl protein phosphatase with an apparent molecular weight of 18 000. Undoubtedly aided by the reducing environment in *E. coli*, we were able to produce a large amount of active BHPTP, which is known to possess eight free cysteines and no disulfide bonds (Zhang & Van Etten, 1990). We also developed a more rapid purification procedure compared to the tedious method that was needed to isolate the enzyme from bovine heart tissue.

Several lines of evidence establish that pVEBH4 contains the coding sequence of the desired phosphatase. First, the catalytic activity of the expressed enzyme is nearly identical to that of the enzyme isolated from tissue. This is of particular interest given that the enzyme isolated from liver and from heart is acetylated on the N-terminal alanine (Camici et al., 1989; A. McCormack, D. Hunt, R. L. Van Etten, and J. Davis, unpublished observations). Although the expressed protein is not N-acetylated, its activity does not differ from that of the tissue enzyme. Moreover, the fact that a fusion protein having seven additional amino acids at the N-terminal end is also active indicates that variations at the N-terminal end do not easily disrupt the tertiary structure. Second, both the expressed BHPTP and the enzyme isolated from tissue behave similarly in immunological analysis; i.e., they both cross-react with antisera to bovine heart and human placental enzymes. Third, the initial amino acid sequence of HPLC-purified, recombinant BHPTP completely matches the sequence of BHPTP from tissue. For the further analysis of structure and function of BHPTP, the present work also provides a convenient enzyme source as well as sequence information that represents an important entry to the genomic sequencing and structure of this class of enzyme. The present results show that the failure of a recent attempt to produce more than a very low yield of enzyme with a much lower specific activity by expression using an artificial gene is not due to protein instability in the bacterial cell, to partially incorrect folding, or to the lack of N-acetylation, as has been suggested (Raugei et al., 1991). Instead, the problem is likely to lie in codon usage or in the sequencing error that we have previously noted.

The chemical stabilities of typical phosphate derivatives of proteins are summarized in Table III. Thus, intermediate cysteine *S*-phosphates or phosphohistidine derivatives should be stable in strong base, while acyl phosphates are very base labile. In the present case, it was clear that the trapped phosphoenzyme intermediate could not be an acyl phosphate linkage, since an acyl phosphate would not be stable under the quenching conditions that were employed. Thiol phosphate linkages can be differentiated from phosphoramidates not only by their different pH stability (thiol phosphates show bell-shaped stability profiles; Herr & Koshland, 1957; Akerfeldt, 1960), but also by their sensitivity to I_2. Thiol phosphates are specifically decomposed by iodine at neutral pH (Akerfeldt, 1960) in a reaction involving the formation of a sulfenyl iodide–phosphate intermediate (Pigiet & Conley, 1978). The chemical stability studies indicated that the residue being labeled by the [^{32}P]*p*NPP was most likely to be a cysteine, because of the pH stability and the sensitivity to I_2 of the phosphoenzyme intermediate. A definitive identification was achieved by ^{31}P NMR spectroscopy (Figure 7).

Table III: Chemical Stabilities of Phosphorylated Residues in Proteins

phosphate–amino acid linkage	chemical stability
O-phosphoserine[a] and *O*-phosphothreonine[a]	acid stable, base labile
O-phosphotyrosine[a]	base stable, acid stable
carboxyl phosphate[b]	labile at extremes of pH, sensitive to NH_2OH
phosphohistidine[c]	acid labile, base stable, increasing lability as the pH decreases from 7 to 0, sensitive to nucleophiles
thiol phosphate[d]	labile at pH 2–6, maximal lability at pH 4, base stable, sensitive to I_2

[a] Eckhart et al. (1979), Witte et al. (1980), Cooper et al. (1983), and Martensen (1984). [b] Bitte and Kabat (1974), Walsh and Spector (1969), and Di Sabato and Jencks (1961). [c] Bitte and Kabat (1974), Smith et al. (1978), Jencks and Gilchrist (1965), Hultquist et al. (1966), Di Sabato and Jencks (1961), Boyer et al. (1962), and Hultquist (1968). [d] Akerfeldt (1960), Herr and Koshland (1957), and Pigiet and Conley (1978).

The trapping, isolation, and identification of a phosphoenzyme intermediate provide direct evidence in support of the conclusion reached on the basis of kinetic results that the low molecular weight phosphotyrosyl protein phosphatase employs covalent catalysis (Zhang & Van Etten, 1991) as has been shown in other classes of phosphatases. However, in contrast to the nucleophilic histidine that is present in human prostatic, lysosomal, yeast, and *E. coli* acid phosphatases (Van Etten et al., 1991), the present study provides one of two examples of phosphotyrosyl phosphatases that have been directly demonstrated to employ a cysteine residue as a nucleophile in the enzyme action.[4] Other thiol phosphate derivatives are known. An altered alkaline phosphatase with its active site Ser-102 replaced by Cys-102 was constructed using site-directed mutagenesis (Ghosh et al., 1986). It was subsequently shown that the hydrolysis of phosphate monoesters by this mutant enzyme proceeds by way of a covalent phosphoenzyme intermediate in the same manner as the wild-type alkaline phosphatase, but the rate-limiting step of the mutant enzyme was changed to the phosphorylation of the enzyme (Butler-Ransohoff et al., 1988, 1989). A thiol phosphate linkage has also been identified in phosphothioredoxin (Pigiet & Conley, 1978).

On the basis in part of results obtained in an accompanying study (Zhang et al., 1992), a model for the active site structure and the mechanism of the present enzyme may be proposed. The enzyme active site contains a hydrophobic pocket and is composed of at least four other functional groups: one arginine, one histidine, and two cysteine residues. The hydrophobic pocket defines an important element of substrate specificity, since substrates with an aromatic moiety are preferentially bound (Zhang & Van Etten, 1990). The function of the arginine residue is presumably to provide an ionic interaction to the phosphate and thus to anchor and orient the substrate. The cysteine residue with a pK_a value of 7.5 that is measured using iodoacetamide or active site-directed epoxides (Zhang et al., 1992) is proposed to be the active site nucleophile that is phosphorylated during enzyme turnover. It may participate in a His^+/Cys^- ion-pair structure in the enzyme–substrate complex. A histidine residue or other po-

[4] In work done contemporaneously with the present study, Guan and Dixon have utilized site-directed mutagenesis and ^{32}P trapping experiments to show that a structurally unrelated, 322-residue fragment of rat brain PTP1 also utilizes a cysteine phosphate mechanism (Guan & Dixon, 1991).

tential general acid may act as a general acid to donate a proton to the oxygen of the leaving group to facilitate the departure of the phenol or alcohol moiety. A comparable catalytic role is necessary in dephosphorylation, since an isolated cysteinyl phosphate linkage is not very reactive. This is apparent from results of chemical stability studies of the ^{32}P-labeled enzyme and model compound studies (Herr & Koshland, 1957; Akerfeldt, 1960). The enzyme typically has a turnover number of 10–100 s^{-1} at 37 °C and pH 5–7, while the uncatalyzed decomposition of a thiol phosphate bond is many orders of magnitude slower. At least part of the rate enhancement caused by the enzyme may be due to the orientational and polarization effects caused by the arginine, since these would be expected to facilitate nucleophilic attack by water on the phosphoenzyme.

It has been proposed that a system of nomenclature similar to that used for proteolytic enzymes could be useful in the classification of phosphatases (Van Etten, 1982). Thus, alkaline phosphatase, human prostate phosphatase, and the low molecular weight phosphotyrosyl protein phosphatase should be called serine, histidine, and cysteine phosphatases, respectively. Distinctive, iron-containing phosphatases are also known, and recent evidence indicates that they may function by covalent catalysis involving a phosphohistidine (Vincent et al., 1991).

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Characterization of a Calcium- and Lipid-Dependent Protein Kinase Associated with the Plasma Membrane of Oat†

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ABSTRACT: A protein kinase that is activated by calcium and lipid has been partially purified from the plasma membrane of oat roots. This protein kinase cross-reacts with four monoclonal antibodies directed against a soluble calcium-dependent protein kinase from soybean described previously [Putnam-Evans, C. L., Harmon, A. C., & Cormier, M. J. (1990) *Biochemistry 29*, 2488–2495; Harper, J. F., Sussman, M. R., Schaller, G. E., Putnam-Evans, C., Charbonneau, H., & Harmon, A. C. (1991) *Science 252*, 951–954], indicating that the oat enzyme is a member of this calcium-dependent protein kinase family. Immunoblots demonstrate that the membrane-derived protein kinase is slightly larger than that observed in the cytosolic fraction of oat. Limited digestion of the membrane-derived kinase with trypsin generates a smaller water-soluble kinase that is still activated by calcium but is no longer activated by lipid. When posthomogenization proteolysis is minimized, the bulk of the immunoreactive kinase material is localized in the membrane. These results suggest that a calcium-dependent protein kinase observed in the supernatant fraction of oat extracts may originate in situ from a calcium- and lipid-dependent protein kinase which is associated with the oat plasma membrane. They further indicate that, in contrast to animal cells, the predominant calcium- and lipid-dependent protein kinase associated with the plasma membrane of plant cells has biochemical properties and amino acid sequence unlike protein kinase C.

In higher plants, as in other eukaryotes, calcium plays an important role as a second messenger, and has been implicated in cell elongation and division, protoplasmic streaming, and hormone action [Reviewed in Marmé and Dieter (1983), Hepler and Wayne (1985), and Carofoli (1987)]. In contrast, the role of cAMP in plants is questionable (Spieteri et al., 1989). Despite the recognition that calcium plays a second-messenger role in plants, little is known concerning the molecular mechanism by which changes in cytoplasmic calcium act.

This past year, the predominant calcium-dependent protein kinase in the cytosolic fraction of soybean cells was purified to homogeneity (Putnam-Evans et al., 1990), and a cDNA clone encoding the kinase was also isolated (Harper et al., 1991). The kinase is different from any previously identified plant or animal kinase in that it requires calcium but not calmodulin or phosphatidylserine for activity (Harmon et al., 1987; Putnam-Evans et al., 1990). The protein has an amino-terminal kinase catalytic domain fused to a carboxy-terminal domain which shows greatest homology to calmodulin and contains four calcium-binding sites (Harper et al., 1991). As such, this kinase is capable of binding calcium directly and represents the prototype for a new family of calcium-regulated protein kinases.

The studies reported in this paper were prompted by the observation that the predominant kinase activity associated with the plasma membrane of higher plants is also calcium-dependent (Schaller & Sussman, 1988; Klucis & Polya, 1988; Klimczak & Hind, 1990). Here we demonstrate that a protein kinase found in both the soluble and the plasma membrane fraction of oat roots is immunologically related to the cytosolic calcium-dependent protein kinase from soybean (Putnam-Evans et al., 1990). Our results indicate that in oat this plasma membrane bound kinase is dependent upon both calcium and lipid and suggest that limited proteolysis of the membrane-

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