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Mutational study of human phosphohistidine phosphatase: Effect on enzymatic activity ☆

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Abstract

Although protein histidine phosphorylation is estimated to account for about 6% of total protein phosphorylation in eukaryotes, knowledge on histidine phosphorylation and dephosphorylation is still limited. Recently, a few reports have appeared on a mammalian 14-kDa phosphohistidine phosphatase, also named protein histidine phosphatase. Molecular cloning of the protein has opened possibilities for exploring its properties and physiological role. In the present work, we have searched for potential active site residues in the human phosphohistidine phosphatase by point mutations of conserved histidine and arginine residues to alanine. When assayed by the phosphohistidine-containing peptide succinyl-Ala-His(P)-Pro-Phe-*p*-nitroanilide, mutants H53A and H102A showed no detectable activity. Compared to the wild-type recombinant enzyme, the specific activity of mutant R45A was decreased by one order of magnitude, that of mutant R78A was decreased by about 30%, while that of mutant H81A was essentially unchanged. These results will facilitate future studies of the reaction mechanism, substrate binding, and molecular structure of the phosphohistidine phosphatase.

Keywords: Point mutation; Phosphohistidine phosphatase; Protein histidine phosphatase; Dephosphorylation

Phosphohistidine in eukaryotic protein was first described by Boyer and coworkers already in 1962 [1] and was shown by them to be derived from mitochondrial succinyl-CoA synthetase [2]. Despite the fact that phosphohistidine represents a substantial fraction of protein-bound phosphate [3], the research on eukaryotic histidine phosphorylation has so far been of low extent when compared to that of serine/threonine and tyrosine phosphorylation. This difference has, at least partially, been explained by the high lability of the two phosphohistidine isomers, 1-phosphohistidine and 3-phosphohistidine; for recent reviews, see [3–7].

Essentially two aspects of eukaryotic histidine phosphorylation and dephosphorylation have been studied. The first is the intermediary phosphorylation (autophosphorylation) of the active site of enzymes, e.g., nucleoside diphosphate kinase, which gives rise to 1-phosphohistidine [8–11], and ATP-citrate lyase, which gives rise to 3-phosphohistidine [12–15]. The dephosphorylation in this case occurs as part of the continued enzyme reaction. The other aspect is the regulatory phosphorylation of various proteins by histidine kinases [3–7,16]. A counteracting dephosphorylation may, at least for histone H4, be achieved by the well-studied serine/threonine protein phosphatases 1, 2A, and 2C [17].

However, in 2002, a 14-kDa phosphohistidine-specific mammalian phosphatase (PHP) was described independently

☆ *Abbreviations:* PHP, phosphohistidine phosphatase (or protein histidine phosphatase); PHPT1, human phosphohistidine phosphatase (as designated by the HUGO Gene Nomenclature Committee); MDEA, *N*-methyl-diethanolamine.

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by us [18] and Klumpp et al. [19]. The latter group has since contributed further by the identification of ATP-citrate lyase [20] and the β_1-subunit of G-proteins [21] as potential substrates of PHP. In particular, the last report seems to give additional weight to further studies on this enzyme.

In the present work, we have studied the human phosphohistidine phosphatase (PHPT1) by point-directed mutagenesis of conserved arginine and histidine residues, with the aim to identify amino acid residues of immediate importance to the catalytic mechanism. The results indicate that Arg^{45}, His^{53}, and His^{102} are of special interest in this context.

Materials and methods

Materials. LA *Taq* polymerase, T_4 DNA ligase, and restriction enzymes were from TaKaRa. L-Arabinose was purchased from SIGMA (St. Louis). Competent TOP10 cells and pBAD vector were from Invitrogen Life Technologies (Carlsbad, CA). Plasmid Midi Kit and Gel Extraction Kit were from QIAgen (Valencia, CA). Succinyl-Ala-His-Pro-Phe-*p*-nitroanilide was purchased from Bachem AG (Switzerland). MDEA was bought from Riedel de Haën (Sweelze, Germany). LB-Medium was bought from Q-BIOgene and Bacto Agar was obtained from Becton–Dickinson (Sparks, MD). Mono Q column was a product of GE Health Care (Uppsala, Sweden).

Preparation of substrate and analysis of enzyme activity. The substrate succinyl-Ala-His(P)-Pro-Phe-*p*-nitroanilide was prepared, and the phosphohistidine phosphatase activity was analyzed as described previously [18] with 66 μM substrate and incubation for 30 min. The *p*-nitroanilide-containing substrate and product peptides were separated on a Mono Q column connected to a HPLC system. The absorbance at 315 nm was monitored and the degree of dephosphorylation was calculated from the peak areas of phosphorylated and dephosphorylated peptides.

Expression of wild-type PHPT1. The wild-type PHPT1 cDNA [18] was transferred from a pCR-II TOPO cloning vector to a pBAD/Myc-His A vector (Invitrogen). This was performed by Crosslink BT, Budapest, Hungary. The purified construct was transformed to TOP10 cells and selected with Carbenicillin. Positive colonies were grown in LB-medium and induced with 0.2% L-arabinose for different time periods at 37 °C. It was found that maximum expression was obtained by an induction for 16 h. This condition was also used for expression of the mutants.

Mutant design and construct preparation. Point mutations of histidine or arginine residues to alanine, was performed by PCR using the primers listed in Table 1. PCR-1 and PCR-2 reactions were performed at the following conditions: denaturing at 94 °C for 3 min, 25 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. PCR-3 conditions: 94 °C for 3 min for denaturing followed by 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s for 33 cycles. For each mutation, two fragments were amplified in two separate PCRs using the wild-type PHPT1 in a pBAD plasmid vector as template. The first PCR amplified the fragment that contained the mutation site together with the upstream sequence, utilizing a reverse primer containing the mutation and a forward primer, pBAD-F. The second PCR amplified the DNA fragment that contained the mutation site together with the downstream sequence, utilizing a forward primer containing the mutation and a reverse primer, pBAD-R. The two PCR-purified fragments were mixed, denatured, annealed, and extended, and the product was amplified in a third PCR using the pBAD-F and pBAD-R primers. Final PCR products were cleaved with *Bam*HI and *Sal*I, and the digests were purified by agarose gel electrophoresis. The purified fragments were cloned into the pBAD/Myc-His A vector that had been linearized by *Bam*HI and *Sal*I. The mutation was confirmed by sequencing the mutant construct using pBAD-F and pBAD-R primers, and the BigDye terminator method from Perkin-Elmer. Sequencing was carried out in ABI Prism 310 Genetic Analyser (Perkin-Elmer).

Table 1
Primers for PCR cloning of PHPT1 mutants

Mutants	Primers
Mut1-F	5′-atcgtgGCTggctacaag-3′
Mut1-R	5′-gtagccAGCcacgatctc-3′
Mut2-F	5′-gagtacGCTgcggacatc-3′
Mut2-R	5′-gtccgcAGCgtactcagc-3′
Mut3-F	5′-ggaGCTatctctcaccagagt-3′
Mut3-R	5′-agatAGCcccgccgcc-3′
Mut4-F	5′-atctccGCTcagagtcag-3′
Mut4-R	5′-actctgAGCggagatgcg-3′
Mut5-F	5′-gcccagGCTgccatttca-3′
Mut5-R	5′-atggcAGCctgggcag-3′
pBAD-F	5′-gcatcagacattgccgtcac-3′
pBAD-R	5′-cgccaggcaaattctgtttt-3′

Analysis of PHPT1 mutant expression. The mutant clones were expressed in TOP10 bacterial cells. After transformation, positive clones were selected on LB-agar plates containing ampicillin (0.1 mg/ml). From each mutant clone, five single colonies were picked and cultured separately in 3 ml LB medium at 37 °C overnight. The cell cultures were centrifuged and the supernatants were discarded. The cell pellets were suspended in 50 μl water and 50 μl SDS–PAGE sample buffer and denatured at 95 °C for 5 min. After centrifugation, samples of 20 μl were applied to a 15% SDS–PAGE [22] for analysis. The clones having the highest expression levels of the 14-kDa protein were selected from each mutant for preparative expression. Protein was estimated by the Bio-Rad D_C protein assay using bovine serum albumin as standard.

Preparative expression of PHPT1 mutant proteins. Fresh LB medium of 50 ml was inoculated with 100 μl of overnight cultures, obtained as described above, and grown at 37 °C to OD_{600nm} 0.4–0.5. After addition of L-arabinose to a final concentration of 0.2% in the culture, the cells were grown for additional 16 h. The cell suspension was centrifuged at 3000*g* for 5 min and the supernatant was discarded. The cell pellet was suspended in 2 ml of 25 mM Hepes (pH 7.5) containing 5 mM EDTA, 0.01 mg/ml pepstatin A, 0.25 mg/ml pefa-bloc, and 0.1 mg/ml lysozyme, and immediately frozen at −70 °C. After 10 min, the cells were thawed on ice and disrupted by sonication in a SONICS Vibra Cell sonicator for 3 min with about 20 pulses of 4 s at amplitude 30. The cell lysate was centrifuged at 13,000*g* for 30 min and the resulting supernatant was used for partial purification of the PHPT1 mutant protein.

Partial purification of the recombinant PHPT1 wild-type and mutant proteins. For each protein, a 1-ml sample of the supernatant of the bacterial lysate was applied to a 1 ml Mono Q column equilibrated with 25 mM Hepes, pH 7.5. Elution was carried out with 2 ml of the equilibration buffer, followed by an 18-ml linear gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 1 ml/min and 1-ml fractions were collected. The location of the 14-kDa protein in the chromatograms was determined by SDS–PAGE stained with Colloidal Coomassie and the phosphohistidine phosphatase activity was analyzed in the fractions containing this protein.

Western blot analysis. Polyclonal antibodies against PHPT1 were produced in rabbits using purified recombinant full length PHPT1as antigen by Innovagen (Lund, Sweden). Samples of 0.5 μg protein were separated in 15% SDS–PAGE and subsequently blotted to a nitrocellulose membrane. The membrane was incubated with the anti-PHPT1 serum (1:200 dilution) followed with horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology). The signal was detected using 3,3′-diaminobenzidine (DAB, from Sigma) as substrate.

Results and discussion

Optimizing the expression of wild-type PHPT1

In our early work [18], we expressed the human PHPT in BL21 (DE3) cells using pET24a(+) vector. Upon new

attempts to express the enzyme, we found that the expression level was low, likely due to the fact that the plasmid construct was not stable. To improve the expression level of the recombinant protein, commercially available expression vectors, pETMalP2E, pETMalC2E, pET39, pET22b, and pBAD/Myc-His A, were tested. Only the pBAD/Myc-His A construct gave a satisfying expression and was found to be stable upon storage different expression systems were investigated. To exclude the potential interference of the His-tag with PHP activity, the PHPT1 was inserted without fusion with the His-tag.

Selection of PHPT1 mutants

From reports on the active site of a few other phosphatases [23,24], we supposed that mutation of conserved arginine and histidine residues in PHPT1 would have the greatest chance of causing changes of the enzyme activity. To identify such residues we first compared the sequence of PHPT1 with orthologous proteins using ClustalW program (European Bioinformatics Institute, EBI). Two arginine and three histidine residues were found to be conserved throughout the animal kingdom (Fig. 1A). We

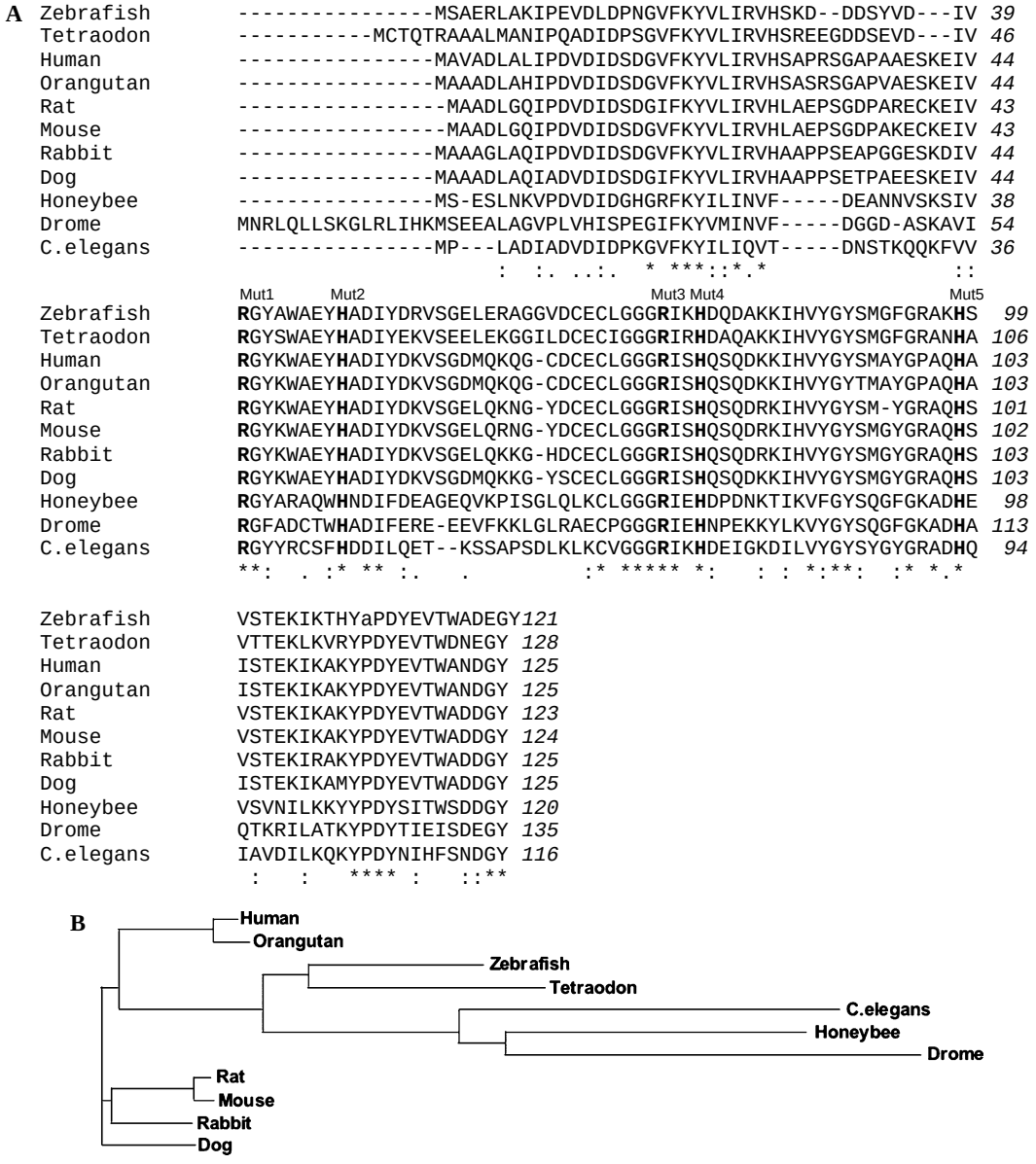

Fig. 1. Multiple alignment analysis of human phosphohistidine phosphatase (PHPT1) and orthologous proteins. (A) Peptide sequence alignment using ClusterlW (EMBL-EBI) shows that the amino acid residues Arg^{45}, His^{53}, Arg^{78}, His^{81}, and His^{102} of PHPT1 are conserved throughout the animal kingdom (boldface). (∗) Indicates identical residues; (:) indicates conserved substitutions; (·) indicates semi-conserved substitutions. The mutant numbers correlate to those shown in Tables 1, Figs. 2 and 3. (B) A phylogenic diagram constructed based on their amino acid sequences shows the relationship among the species with respect to PHPT1 and orthologous proteins. The branch length indicates the evolutionary distances.

considered mutation of these five sites to alanine worthwhile. The PCR approaches using the primers listed in Table 1 have generated the mutants as designed, which were confirmed by DNA sequencing. The mutant cDNAs were inserted into the pBAD/Myc-His A vector without fusion with His-tag.

Expression and catalytic activity of wild-type and mutant PHPT1

For each mutant, we screened five colonies. All screened colonies were found to express the recombinant 14-kDa protein. For further study we selected, from each mutation, the clone that had the highest expression level. The expression levels of the selected clones differed slightly. As judged from SDS–PAGE (Fig. 2), Mut2 and Mut3 had a expression higher than that of the wild-type clone, while Mut1, Mut4, and Mut5 produced less recombinant protein than the wild-type PHPT1.

Only Mut4 (H81A) had a specific activity similar to that of the wild-type. Mut3 (R78A) displayed a specific activity that was about 30% lower than that of the wild-type, while that of Mut1 (R45A) was one order of magnitude lower. Mut2 (H53A) and Mut5 (H102A) displayed no detectable activity (Table 2). The low or undetectable activities could not be explained by different amounts of recombinant protein expressed. In fact, Mut2 (H53A) had the highest expression level among all the clones analyzed, including wild-type (Fig. 2).

In order to control whether the decreased activity of mutants with low or undetectable activity was possibly due to interaction with other protein(s) in the crude supernatants of the cell lysates, the recombinant proteins were partially purified on a Mono Q column that was eluted with a linear gradient of NaCl. The enzyme activity was eluted at about 0.25 M NaCl (fraction 11) and coincided with the position of the 14-kDa protein, as tested by SDS–PAGE (Fig. 3A).

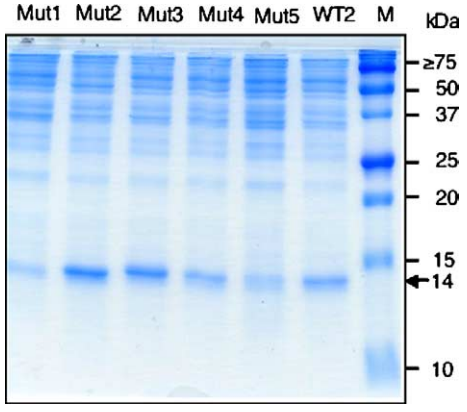

Fig. 2. Analysis of PHPT1 expression by SDS–PAGE. The supernatants of the cell lysate (5 μg protein in SDS–PAGE sample buffer) were denatured at 95 °C for 5 min. After centrifugation, the supernatants were applied to a 15% SDS–PAGE. The gel was stained with Colloidal Coomassie. The molecular weight standards are Precision Plus Protein Standards (Bio-Rad, CA). The arrow indicates PHPT1.

Table 2
Enzyme activity of PHPT1 wild-type and mutants

Mutants	Specific activity (μmol/min/mg)	
	Cell lysate	Purified
Wild-type	0.4	1.8
Mut1 (R45A)	0.06	0.2
Mut2 (H53A)	n.d.	n.d.
Mut3 (R78A)	0.3	0.9
Mut4 (H81A)	0.4	1.4
Mut5 (H102A)	n.d.	n.d.

n.d., not detectable.

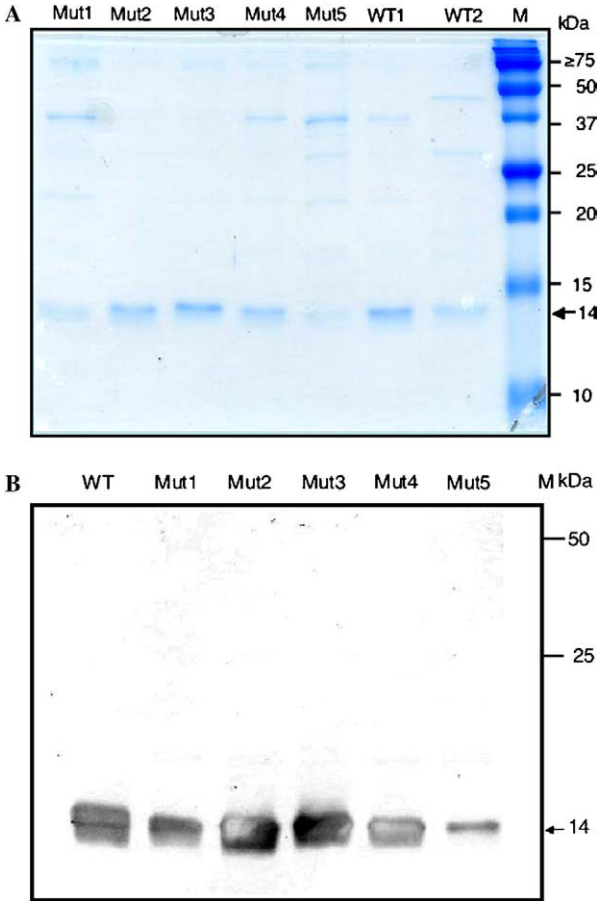

Fig. 3. Analysis of partially purified mutants and wild-type PHPT1 by SDS–PAGE and Western blotting. (A) Samples (fraction 11 after Mono Q chromatography) of partially purified recombinant protein (1.5 μg) were analyzed as described in Fig. 2. Lane WT2 represents a positive control of wild-type PHPT1 prepared according to the procedure described in [18]. (B) The same samples (0.5 μg) were subjected to the SDS–PAGE as in (A) and blotted to a nitrocellulose membrane that was incubated with antibodies against PHPT1. The signals were visualized using secondary antibodies conjugated to horseradish peroxidase with DAB as substrate.

In addition, a Western blotting analysis, where antiserum from a rabbit immunized with wild-type PHPT1 was used, confirmed this position of the PHPT1 protein (Fig. 3B). Apparently, the point mutation from a basic amino acid to a neutral amino acid did not significantly alter the binding of the proteins to the anion exchange matrix.

The one-step partial purification of PHPT1 enabled us to rapidly process all the mutant samples, accordingly minimizing the loss of enzyme activity during purification process, which was advantageous for comparison of the specific activities of the wild-type PHPT1 and the different mutants. It is concluded from Table 2 that the partial purification on Mono Q increased the specific activity of the wild-type and the active mutants to approximately the same extent. The data also suggest that the low specific activity of mutant R45A, and the undetectable activity of H53A and H102A in the crude extracts were true properties of these mutants, since a 10-fold amount of the partially purified recombinant protein was used for analysis of Mut2 (H53A) and Mut5 (H102A), and failed to detect activity.

In conclusion, PHP is a conserved protein in animal kingdom, including those of non-mammals (Fig. 1A), with a relatively close distance (Fig.1B). Conservation of the protein indicates a fundamental role of the enzyme in animal development and homeostasis. The access to the PHPT1-mutants with decreased enzyme activity described in the present work will be of great value in future studies on the function, reaction mechanism, and substrate binding, as well as in ongoing studies on the 3-D structure of the human 14-kDa phosphohistidine phosphatase.

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