



HAL
open science

The HDHD1 gene, often deleted in X-linked ichthyosis, encodes a pseudouridine-5'-phosphatase

Alice Preumont, Rim Rzem, Didier Vertommen, Emile van Schaftingen

► **To cite this version:**

Alice Preumont, Rim Rzem, Didier Vertommen, Emile van Schaftingen. The HDHD1 gene, often deleted in X-linked ichthyosis, encodes a pseudouridine-5'-phosphatase. *Biochemical Journal*, 2010, 431 (2), pp.237-244. 10.1042/BJ20100174 . hal-00521555

HAL Id: hal-00521555

<https://hal.science/hal-00521555>

Submitted on 28 Sep 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

The HDHD1 gene, often deleted in X-linked ichthyosis, encodes a pseudouridine-5'-phosphatase

Alice Preumont, Rim Rzem, Didier Vertommen, Emile Van Schaftingen

From the de Duve Institute, Université Catholique de Louvain, B-1200 Brussels, Belgium

Address correspondence to: Emile Van Schaftingen, de Duve Institute, Université catholique de Louvain, Avenue Hippocrate 75, B-1200 Brussels, Belgium. Tel.: 3227647564; Fax: 3227647598; E-mail: emile.vanschaftingen@uclouvain.be

The abbreviations used are: 5'-PsiMP, pseudouridine 5'-phosphate; 5'-PsiMPase, pseudouridine-5'-phosphatase; DTT, dithiothreitol; FMNPase, FMN phosphatase ; LB, Luria-Bertani.

REVISED VERSION

Short title : Pseudouridine 5'-phosphate phosphatase

Keywords : pseudouridine, RNA metabolism, nucleotidase, ichthyosis, phosphatase

Pseudouridine, the fifth most abundant nucleoside in RNA, is not metabolised in mammals, but is excreted intact in urine. The purpose of the present work was to search for an enzyme that would dephosphorylate pseudouridine 5'-phosphate, a potential intermediate in RNA degradation. We show that human erythrocytes contain a pseudouridine-5'-phosphatase displaying a $K_m \leq 1 \mu\text{M}$ for its substrate. The activity of the partially purified enzyme was dependent on Mg^{2+} , and was inhibited by Ca^{2+} and by vanadate, suggesting that it belonged the 'haloacid dehalogenase' family of phosphatases. Its low molecular mass (26 kDa) suggested that this phosphatase could correspond to the protein encoded by the *HDHD1* (previously called *GSI*) gene, present next to the *STS* (steroid sulfatase) gene on human chromosome Xp22. Purified human recombinant HDHD1 dephosphorylated pseudouridine 5'-phosphate with a k_{cat} of 1.6 s^{-1} , a K_m of $0.3 \mu\text{M}$, and a catalytic efficiency at least 1000 fold higher than that on which it acted on other phosphate esters, including 5'-UMP. The molecular identity of pseudouridine-5'-phosphatase was confirmed by the finding that its activity was negligible (< 10% of controls) in extracts of B-cell lymphoblasts or erythrocytes from X-linked ichthyosis patients harbouring a combined deletion of the *STS* gene (the X-linked ichthyosis gene) and the *HDHD1* gene. Furthermore, pseudouridine-5'-phosphatase activity was 1.5-fold higher in erythrocytes from women compared to men, in agreement with the *HDHD1* gene undergoing only partial inactivation in females. In conclusion, HDHD1 is a phosphatase specifically involved in dephosphorylation of a modified nucleotide present in RNA.

INTRODUCTION

Pseudouridine, the most abundant modified nucleoside present in RNA, is formed post-transcriptionally in transfer RNAs, ribosomal RNAs, and small nuclear RNAs through isomerization of uridine by pseudouridine synthases [1]. Free pseudouridine, which results from the breakdown of RNAs, is metabolised in *Escherichia coli* by a specific kinase (YeiC) that phosphorylates the 5' carbon and a specific glycosidase (YeiN) that hydrolyzes the unique C-C glycosidic bond of pseudouridine 5'-phosphate (5'-PsiMP) [2-4]. Homologues of

the kinase and the glycosidase are found in many bacteria and in many eukaryotes. In the latter, the two enzymes belong to a single bifunctional protein. Analysis of genomes indicates that the gene encoding this bifunctional protein is absent from mammalian genomes, accounting for the fact that pseudouridine is not metabolised in man, but is excreted intact in urine. Its urinary excretion is increased in cancerous patients, and serves therefore as a cancer marker [5-7]. The reason for this rise is not well understood, but it is probably due to an increase in RNA turnover. To avoid random incorporation in RNAs, pseudouridine is not reutilized but excreted from cells, as most other modified nucleosides [8].

Little is known about the way free pseudouridine is formed. It is likely that breakdown of RNA leads to the formation of both 3'-PsiMP and 5'-PsiMP, which are dephosphorylated before leaving the cells. The availability of purified *E. coli* pseudouridine 5'-kinase allowed for the easy synthesis of radiolabelled 5'-PsiMP, which we used to check for the presence of a phosphatase acting on this substrate. We found that human erythrocyte extracts indeed contain a rather specific phosphatase for this substrate. The purpose of the present article is to report the characterization of this phosphatase and the determination of its molecular identity.

EXPERIMENTAL

Materials

Reagents, of analytical grade whenever possible, were from Sigma (St-Louis, MO, USA), Acros (Geel, Belgium), Roche Applied Sciences (Mannheim, Germany), Perkin-Elmer (Coignieres, France) or Merck (Darmstadt, Germany). DEAE-Sepharose, Q-Sepharose, HisTrap, HisSpinTrap, Nap-5, PD-10 and Superdex-200 16/60 columns were purchased from GE Healthcare (Diegem, Belgium). Pseudouridine and its N1-methyl derivative was from Berry and Associates (Dexter, MI, USA). Other nucleosides and nucleotides were from Sigma. Vivaspin-15 centrifugal concentrators were from Vivascience (Göttingen, Germany). Dowex 1-X8 (100–200 mesh) was purchased from Acros. Enzymes were purchased from Sigma, Roche Applied Sciences, Finnzymes (Espoo, Finland) or Fermentas (St Leon-Rot, Germany). B-cell lymphoblast cell lines from patients were obtained from the European Collection of Cell Culture (ECACC) (Salisbury, UK) and control B-cell lymphoblast cell lines were kindly provided by Nathalie Demotte, Ludwig Institute for Cancer Research, Brussels.

Preparation of 5'-PsiMP

Unlabeled 5'-PsiMP was synthesised by phosphorylation of pseudouridine with purified YeiC (as described in [4]). For the synthesis of [³²P]5'-PsiMP, 100 μM pseudouridine was incubated for 20 min at 30°C with 0.5 μg YeiC in a solution (final volume: 100 μl) containing 25 mM Hepes, pH 7.1, 25 mM KCl, 1 mM MgCl₂, 10 μM ATP-Mg and 5 x 10⁷ cpm of [^γ³²P]-ATP. The incubation was stopped by the addition of 25 μl of ice-cold 10% (w/v) HClO₄. After neutralization with K₂CO₃, the salts were removed by centrifugation and the supernatant was diluted with water. The sample (1 ml) was applied onto a 1 ml Q-Sepharose column to purify [³²P]5'-PsiMP; this compound was eluted with a NaCl gradient at neutral pH. 3'-UMP was prepared by digestion of polyuridylic acid with pancreatic RNase A and purified by chromatography on the anion exchanger AG1-X8 using a NaCl gradient at neutral pH.

Measurement of Phosphatase Activities

Unless otherwise stated enzymatic assays were performed at 30°C in a medium containing 25 mM Hepes, pH 7.1, 25 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin, and the indicated concentration of substrate. Assays using unlabeled substrates were performed in 50 µl and stopped by adding 100 µl of 10 mM HCl. Inorganic phosphate was then assayed as described by Itaya and Ui [9]. Assays with ³²P-labeled 5'-PsiMP were performed in a solution containing, unless otherwise stated, 1 µM 5'-PsiMP (50000 cpm). The reaction was stopped by the addition of 100 µl ice-cold 5% (w/v) trichloroacetic acid. The samples were centrifuged for 10 min at 10000 *g* and ³²P-inorganic phosphate was isolated as a phosphomolybdic complex [10] before being counted.

Partial purification of pseudouridine-5'-phosphatase from human erythrocytes

300 ml of packed erythrocytes were washed three times with 150 mM NaCl and diluted in 1200 ml of lysis buffer (5 mM Hepes, pH 7.1, 1 mM DTT, 1 µg/ml leupeptin, 1 µg/ml antipain). The haemolysate was centrifuged for 20 min at 11000 *g*. The supernatant (1300 ml) was applied to a DEAE-Sepharose column (200 cm³) equilibrated with buffer A (20 mM Hepes, pH 7.1, 1 mM DTT, 1 µg/ml leupeptin, and 1 µg/ml antipain). The column was washed with 400 ml of buffer A and protein was eluted with a 0-0.5 M NaCl gradient in 900 ml of buffer A. Fractions of 7 ml were collected. Fraction 62 (3.5 ml), which contained the peak of 5'-PsiMPase activity (see Results), was concentrated to 1 ml and applied to a Superdex-200 16/60 column equilibrated with buffer B (25 mM Hepes, pH 7.1, 1 mM DTT, 1 µg/ml leupeptin, 1 µg/ml antipain, 200 mM NaCl). Fractions of 1 ml were collected.

Expression and purification of putative phosphatases

For HDHD1 a 5' primer containing the initiator ATG codon (ACCTCGCATATGGCGGCGCCCCGC) in a NdeI site (in bold) and a 3' primer containing the stop codon (ACCTCGGGATCCTCACTCATAGGAGGGCAAACCA) flanked by a BamHI site were used to PCR-amplify cDNA from human brain with *Pwo* polymerase. An ~700-bp product was obtained. For PDXP we used a 5' primer containing the initiator ATG codon (CCATATGGCGGCTGCGAGAGGCTG) in an NdeI site (in bold) and a 3' primer containing the stop codon (AGGATCCTCAGTCTCCAACCCCTCTGTC) near to a BamHI site to PCR-amplify cDNA from human liver with *Pwo* polymerase. An ~900 bp product was obtained, which was subcloned in pBlueScript. For PHOSPHO2, a forward primer containing the start codon (ACCTCGCATATGAAAGTTCTGTTGGTGTGGTGGAC) in a NdeI site (in bold) and a reverse primer with the stop codon (ACCTCGGGATCCTCACATCTTTATTAGAAATTGTAAA) flanked by a BamHI site were used to PCR-amplify cDNA from human brain with *Pwo* polymerase. An ~700-bp product was obtained. For NT5C3L a 5' primer containing the initiator ATG codon (ACCTCGCATATGGCGGAGGAGGTAAGCAGC) in a NdeI site (in bold) and a 3' primer containing the stop codon (ACCTCGCTCGAGTTAAGAGCCCTGGAGCTCCA) flanked by a XhoI site were used to PCR-amplify cDNA from mouse brain with Phusion polymerase. An ~900-bp product was obtained.

PCR-products were incubated with the indicated restriction enzymes and the fragments were ligated in pET-15b. The recombinant plasmids were used to transform *E. coli* BL21 pLysS and their inserts were checked by sequencing. The resulting bacteria were grown in Luria-Bertani (LB) medium containing 100 mg/l ampicillin. The cultures were grown at 37 °C until A₆₀₀ reached 0.5 – 0.6. They were then cooled on ice for 20 min and the inducer isopropyl-1-thio-beta-D-galactopyranoside was added to a final concentration of 0.4 mM.

After overnight incubation at 37 °C, the cells were collected by centrifugation, resuspended in a buffer containing 20 mM Hepes, pH 7.1, 0.5 mM phenylmethylsulphonyl fluoride, 5 mM EDTA, 5 µg/ml leupeptin, 5 µg/ml antipain, 1 mg/ml lysozyme and submitted to three cycles of freezing and thawing. The bacterial extracts were incubated on ice for 1 h with 0.5 mg/ml DNaseI in the presence of 10 mM MgSO₄, and were then centrifuged for 30 min at 10000 g. The resulting supernatants were used to purify the His-tagged recombinant proteins by chromatography on HisTrap HP columns (1 ml), as previously described [11].

Preparation of B-cell lymphoblast and red blood cell extracts for activity measurements

B-cell lymphoblasts were cultured in Iscove's modified Dulbecco's medium (IMDM) (Life Technologies) supplemented with 10% (v/v) fetal calf serum, 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. They were collected by centrifugation of 8 ml cultures for 10 min at 2000 g and 4°C. Cell pellets were washed twice with ice-cold PBS and resuspended in 300 µl of buffer C (25 mM Hepes, pH 7.1, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM DTT, 5 µg/ml leupeptin, 5 µg/ml antipain). After two cycles of freezing and thawing, extracts were submitted to a 10 min centrifugation and the 5'-PsiMPase activity was measured in the supernatants.

Blood was collected in EDTA-tubes and centrifuged for 10 min at 2000 g and 4°C. The plasma was discarded and the buffy coat was isolated and washed twice with 150 mM NaCl. Contaminating erythrocytes were eliminated by two washings with 50 mM NaF, and leukocytes were then washed twice with 150 mM NaCl and stored at -70°C before DNA extraction. The erythrocyte pellet was washed twice with 150 mM NaCl and stored at -70°C before use. One volume of washed erythrocytes was diluted in 3 vol of buffer C. After a 10 min centrifugation, 5'-PsiMPase activity was assayed in the supernatant.

DNA extraction and verification of the absence of the HDHD1 gene

Genomic DNA was extracted from frozen leukocytes isolated from 5 ml of EDTA-blood or from B-cell lymphoblasts using the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany). Exons 1 and 3 of the *HDHD1* gene were PCR-amplified. Primers (sequences available on request) for the amplification were designed in such a way as to avoid amplification of pseudogenes related to *HDHD1*.

RESULTS

Pseudouridine-5'-phosphatase activity in RBC

Using [³²P]5'-PsiMP as a substrate, we found that human red blood cell extracts catalysed the dephosphorylation of this nucleotide. The dephosphorylation of radiolabelled 5'-PsiMP was inhibited by unlabelled 5'-PsiMP at concentrations in the micromolar range (apparent *K_i* of ≈ 1 µM), suggesting that the phosphatase involved in this reaction had a low *K_m* for its substrate. Computation of these data indicated indeed that the phosphatase acting on 5'-PsiMP had a *K_m* of about 1 µM and a *V_{max}* of ≈ 7 nmol/min/mg haemoglobin. Interestingly, the phosphatase activity measured on [³²P]5'-PsiMP was not inhibited by 5'-UMP at a concentration of 300 µM, i.e. 300 fold higher than the *K_m* for 5'-PsiMP. This indicated that 5'-UMP was at best a poor substrate for this enzyme. As 5'-UMP is a close structural analogue of 5'-PsiMP, differing mainly in its accessible portion, by the replacement of a

protonated carbon by a protonated nitrogen in structurally equivalent positions on the uracil base, these findings suggested that the phosphatase might be specific for 5'-PsiMP.

Pseudouridine-5'-phosphatase (5'-PsiMPase) was partially purified by chromatography on DEAE-Sepharose and gel filtration on Superdex-200 16/60. 5'-PsiMPase nearly coeluted with a peak of uridine-5'-phosphatase (5'-UMPase) activity in the anion exchange step (Fig. 1A), but was largely separated from it in the subsequent gel filtration step (Fig. 1B). Its apparent molecular size, as determined by comparison with the elution profile of molecular mass markers, was 26 kDa, whereas the enzyme that hydrolysed 5'-UMP had a molecular mass of ≈ 35 kDa, in agreement with it corresponding to 5'-UMPase (also designated cytosolic 5'-nucleotidase III and encoded by the *NT5C3* gene [12]). Further characterization of the partially purified enzyme confirmed the low K_m for 5'-PsiMP (≈ 0.8 μM , not shown) and indicated that the activity was dependent on the presence of Mg^{2+} and inhibited by Ca^{2+} and by vanadate (Fig. 2).

Vanadate inhibits phosphatases that form a phosphoenzyme during catalysis [13]. Among these, dependency on Mg^{2+} and inhibition by low concentrations of Ca^{2+} point to phosphatases of the HAD (haloacid dehalogenase) family [11]. These enzymes, which share structural homology with haloacid dehalogenase [14], form a phosphoaspartate during their catalytic cycle [15]. They comprise many specific phosphatases acting on different substrates, such as L-serine 3-phosphate [15], purine and pyrimidine 5'-nucleotides [16], N-acetylneuraminate 9-phosphate [11] and glucose 1,6-bisphosphate [17], as well as eukaryotic phosphomannomutase [18] and prokaryotic beta-phosphoglucomutase [19]. They are characterized by the presence of three conserved motifs, the first one comprising a characteristic N-terminal DXDXT/V motif in which the first aspartate serves as phosphate acceptor during catalysis [14, 15]. Due to these motifs, they are easily identified by BLAST searches.

5'-PsiMPase corresponds to HDHD1

By performing BLAST searches, we identified four human proteins of the HAD family for which the function was unknown and which had a molecular size compatible with that of human erythrocyte 5'-PsiMPase (Table 1). These proteins were expressed in *E. coli* as fusion proteins with a polyHis tag at their N-terminus. They were purified to homogeneity (Fig. 3A) and their phosphatase activity was determined with 5'-PsiMP as a substrate (Fig. 3B). Only the protein encoded by the *HDHD1* gene was found to have a substantial 5'-PsiMPase activity, whereas the other three proteins were either inactive or poorly active on this substrate.

To check the specificity of this enzyme, the activity of HDHD1 was tested on different phosphate esters, which included classical and modified nucleoside monophosphates, as well as several other derivatives (Table 2A and B). The assay was based on the formation of inorganic phosphate as determined with the sensitive method of Itaya and Ui [9] and in the case of 5'-PsiMP also through the formation of radiolabelled Pi from [^{32}P]5'-PsiMP. With the first method we found that the phosphatase activity of HDHD1 had already reached its V_{max} with the lowest concentration of 5'-PsiMP that we used (20 μM), indicating that the K_m of HDHD1 must be at least one order of magnitude lower than this concentration. Accordingly, the K_m value determined with the radiochemical method amounted to 0.3 μM , whereas the V_{max} was similar to that obtained with the chemical phosphate assay.

The chemical phosphate assay was suitable to determine the kinetic properties for all other tested substrates, because the K_m was in all cases higher than the lowest concentration of substrate used (20 μM). Using the catalytic efficiency as a criterion, all tested substrates were poorer by at least three orders of magnitude compared to 5'-PsiMP. Interestingly 3'-

AMP was the second best substrate in our series and was much better than 5'-AMP. 3'-UMP was also better than 5'-UMP, though it was not as good as 3'-AMP. It would have been of interest to test 3'-PsiMP. However, this compound was not commercially available. We tried to prepare it by digestion of synthetic polypseudouridylic acid (5-mer, produced by Eurogentec), but this polymer was apparently resistant to hydrolysis by pancreatic RNase A (not shown). None of the modified nucleoside monophosphate that we tested was a good substrate for HDHD1. As mentioned below HDHD1 is related to plant flavin-monomonucleotide-phosphatase (FMNPase). The catalytic efficiency of human HDHD1 on this substrate was, however, more than 30,000 fold lower than that observed with 5'-PsiMP.

Eukaryotic ribosomal RNA comprises N1-methylpseudouridine, a precursor for the hypermodified nucleoside 1-methyl-3-(3-amino-3-carboxypropyl)-pseudouridine [20]. Our attempts to prepare N1-methylpseudouridine 5'-phosphate from commercial N1-methylpseudouridine failed, because this compound was not phosphorylated by *E. coli* pseudouridine kinase (not shown). We found, however, that whereas pseudouridine inhibited the HDHD1 activity measured either on 5'-PsiMP or 3'-AMP (Fig. 4A and B), 1-methylpseudouridine barely affected this activity, suggesting that the methyl group on N1 prevents recognition of pseudouridine by the catalytic site of HDHD1. No inhibition was observed with uridine and adenosine, which were used as controls.

5'-PsiMPase activity in X-linked ichthyosis and according to sex

The gene coding for HDHD1 is present on chromosome X next to the *STS* gene [21]. The latter encodes steroid sulfatase (arylsulfatase C), the enzyme that is deficient in X-linked ichthyosis, a disorder that is most often due to a submicroscopic deletion of the X chromosome encompassing both the *STS* gene and the *HDHD1* gene [22]. We obtained B-cell lymphoblasts or erythrocytes from four patients with X-linked ichthyosis and verified that they had a deletion of the *HDHD1* gene by showing that no amplification product could be obtained when their DNA was submitted to PCR reactions with appropriate primers. As shown in Fig. 5, B-cell lymphoblasts from 3 patients and erythrocytes from a fourth one were devoid of low K_m 5'-PsiMPase activity, confirming that *HDHD1* indeed encodes this enzyme.

The *STS* and *HDHD1* genes are present in a region of chromosome X that partially escapes X-inactivation, as confirmed by activity measurements of the steroid sulfatase activity in female subjects compared to male subjects [23, 24] and, for the *HDHD1* gene, by analysis of its mRNA level with microarrays [25]. As shown in Fig. 5B, the pseudouridine phosphatase activity of erythrocyte extracts was about 50% higher as a mean in female subjects compared to male subjects.

Sequence comparison and distribution of HDHD1

Homologues of HDHD1 are widely distributed. Proteins sharing more than 40% identity are indeed found in virtually all eukaryotes, including vertebrates, invertebrates, plants and fungi. The *HDHD1* gene is encoded by a multiexon gene present next to the *STS* gene on chromosome X in man and several other mammals (*Bos*, *Equus*, *Canis*), but on an autosome in birds and fishes. The mouse and rat homologues are encoded by a single-exon gene on chromosome 18 in both cases. This gene presumably resulted from a retrotransposition event. *Arabidopsis thaliana* and other plants comprise two homologues of HDHD1. One of them, more distant (34% sequence identity with the human enzyme; Fig. 6), forms a fusion protein with riboflavin kinase and was shown to act as an FMN phosphatase (FMNPase). We speculate that the function of the other one, which shares 50% sequence identity with HDHD1

(Fig. 6), is to catalyse the hydrolysis of 5'-PsiMP. As mentioned above, human HDHD1 has an extremely low FMNPase activity.

The alignment displayed in Fig. 6 shows that the HDHD1 proteins possess the three motifs known to be conserved in phosphatases of the HAD family, though the usual valine or threonine at the end of the DXDXT/V motif is replaced by a leucine. These comprise the two aspartates of the first motif, the threonine (or serine) of the second motif and a lysine and an aspartate in the third motif. These residues are shared with *A. thaliana* FMNPase.

DISCUSSION

Identification of 5'-PsiMPase as of HDHD1

We show in this paper that human erythrocytes contain a phosphatase that acts with very high affinity on a modified nucleotide, 5'-PsiMP, and much less on classical nucleoside monophosphates and other phosphate esters. We identified this enzyme as being the product of the *HDHD1* (also known as *HDH1A* or *GSI*) gene, a putative phosphatase of the HAD family for which the function has long remained unknown. This identification is based on the findings that (1) human 5'-PsiMPase has kinetic properties typical of phosphatases of the HAD family [11] (inhibition by vanadate, Mg^{2+} dependency, inhibition by Ca^{2+} , which replaces Mg^{2+} in the catalytic site and perturbs the orientation of the nucleophilic carboxylate [26]); (2) gel filtration shows it to have a size in agreement with that of HDHD1; (3) recombinant HDHD1 catalyses the hydrolysis of 5'-PsiMP with a K_m of 0.3 μM , similar to the 1 μM value observed with the enzyme purified from erythrocytes, and shows a much lower affinity for 5'-UMP than for 5'-PsiMP; (4) 5'-PsiMPase activity is absent from human cells obtained from male X-linked ichthyosis patients, in which the *HDHD1* gene is deleted due to partial deletion of the X-chromosome; (5) this activity is higher in erythrocytes of women than of men, consistent with the *HDHD1* gene being, like the *STS* gene, more highly expressed in cells of women than of men due to incomplete inactivation of the second copy [25].

Specificity of HDHD1

The HAD family is the most important family of phosphatases acting on low-molecular-weight compounds. Some of the enzymes from this family are specific, in the sense that they act on one substrate with a catalytic efficiency that is at least three or four orders of magnitude higher than on other substrates (see [27, 28] for phosphoethanolamine-phosphocholine phosphatase; see [11] for N-acetylneuraminase phosphatase).

The study of the substrate specificity of HDHD1 indicates that this enzyme dephosphorylates a series of phosphate esters, but acts on 5'-PsiMP with a much (> 1000 -fold) higher catalytic efficiency than on all other phosphate esters that we could test. It is 10^5 times less active on 5'-UMP, which is structurally very close to 5'-PsiMP since it essentially differs through the presence of a protonated nitrogen (N1 in pseudouridine) instead of a protonated carbon (C5 in uridine). It is likely that specific recognition of 5'-PsiMP involves hydrogen bonding with this N1-proton. This is further indicated by the conclusion that N1-methyl-pseudouridine 5'-phosphate is most likely not a good substrate, as deduced from the finding that N1-methyl-pseudouridine is a much poorer inhibitor of HDHD1 activity than non-methylated pseudouridine. The catalytic efficiency observed when HDHD1 acts on 5'-PsiMP ($4.2 \cdot 10^6 \cdot s^{-1} \cdot M^{-1}$) favourably compares with the values observed with other specific phosphatases of HAD family acting on their physiological substrate : $0.75 \cdot 10^6 \cdot s^{-1} \cdot M^{-1}$ for phosphoethanolamine phosphatase [27]; $0.62 \cdot 10^6 \cdot s^{-1} \cdot M^{-1}$ for N-acetylneuraminase phosphatase

[11] ; $0.68 \cdot 10^6 \cdot \text{s}^{-1} \cdot \text{M}^{-1}$ for *E. coli* phosphoserine phosphatase [29]; $3 \cdot 10^6 \cdot \text{s}^{-1} \cdot \text{M}^{-1}$ for glucose-1,6-bisphosphatase [17]. Taken together, these findings suggest that HDHD1 may be a 'specific' 5'-PsiMPase.

Remarkably, the second best substrate that we found for HDHD1 is 3'-AMP, which, though being 1,300 fold poorer than 5'-PsiMP (based on the catalytic efficiency), is about 600-fold better than 5'-AMP. The finding that the activity on 3'-AMP was inhibited much more by pseudouridine than by other nucleosides confirmed that this activity was contributed by HDHD1 and not by a different phosphatase, originating from *E. coli*, which would hypothetically contaminate our purified preparation of HDHD1. 3'-UMP was also found to be a better substrate than 5'-UMP, though in this case, the ratio of the two catalytic efficiencies amounted to ≈ 3 . We interpret this as being due to the fact that non-optimal substrates do not bind to the catalytic site in the exact same manner as the best substrate(s). Therefore, the presence of a bulkier base (as in 3'- and 5'-AMP) may make that a 3'-phosphate is better positioned for hydrolysis than a 5'-phosphate, explaining that 3'-AMP is a better substrate than 5'-AMP (yet both of them are poor substrates compared to 5'-PsiMP). It would have been interesting to test 3'-PsiMP, but this couldn't be done due to the lack of availability of this substrate. Our expectation is that 3'-PsiMP would be a poorer substrate than 5'-PsiMP : correct positioning of the uracil moiety of pseudouridine in the catalytic site of HDHD1 presumably makes that only a 5'-phosphate can be in the best position to be attacked by the nucleophilic aspartate.

Role in RNA degradation

The role of HDHD1 is most probably to degrade 5'-PsiMP and maybe also its 2'-O methyl-derivative, which is also present in ribosomal RNA [30]. Experiments with cell free systems indicate that 5'-PsiMP is further phosphorylated to pseudouridine di- and tri-phosphate [31]. This conversion probably involves uridine/cytidine kinases, which have a broad substrate specificity [32] and nucleotide diphosphate kinases, which act on a variety of nucleotide diphosphates [33]. The accumulated pseudouridine 5'-triphosphate can be utilised instead of UTP by various enzymes, such as RNA polymerases and UDP-glucose pyrophosphorylase, as suggested by Goldberg and Rabinowitz [34, 35]. The presence of an enzyme that acts on 5'-PsiMP with high affinity is presumably an efficient means to prevent aberrant incorporation of pseudouridine into RNA or nucleotide diphosphate sugars.

The role of many phosphatases of the HAD family remains unknown. Kuznetsova *et al.* studied the catalytic activity of a series of phosphatases of the HAD family encoded by the *E. coli* genome [29] and found that most of these enzymes acted with a low catalytic efficiency on a series of phosphate esters. We speculate that some of them may act with a much higher catalytic efficiency on 5'-PsiMP or on other modified nucleoside 5'- or 3'-monophosphate that result from the degradation of RNAs. This dephosphorylation is presumably a prerequisite for the excretion of the modified nucleosides from cells.

Potential role of HDHD1 deficiency in X-linked ichthyosis

The *HDHD1* gene was first identified (as the *GSI* gene) because of its close proximity to the STS (steroid sulfatase) gene on human chromosome X [21]. Inactivating mutations or, more often, deletion of the latter gene is responsible for X-linked ichthyosis, which is characterized by scaly skin on the scalp, trunk and limbs, often accompanied by corneal opacities, delayed birth, difficult labor, undescended testis and a higher frequency of testis tumors [22]. Most of these pathological findings are the consequence of the defect in the sulfatase activity and the consequent changes in the concentration of estriol, estrol-sulfate and cholesterol sulfate. To

the best of our knowledge, cryptorchidism and testis tumors have not been reported in cases with specific mutations (point mutations, intragenic deletions) of the *STS* gene or in multiple sulfatase deficiency, a disorder due to a deficiency in the enzyme that converts cysteine to formylglycine in the catalytic site of sulfatases and leads to inactivity of all sulfatases including steroid sulfatase. We may therefore not exclude that the absence of 5'-PsiMPase activity plays a role in the pathophysiology of cryptorchidism or in the development of testis cancer. Though we have no simple hypothesis to explain this potential link, it would be interesting to examine more systematically if 5'-PsiMPase activity is absent in X-linked ichthyosis patients with cryptorchidism or testis cancer.

ACKNOWLEDGEMENTS

We thank J. Fortpied, M.-F. Vincent, S. Marie, N. Demotte and P. van der Bruggen for their helpful suggestions. We thank also Pr Tennstedt and one of his patients for providing us red blood cells.

FUNDING

This work was supported by the Directorate General Higher Education and Scientific Research, French Community of Belgium, the Fund for Medical Scientific Research, the Interuniversity Attraction Poles Program (Network P6/05), Belgian Science Policy. A.P. is a Télévie fellow (grant F.N.R.S – Télévie).

REFERENCES

- 1 Charette, M. and Gray, M. W. (2000) Pseudouridine in RNA: what, where, how, and why. *IUBMB Life* **49**, 341-351
- 2 Breitman, T. R. (1970) Pseudouridylate synthetase of *Escherichia coli*: correlation of its activity with utilization of pseudouridine for growth. *J. Bacteriol.* **103**, 263-264
- 3 Solomon, L. R. and Breitman, T. R. (1971) Pseudouridine kinase of *Escherichia coli*: a new enzyme. *Biochem. Biophys. Res. Commun.* **44**, 299-304
- 4 Preumont, A., Snoussi, K., Stroobant, V., Collet, J. F. and Van Schaftingen, E. (2008) Molecular identification of pseudouridine-metabolizing enzymes. *J. Biol. Chem.* **283**, 25238-25246
- 5 Feng, B., Zheng, M. H., Zheng, Y. F., Lu, A. G., Li, J. W., Wang, M. L., Ma, J. J., Xu, G. W., Liu, B. Y. and Zhu, Z. G. (2005) Normal and modified urinary nucleosides represent novel biomarkers for colorectal cancer diagnosis and surgery monitoring. *J. Gastroenterol. Hepatol.* **20**, 1913-1919
- 6 Masaki, Y., Itoh, K., Sawaki, T., Karasawa, H., Kawanami, T., Fukushima, T., Kawabata, H., Wano, Y., Hirose, Y., Suzuki, T., Sugai, S. and Umehara, H. (2006) Urinary pseudouridine in patients with lymphoma: comparison with other clinical parameters. *Clin. Chim. Acta* **371**, 148-151
- 7 Seidel, A., Brunner, S., Seidel, P., Fritz, G. I. and Herbarth, O. (2006) Modified nucleosides: an accurate tumour marker for clinical diagnosis of cancer, early detection and therapy control. *Br. J. Cancer* **94**, 1726-1733
- 8 Marvel, C. C., Del Rowe, J., Bremer, E. G. and Moskal, J. R. (1994) Altered RNA turnover in carcinogenesis. The diagnostic potential of modified base excretion. *Mol. Chem. Neuropathol.* **21**, 353-368
- 9 Itaya, K. and Ui, M. (1966) A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta* **14**, 361-366
- 10 McClard, R. W. (1979) Synthesis and purification of [1-32p]fructose-1,6-bisphosphate with high specific radioactivity. *Anal. Biochem.* **96**, 500-503

- 11 Maliekal, P., Vertommen, D., Delpierre, G. and Van Schaftingen, E. (2006) Identification of the sequence encoding N-acetylneuraminase-9-phosphate phosphatase. *Glycobiology* **16**, 165-172
- 12 Amici, A. and Magni, G. (2002) Human erythrocyte pyrimidine 5'-nucleotidase, PN-I. *Arch. Biochem. Biophys.* **397**, 184-190
- 13 Macara, I.G. (1980) Vanadium, an element in search of a role. *Trends Biochem. Sci.*, **5**, 92-94
- 14 Aravind, L., Galperin, M. Y. and Koonin, E. V. (1998) The catalytic domain of the P-type ATPase has the haloacid dehalogenase fold. *Trends Biochem. Sci.* **23**, 127-129
- 15 Collet, J. F., Stroobant, V., Pirard, M., Delpierre, G. and Van Schaftingen, E. (1998) A new class of phosphotransferases phosphorylated on an aspartate residue in an amino-terminal DXDX(T/V) motif. *J. Biol. Chem.* **273**, 14107-14112
- 16 Hunsucker S. A., Mitchell B. S. and Spychala J. (2005). The 5'-nucleotidases as regulators of nucleotide and drug metabolism. *Pharmacol. Ther.* **107**, 1-30
- 17 Veiga-da-Cunha, M., Vleugels, W., Maliekal, P., Matthijs, G. and Van Schaftingen E. (2008) Mammalian phosphomannomutase PMM1 is the brain IMP-sensitive glucose-1,6-bisphosphatase. *J Biol. Chem.* **283**, 33988-93
- 18 Kepes, F. and Schekman, R. (1988) The yeast SEC53 gene encodes phosphomannomutase. *J. Biol. Chem.* **263**, 9155-61
- 19 Lahiri, S.D., Zhang, G., Dunaway-Mariano, D. and Allen, K.N. (2002) Caught in the act: the structure of phosphorylated beta-phosphoglucomutase from *Lactococcus lactis*. *Biochemistry* **41**, 8351-9
- 20 Brand, R.C., Klootwijk, J., Planta, R.J., Maden, B.E. (1978) Biosynthesis of a hypermodified nucleotide in *Saccharomyces carlsbergensis* 17S and HeLa-cell 18S ribosomal ribonucleic acid. *Biochem. J.* **169**, 71-7
- 21 Yen, P. H., Ellison, J., Salido, E. C., Mohandas, T. and Shapiro, L. (1992) Isolation of a new gene from the distal short arm of the human X chromosome that escapes X-inactivation. *Hum. Mol. Genet.* **1**, 47-52
- 22 Hernandez-Martin, A., Gonzalez-Sarmiento, R. and De Unamuno, P. (1999) X-linked ichthyosis: an update. *Br. J. Dermatol.* **141**, 617-627
- 23 Muller, C. R., Migl, B., Traupe, H. and Ropers, H. H. (1980) X-linked steroid sulfatase: evidence for different gene-dosage in males and females. *Hum. Genet.* **54**, 197-199
- 24 Willems, P. J., de Bruijn, H. W., Groenhuis, A., Mooyart, B. R. and Berger, R. (1986) Partial lyonisation of steroid sulphatase gene in single hair roots. *J. Inherit. Metab. Dis.* **9**, 156-162
- 25 Johnston, C. M., Lovell, F. L., Leongamornlert, D. A., Stranger, B. E., Dermitzakis, E. T. and Ross, M. T. (2008) Large-scale population study of human cell lines indicates that dosage compensation is virtually complete. *PLoS Genet.* **4**, e9
- 26 Peeraer, Y., Rabijns, A., Collet, J.F., Van Schaftingen, E. and De Ranter C (2004) How calcium inhibits the magnesium-dependent enzyme human phosphoserine phosphatase. *Eur. J. Biochem.* **271**, 3421-7
- 27 Roberts, S.J., Stewart, A.J., Sadler, P.J. and Farquharson, C. (2004) Human PHOSPHO1 exhibits high specific phosphoethanolamine and phosphocholine phosphatase activities. *Biochem. J.* **382**, 59-65
- 28 Roberts, S.J., Stewart, A.J., Schmid, R., Blindauer, C.A., Bond, S.R., Sadler, P.J. and Farquharson, C. (2005) Probing the substrate specificities of human PHOSPHO1 and PHOSPHO2. *Biochim. Biophys. Acta.* **1752**, 73-82
- 29 Kuznetsova, E., Proudfoot, M., Gonzalez, C. F., Brown, G., Omelchenko, M. V., Borozan, I., Carmel, L., Wolf, Y. I., Mori, H., Savchenko, A. V., Arrowsmith, C. H., Koonin, E. V., Edwards, A. M. and Yakunin, A. F. (2006) Genome-wide analysis of

- substrate specificities of the *Escherichia coli* haloacid dehalogenase-like phosphatase family. *J. Biol. Chem.* **281**, 36149-36161
- 30 Gray, M.W. and Keddy, G.M. (1974) Presence of O^{2'}-methylpseudouridine in the 18S + 26S ribosomal ribonucleates of wheat embryo. *Biochemistry* **13**, 5453-5463.
- 31 Goldberg, I. H. and Rabinowitz, M. (1961) The enzymic synthesis of pseudouridine triphosphate. *Biochim. Biophys. Acta* **54**, 202-204
- 32 Van Rompay, A. R., Norda, A., Linden, K., Johansson, M. and Karlsson, A. (2001) Phosphorylation of uridine and cytidine nucleoside analogs by two human uridine-cytidine kinases. *Mol. Pharmacol.* **59**, 1181-1186
- 33 Postel, E. H. (1998) NM23-NDP kinase. *Int. J. Biochem. Cell Biol.* **30**, 1291-1295
- 34 Goldberg, I. H. and Rabinowitz, M. (1961) The incorporation of 5-ribosyluracil triphosphate into RNA in nuclear extracts of mammalian cells. *Biochem. Biophys. Res. Commun.* **6**, 394-398
- 35 Rabinowitz, M. and Goldberg, I. H. (1961) The formation of 5-ribosyluracil diphosphoglycosyl compounds from 5-ribosyluracil triphosphate. *J. Biol. Chem.* **236**, PC79-PC80

Gene symbol	Name	Chromosomal localisation	Size of the polypeptide chain
NT5C3L	NT5C3-like	17q21.2	34 kDa
PDXP	Pyridoxal phosphatase	22cen-q12.3	31 kDa
PHOSPHO2	phosphatase, orphan 2 pyridoxal-phosphatase activity	2q31.1	28 kDa
HDHD1	family with sequence similarity 16, member A, X-linked	Xp22.32	26 kDa

Table 1. Human members of the HAD family of phosphatases investigated in the present study. The chromosomal localisation of the gene and the size of the encoded protein are indicated.

Table 2A

Substrate	K_m (mM)	k_{cat} (s ⁻¹)	Catalytic efficiency k_{cat}/K_m (s ⁻¹ .M ⁻¹)
5'-PsiMP	0.00038	1.59	4.2 10 ⁶
3'-AMP	1.5	4.71	3250
Fructose-1,6-P2	5.9	1.09	186
Fructose-6-P	6.9	1.05	152
5'-UMP	9.4	0.41	44
FMN	0.3	0.04	133

Table 2B

Catalytic efficiency k_{cat}/K_m	Nucleotide	Other phosphate esters
Between 10 and 100 s ⁻¹ .M ⁻¹	5'-dGMP 5'-dIMP 5'-dUMP 5'-GMP 5'-IMP 5'-UMP 4-thiouridine-5'-P	Glycerol-3-P
Lower than 10 s ⁻¹ .M ⁻¹	5'-AMP 5'-dAMP 5'-CMP Cytosine β-D-arabinofuranoside-5'-P 5'-dCMP N6-Methyladenosine-5'-P 5'-TMP	Glucose-1-P L-3-phosphoserine Phosphoglycolate Phosphoenolpyruvate Phosphorylcholine

Table 2 A and B. Activity of recombinant human HDHD1A on different phosphate esters.

In Table 2A, the activity was measured with different concentrations of the indicated substrates and K_m , k_{cat} and catalytic efficiencies were computed from these data. For the poorer substrates (Table 2B), the catalytic efficiency was estimated from the activity measured at 1 mM substrate by assuming that the K_m was much higher than 1 mM and using the approximate formula $v = [\text{Enzyme}] \cdot k_{cat}/K_m \cdot [\text{Substrate}]$. The error due to this assumption amounts to 33 % if the K_m is 2 mM and is lower if the K_m is higher. All substrates were also tested at a concentration of 5 mM; the activities observed at this concentration indicated that the K_m values were always > 2 mM. In addition, all substrates were also tested at 1 mM in combination with 1 mM 3'AMP to check if they caused inhibition of the activity on this substrate. Less than 10 % inhibition was observed in all cases. This indicated that the low activities observed with these substrates were not due to substrate inhibition at 'high' concentration. All assays were performed in triplicate.

FIGURE LEGENDS

Fig. 1. Partial purification of 5'-PsiMPase from human erythrocytes by anion-exchange chromatography and gel filtration.

(A) A human erythrocyte extract was applied onto a DEAE-Sepharose column and a linear NaCl gradient was applied. A₄₁₅ (to monitor haemoglobin; dotted line), 5'-PsiMPase (▲), 5'-UMPase (□) and NaCl (full line) were assayed. (B) Fraction 62 of DEAE was further purified on a Superdex-200 16/60 column. Activities were measured on 1 μM 5'-PsiMP (³²P-labeled 5'-PsiMP) and on 1 mM 5'-UMP.

Fig. 2. 5'-PsiMPase activity is due to an enzyme of the HAD family.

The activity of 5'-PsiMPase present in Fraction 70 of the Superdex-200 16/60 column was assayed in the presence of 1 μM 5'-PsiMP and 0 (*) or 1 (all other symbols) mM MgCl₂, as well as the indicated concentrations of vanadate (▽), CaCl₂ (◇) or EDTA (○).

Fig. 3. Expression of four mammalian phosphatases of the HAD family and their activity on 5'-PsiMP.

Human HDHD1, human PDXP, mouse PHOSPHO2, and mouse NT5C3L were expressed in *E. coli* as fusion proteins with a His-tag and purified by affinity chromatography. (A) SDS-PAGE gel of purified recombinant proteins (1 μg of protein per lane). (B) 5'-PsiMPase activity at 1 μM 5'-PsiMP.

Fig. 4. Effect of pseudouridine, N1-methylpseudouridine, adenosine and uridine on the activity of HDHD1.

The activity of HDHD1 was measured with 1 mM 5'-PsiMP (A) or 10 mM 3'-AMP (B) in the presence of the indicated concentrations of pseudouridine (■), N-1-methylpseudouridine (◆), uridine (▲), and adenosine (▼). Values shown are the means ± SEM for n = 3 determinations.

Fig. 5. Activity of pseudouridine-5'-phosphatase in cell extracts from male and female subjects and from patients with X-linked ichthyosis.

The activity was measured in triplicate in extracts of B-cell lymphoblasts (A) or human erythrocytes (B) through the release of [³²P]Pi from radiolabelled 5'-PsiMP; 2 μM 5'-PsiMP and 500 μM 5'-UMP were present in the assays. 5'-UMP was added to inhibit the non-specific activity of cytosolic 5'-UMPase on 5'-PsiMP and assay the phosphatase activity of HDHD1 more specifically. Values are the means ± SEM for the indicated number of subjects.

Fig. 6. Alignment of human HDHD1 with orthologous sequences and with FMN phosphatase from *Arabidopsis thaliana*. The sequences shown are *Homo sapiens* HDHD1 (gi|119619152|gb|EAW98746.1), *Bos taurus* HDHD1 (gi|114051249|ref|NP_001040060.1), their orthologue from *A. thaliana* (gi|26453252|dbj|BAC43699.1) and the first 232 residues of *A. thaliana* FMNPase/riboflavin kinase (gi|42567011|ref|NP_193878.2). The residues that are identical to those of human HDHD1 are indicated in bold. Asterisks indicate the conserved residues of the three conserved motifs of the HAD-family.

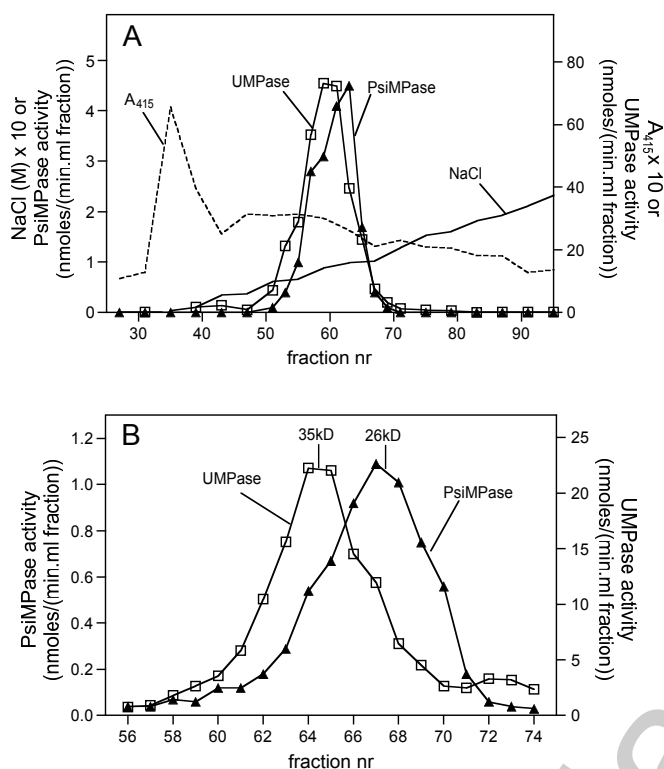


Fig. 1.

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20100174

Accepted Manuscript

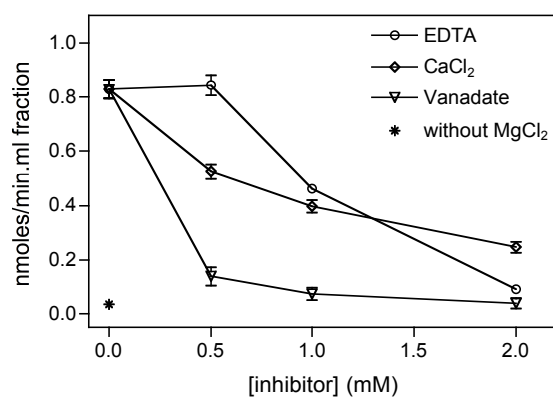


Fig. 2.

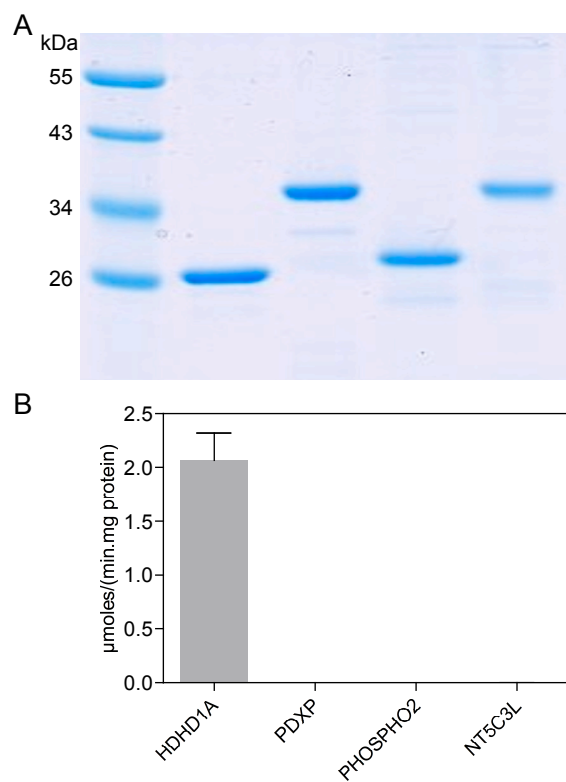


Fig. 3.

Accepted Manuscript

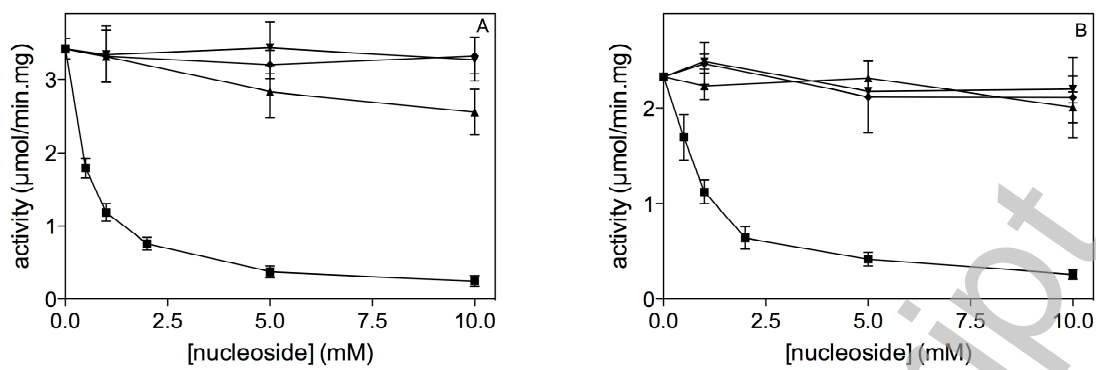


Fig.4.

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20100174

Accepted Manuscript

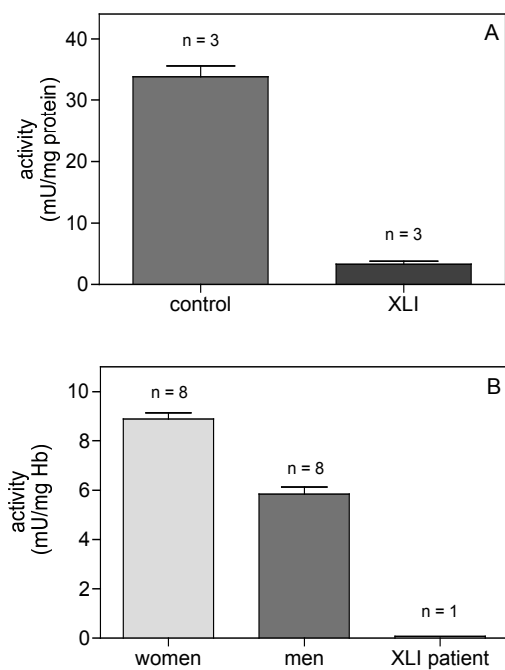


Fig. 5.

```

Hsapiens  MAAP-----POPVTHLIFDMDGLLLDTERLYSVVFQEICNR 36
Btaurus   MAAPVP-----LPRPVTHLLFDMDGLLLDTERLYSAVFEDICGR 39
Athaliana MSTPAAAVNATVTVTDAGRGSITHVIFDMDGLLLDTEKFYTEVQEKILAR 50
AtFMNPase MSMSNS-----LKKLSSCVLIDLDTLINTDGVVVDILRKYLCK 39
          * *

Hsapiens  YDKKYSWDVKSLVMGKKALEAAQIIIDVLQLP--MSKEELVEESQTKLKE 84
Btaurus   YGKKYSWDVKSLVMGKKALEAAQLIRDTLQLP--MSAEELVEVSQAKLKE 87
Athaliana YNKTFDWSLKAKMMGRKAIEAARLFVDESGISDSLAEDFIVERESMLQD 100
AtFMNPase YGKQWDGRESLKIVGKTPVEAATTIVEDYELP--CKVDEFNSEFYPLFSA 87

Hsapiens  VFPMAALMPGAEKLIHLRKHGIPFALATSSGSASFDMKTSRHKEFFSLF 134
Btaurus   VFPTAALMPGVEKLIHLRKHVDVPCAVATSSGTASFQLKTSRHQDFFGLF 137
Athaliana LFPTSDLMPGASRLRLRHLHGKGIPICIATGTHTRHFDLKTQRHRELFSLM 150
AtFMNPase QMDKIKSLPGANRLIRHLKCHGVPPVALASNSSRANIESKISYHEGWKECF 137
          *

Hsapiens  SHIVLGDDPEVQHKGKPDPIFLACAKRFSPPPA-MEKCLVFEDAPNGVEA 183
Btaurus   HHVVLGDDPEVRSGKPEPDIFLTCAARRFSPAPP-ANKCLVFEDAPNGVEA 186
Athaliana HHVVRGDDPEVKEGKPADGFLAASRRFEDGPVDPKVLVFEAPSGVQA 200
AtFMNPase SVIVGSD--EVSKGKPSPIFLEAAKRLKKDPA---DCLVIEDSVPGVMA 182
          * *

Hsapiens  ALAAGMQVVMVPDGNLSRDLTTKATLVLNSLQDFQPELFGGLPSYE----- 228
Btaurus   ALAAGMQVVMVPDGNLKPDLTTSKATLVLGSLQDFQPELFGGLPPYD----- 231
Athaliana AKNAGMNVIMVPDSRLDKSYCNVADQVLASLLDFKPEEWGLPSFQDSHN- 249
AtFMNPase GKAAGTKVIAVPSLKPQTHLYTSADEVINSLLDIRLEKWGLPPFQDWIEN 232

```

Fig. 6.