MOLECULAR IDENTIFICATION OF HYDROXYLYSINE KINASE AND OF THE AMMONIOPHOSPHOLYASES ACTING ON 5-PHOSPHOHYDROXY-L-LYSINE AND PHOSPHOETHANOLAMINE*

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Running head: Hydroxylysine and phosphoethanolamine metabolism

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Background: Vertebrate genomes encode AGXT2L1 and AGXT2L2, two related, pyridoxal-phosphate dependent enzymes of unknown function.

Results: Comparison with bacterial genomes suggested that AGXT2L1 and AGXT2L2 are ammoniophospholyases functionally related with AGPHD1. The three recombinant proteins were produced and studied.

Conclusion: AGXT2L1 is O-phosphoethanolamine phospholyase; AGXT2L2 is 5-phosphohydroxy-L-lysine phospholyase; AGPHD1 is 5-hydroxylysine kinase.

Significance: These molecular identifications will help understand some specific neurometabolic diseases.

Summary
The purpose of the present work was to identify the catalytic activity of AGXT2L1 and AGXT2L2, two closely related, putative pyridoxal-phosphate dependent enzymes encoded by vertebrate genomes. The existence of bacterial homologues (40-50 % identity with AGXT2L1 and AGXT2L2) forming bi- or tri-functional proteins with a putative kinase belonging to the family of aminoglycoside phosphotransferases suggested that AGXT2L1 and AGXT2L2 acted on phosphorylated and aminated compounds. Vertebrate genomes were found to encode a homologue (AGPHD1) of these putative bacterial kinases, which was therefore likely to phosphorylate an amino compound bearing a hydroxyl group. These and other considerations led us to hypothesize that AGPHD1 corresponded to 5-hydroxy-L-lysine kinase and that AGXT2L1 and AGXT2L2 catalyzed the pyridoxal-phosphate dependent breakdown of phosphoethanolamine and 5-phosphohydroxy-L-lysine. The three recombinant human proteins were produced and purified to homogeneity. AGPHD1 was indeed found to catalyze the GTP-dependent phosphorylation of 5-hydroxy-L-lysine. The phosphorylation product made by this enzyme was metabolized by AGXT2L2, which converted it to ammonia, inorganic phosphate and 2-aminoadipate semialdehyde. AGXT2L1 catalyzed a similar reaction on phosphoethanolamine, converting it to ammonia, inorganic phosphate and acetaldehyde. AGPHD1 and AGXT2L2 are likely to be the mutated enzymes in 5-hydroxylysinuria and 5-phosphohydroxylysinuria, respectively. The high level of expression of AGXT2L1 in human brain, as well as data in the literature linking AGXT2L1 to schizophrenia and bipolar disorders suggest that these diseases may involve a perturbation of brain phosphoethanolamine metabolism. AGXT2L1 and AGXT2L2, the
first ammoniophospholyases to be identified, belong to a family of aminotransferases acting on ω-amines.

INTRODUCTION
Pyridoxal-phosphate (PLP) dependent enzymes catalyze a series of reactions, mostly on amino compounds, that include the transfer of amine, the decarboxylation and racemization of amino acids, and β- and γ-elimination reactions on amino acids [1]. A recent review reports more than 50 human enzymes or putative enzymes using PLP as a cofactor [2]. Many of these enzymes have a known function, yet the reaction catalyzed by a few of them is still unknown. This is particularly the case for mammalian AGXT2L1 (AGXT2-like 1) and AGXT2L2 (AGXT2-like 2), two enzymes that share ≈36 % sequence identity with mammalian AGXT2 (alanine: glyoxylate aminotransferase 2). AGXT2 is a member of the aminotransferase III subfamily (as defined in the Pfam database [3]), which mostly comprises enzymes that catalyze transamination on ω-amines. It catalyzes the pyruvate-dependent conversion of D-β-aminoisobutyrate, a breakdown product of thymine, to trimethylamine-N-oxide (TMAO) [4]. Unlike AGXT2, AGXT2L1 and AGXT2L2 did not act as transaminases [5].

The purpose of the present work was to identify the function of AGXT2L1 and AGXT2L2. We show how bacterial homologues of these proteins, because of their association with a kinase of unknown function (homologous to a protein named AGHPD1), led us to hypothesize that AGXT2L1 and AGXT2L2 are ammoniophospholyases acting on phosphoethanolamine (PEtN) and (5R)-5-phosphohydroxy-L-lysine (5PHyl).

PEtN, a component and a precursor of phospholipids, is produced through phosphorylation of free ethanolamine by ethanolamine kinase [6, 7], through the breakdown of phosphatidylethanolamine by phospholipase C [8, 9] or through aldolytic cleavage of sphinganine-1-phosphate by sphinganine-1-phosphate lyase [10]. PEtN is metabolized by a PLP-dependent enzyme, of unknown molecular identity, that catalyzes the elimination of inorganic phosphate (Pi) and ammonia, leading to the formation of acetaldehyde [11]. Initially described in mammalian tissues, this activity appears to be present also in bacteria, such as Erwinia carotovora (now called Pectobacterium carotovorum var. atrosepticum) [12]. A distinct PLP-dependent ammoniophospholyase is involved in the breakdown of 5PHyl in mammalian tissues, leading to the formation of ammonia, 2-aminoacidopropionate semialdehyde (2-AASA) and Pi [13]. 5PHyl is produced through the phosphorylation of (5R)-5-hydroxy-L-lysine (5Hyl), a breakdown product of collagen, by a GTP-dependent kinase [14]. The molecular identity of the kinase and of the ammoniophospholyase that are involved in the metabolism of 5Hyl were unknown before we started this work. We describe here the database searches and the experiments that led us to provide the molecular identification of PEtN phosphohydrolase and of the two enzymes involved in 5Hyl metabolism in mammalian tissues.

EXPERIMENTAL PROCEDURES
Materials – (5R)-5-Hydroxy-L-lysine (5Hyl) and 5-hydroxy- DL-lysine were from Sigma, allysine ethylene acetal from Aldrich, and HisTrap HP columns from Qiagen. Optimase polymerase was from Transgenomics. pET expression plasmids were from Novagen. Yeast alcohol dehydrogenase, beef liver glutamate dehydrogenase, rabbit muscle pyruvate kinase and lactate dehydrogenase were from Roche.

Cloning, overexpression and purification of recombinant proteins - The open reading frames of human AGXT2L1, AGXT2L2 and AGPHD1 were PCR-amplified using human cDNA (brain for AGXT2L1 and kidney for AGXT2L2 and AGPHD1) as a template. The PCR fragments obtained in the presence of 1M betaine using the Optimase polymerase were purified, digested with the appropriate restriction enzymes (restriction site included in the primers) and ligated into an adequately restricted expression plasmid. For AGXT2L1 and AGXT2L2, the PCR fragments permitting the expression of fusion proteins with an N-terminal His6-tag were cloned in pET28a and those with a C-terminal His6-tag were cloned in pET24a after incubation with NdeI and NotI restriction enzymes. For AGPHD1, N- and C-terminal fusion proteins were expressed using pET28a plasmids harboring the PCR-fragments corresponding to AGPHD1 cloned in NdeI/NotI or NcoI/NotI restricted plasmids, respectively. When
proteins were fused with a C-terminal His$_6$-tag, the sequence of the primers used in the PCR reaction removed the natural STOP codon. Table 1 shows the sequence of the primers used in the PCR reactions. The ligated plasmids were amplified in *Escherichia coli* XL1 blue, the positive clones were checked by sequencing to rule out any PCR errors and finally transformed in *E. coli* BL21(DE3). All expression experiments were in 2 l flasks containing 500 ml culture media for 18 h (AGXT2L1 and AGXT2L2) or 72 h (AGPHD1) in the presence of 0.4 mM isopropyl ß-D-thiogalactoside. AGXT2L1 and AGXT2L2 with a N-terminal His$_6$-tag were expressed in M9 medium at 20°C. In the case of the more instable AGPHD1, we expressed the C-terminally tagged protein in M9 medium at 16°C.

Preparation of bacterial extracts (25 ml) and their centrifugation was as described previously [15] with two exceptions in the composition of the extraction buffer. (1) For the bacteria expressing AGXT2L1 the extraction buffer contained 1 mM phenylmethylsulphonyl fluoride to reduce proteolysis at the C-terminal end. (2) 0.3 M NaCl was added to all buffers during extraction and purification of AGPHD1 to prevent precipitation of this protein.

The supernatants (20 ml) were supplemented with 0.3 M NaCl, diluted 3-fold in buffer A (50 mM NaPO$_4$ pH 8.0, 0.3 M) and applied onto a 1 ml HisTrap HP column (Ni$^{2+}$ form) previously equilibrated with buffer A. The column was washed with buffer A containing 15 mM imidazole (≈ 25 ml) and the retained proteins were eluted with a 20 ml linear imidazole (0.015 – 0.3 M) gradient in buffer A. Protein elution with imidazole and the purity of the fractions was analysed by SDS-PAGE. All recombinant proteins showed the expected size (≈ 43 kDa for AGPHD1, 57 kDa for AGXT2L1 ≈ 51 kDa for AGXT2L2, including the His$_6$-tag). Three to four selected fractions were pooled, concentrated, desalted as previously described [16] and stored at -70°C in 25 mM Hepes, pH 7.1, containing 1 µg.ml$^{-1}$ leupeptin and antipain, 0.01, 0.1 or 0.3 M NaCl (for AGXT2L2, AGXT2L1 or AGPHD1, respectively) and 10% glycerol. Protein concentration was estimated by measuring A$_{280}^\text{nm}$ and assuming molar absorptivities of 31143 (AGXT2L1), 35090 (AGXT2L2), 43445 (AGPHD1) M$^{-1}$.cm$^{-1}$. From 1 l of culture we obtained on average 10 – 15 mg of pure protein.

Purified AGXT2L1 and AGXT2L2 where yellow, suggesting that they did indeed bind PLP. Since the position of the His$_6$-tag did not interfere with the kinetic properties that we investigated, we chose to report the results obtained for AGPHD1 and AGXT2L1 tagged at the C-terminal end and for AGXT2L2 tagged at the N-terminal end, based on a higher purification yield obtained for these fusion proteins.

Measurement of 5Hyl-kinase and PEtN-phosphorylase activities - The 5Hyl-kinase activity of AGPHD1 was assayed spectrophotometrically at 30°C using a pyruvate kinase/lactate dehydrogenase-coupled assay. The assay mixture (0.6 ml) contained 25 mM Heps, pH 7.1, 0.15 mM NADH, 0.25 mM phosphoenolpyruvate, 1 mM dithiothreitol, 25 mM KCl, 0.2 mM GTP-Mg or 2 mM ATP-Mg, 5 mM MgCl$_2$, 8 µg lactate dehydrogenase and pyruvate kinase, and the indicated concentrations of 5Hyl. The physiological form of hydroxylysine found in collagen) and ≈ 2.5 µg.ml$^{-1}$ of purified recombinant human AGPHD1. When necessary other substrates were tested as indicated in the Results section.

PEtN-phosphorylase activity of AGXT2L1 was routinely assayed spectrophotometrically by monitoring the production of acetaldehyde. The activity was measured at 30°C in a final volume of 0.6 ml containing 50 mM triethanolamine, pH 7.4, 0.15 mM NADH, 5 µM PLP, 1 mM EGTA, 0.5 mg.ml$^{-1}$ bovine serum albumin, 0.2 mg.ml$^{-1}$ desalted yeast alcohol dehydrogenase and 28 µg.ml$^{-1}$ purified recombinant human AGXT2L1.

Synthesis and purification of $[^{32}P]$ and $[^{32}P]5PHyl$ - $[^{32}P]5PHyl$ was produced by the phosphorylation of 5Hyl with purified recombinant human AGPHD1. Practically, 0.1 ml of 25 mM Hepes pH 7.5 containing 5 mM MgCl$_2$, 25 mM KCl, 1 mM DTT, 0.5 mM 5Hyl, 5 µM ATP-Mg, and 1.5 x 10$^8$ cpm [$^{32}$P]ATP was incubated at 30°C in the presence of 5 µg of AGPHD1. After 30 min the reaction was stopped (5 min at 80°C) and $[^{32}P]5PHyl$ was separated from other radioactive compounds present in the reaction mixture ($[^{32}P]$Pi and [$^{32}$P]ATP) using anion exchange chromatography. At neutral pH, the resins
retained negatively charged molecules (Pi and ATP) while 5P\(\text{Hyl}\) was eluted in the washing fractions. The reaction mixture was diluted 10-fold in buffer A (10 mM Hepes pH 7.5) and loaded onto a 1 ml DOWEX AG1X8 equilibrated in the same buffer and washed with 9 ml buffer A to recover radiolabelled 5P\(\text{Hyl}\) (which contained 73% of total radioactivity). The non-radioactive 5P\(\text{Hyl}\) was prepared in a similar way by incubating 1 ml of 50 mM Hepes, pH 7.5, 5 mM MgCl\(_2\), 25 mM KCl, 1 mM DTT, 12 mM 5\(\text{Hyl}\), 14 mM ATP-Mg and 40 \(\mu\)g of AGPHD1. After 3.5 hours the reaction was stopped as above, diluted 3-fold in 10 mM Tris pH 8.0 and chromatographed on a 3 ml DOWEX AG1X8 column, which was then washed with 3 ml of the same buffer. Assays of Pi [17] before and after hydrolysis with alkaline phosphatase indicated that 5P\(\text{Hyl}\) was present in the flow-through and wash fractions, and that it was separated from Pi. The yield of this preparation was close to 100 % compared to the initial 5Hyl.

Radiochemical assay of 5P\(\text{Hyl}\)-phosphohexose - This assay measures the \([^{32}\text{P}]\text{Pi}\) production from \([^{32}\text{P}]\text{P}\text{Hyl}\). The reaction mixture (50 \(\mu\)l) contained 4.5 \(\mu\)g of recombinant human AGXT2L2 (or AGXT2L1) in 20 mM Hepes, pH 7.1, containing 5 \(\mu\)M PLP, 0.5 \(\mu\)g.ml\(^{-1}\) bovine serum albumin, 0.15 \(\times\) \(10^6\) cpm \([^{32}\text{P}]\text{P}\text{Hyl}\) and different concentrations of unlabelled 5P\(\text{Hyl}\). After 10 min at 30°C, the reaction was stopped (5 min at 80°C) and the samples centrifuged at 10000 \(\times\) g for 10 min. \([^{32}\text{P}]\text{Pi}\) was isolated as a phosphomolybdic complex [18] and its radioactivity was counted.

Assay of the reaction products of AGXT2L1 and AGXT2L2 - AGXT2L1 (0.14 mg.ml\(^{-1}\)) was incubated at 30°C in a reaction mixture (0.5 ml) containing 5 mM P\(\text{EtN}\), 50 mM triethanolamine, pH 7.4, 10 \(\mu\)M PLP, 1 mM EGTA, and 0.5 mg.ml\(^{-1}\) bovine serum albumin. AGXT2L2 (0.08 mg.ml\(^{-1}\)) was incubated in 0.05 ml of a mixture comprising 1 mM 5P\(\text{Hyl}\), 25 mM Hepes, pH 7.1, 10 \(\mu\)M PLP, and 0.5 mg.ml\(^{-1}\) bovine serum albumin. In both cases, the reaction was stopped after the indicated time by heating for 5 min at 80°C. The samples were centrifuged for 10 min at 10000 \(\times\) g and 4°C and the reaction products measured. Ammonia was assayed spectrophotometrically [19] and Pi was determined in a microplate assay [17].

Acetaldehyde was assayed spectrophotometrically by adding yeast alcohol dehydrogenase (final concentration 0.7 mg.ml\(^{-1}\)) to a mixture (0.6 ml) containing 50 mM Hepes, pH 7.0, 0.5 mg.ml\(^{-1}\) bovine serum albumin, 0.15 mM NADH, and 30 \(\mu\)l of the sample.

The 2-aminoadipate semialdehyde (2-AASA) formed in the AGXT2L2 reaction was measured as its dihydroquinazolinium derivative [20] by reverse-phase HPLC with a diode-array detector at 465 nm. Ten \(\mu\)l of the reaction mixture was mixed with 10 \(\mu\)l of 8 mM o-aminobenzaldehyde, incubated for 120 min at 40°C and loaded on the column. Quantification was achieved using a standard of allysine ethylene acetal after its hydrolysis in acid conditions.

Assay of the aminotransferase activity of AGXT2L1 and AGXT2L2 - When pyruvate was tested as an amine acceptor, \(\approx\) 20 \(\mu\)g of AGXT2L1 or AGXT2L2 was incubated at 30°C in 0.5 ml of a reaction mixture containing 25 mM Hepes, pH 7.2, 25 mM KCl, 1 mM dithiothreitol, 0.5 mg.ml\(^{-1}\) bovine serum albumin, 5 \(\mu\)M PLP, 0.02-0.5 mM pyruvate as well as 0, 0.1 or 1 mM \(\beta\)-alanine or \(\beta\)-aminoisobutyrate. The reaction was stopped after 30 min by addition of HClO\(_4\) (5% final concentration). The extract was neutralized with 3M K\(_2\)CO\(_3\) and centrifuged (5 min at 4°C at 10000 x g). The remaining pyruvate was assayed in the supernatant by computing the change in absorbance at 340 nm following the addition of rabbit muscle lactate dehydrogenase (5 \(\mu\)g) to a 1 ml cuvette containing 20 mM imidazole buffer pH 7.2, 0.15 mM NADH and 0.1 – 0.4 ml of sample. A glutamate dehydrogenase-coupled assay [21] was used to test if AGXT2L1 or AGXT2L2 catalyzed an aminotransferase reaction between \(\beta\)-alanine, \(\beta\)-aminoisobutyrate (0.1 or 1 mM), L-lysine, L-ornithine or L-aminobutyrate (1 or 25 mM) and \(\alpha\)-ketoglutarate (0.1 or 2 mM).

Calculations - \(V_{\text{max}}\) and \(K_m\) for the enzymatic activities studied were calculated with Prism 4.0 (GraphPad software) using a nonlinear regression.

Phylogenetic analysis - The phylogenetic tree was inferred by maximum likelihood (PhyML, version 3, [22]) for parameter estimation and Bayesian analysis (MrBayES version 3.1.2, [23]). The Whelan And Goldman (WAG) model [24] of evolution
and a gamma rate distribution parameter of 1.91, with 4 substitution rate categories fitted best the data. To estimate Bayesian posterior probabilities, Markov Chain Monte Carlo (MCMC) chains were run for 130,000 generations until convergence and sampled every 100 generations (burn-in: 25,000 generations).

RESULTS

Database searches – Bi-functional or multi-functional enzymes most often comprise domains that catalyze physiologically related reactions. The identification of a function of an orphan enzyme may therefore be greatly facilitated by the identification of homologues that belong to bi- or multi-functional proteins, if one of the components of these composite proteins has a known function. The existence of close bacterial homologues of an orphan enzyme may also be of great help, since the analysis of operons may point to associations with proteins of known function.

Blast searches performed with AGXT2L1 or AGXT2L2, (which share 67% of identical residues) disclosed the existence of bacterial proteins showing 40-50% identity with either AGXT2L1 or AGXT2L2. This degree of identity is higher than that observed when AGXT2L1 or AGXT2L2 is compared with AGXT2s (≈36% identity), their closest mammalian homologues, suggesting that the bacterial homologues were likely to have a function similar to that of AGXT2L1 or AGXT2L2. The close relationship between AGXT2L1 and AGXT2L2 with bacterial sequences is illustrated in the evolutionary tree represented in Figure 1. This tree comprises members of five vertebrate proteins belonging to the aminotransferase III family, defined as: AGXT2, AGXT2L1, AGXT2L2, ornithine amino transferase, γ-aminobutyrate aminotransferase in addition to a few selected bacterial homologues of AGXT2L1 and AGXT2L2. The bacterial homologues (bacterial AGXT2L1/2) stem off from the branch leading to AGXT2L1 and AGXT2L2 after the separation between AGXT2 and AGXT2L1 or AGXT2L2 took place.

Remarkably, a significant fraction of the bacterial homologues were putative tri-functional proteins comprising an aminoglycoside phosphotransferase domain and a peptidase M23 domain in addition to the aminotransferase class III domain (as defined in the Pfam database; [3]). Other genomes contained shorter bi-functional fusion proteins without the M23 peptidase domain. Additional “bacterial AGXT2L1/2” comprised a single aminotransferase class III domain, but their encoding gene was often close to a gene encoding a protein homologous to the aminoglycoside phosphotransferase found in the putative tri-functional or bi-functional proteins (Figure 2). This observation strengthened the physiological significance of the association of the putative phosphotransferase (i.e., kinase) and the “bacterial AGXT2L1/2”.

It should be noted that these aminoglycoside phosphotransferases are very distant from bona fide aminoglycoside phosphotransferases, which serve to phosphorylate and inactivate aminoglycoside antibiotics. Interestingly, Blast searches using these putative aminoglycoside phosphotransferases indicated that mammalian genomes encoded a homologous protein, named AGPHD1 (aminoglycoside phosphotransferase domain 1), that shared about 26% sequence identity with its bacterial homologues (Figure 2). These findings suggested that mammalian AGPHD1 was likely to catalyze a reaction that was physiologically connected with the reaction catalyzed by AGXT2L1, AGXT2L2 or by both of them.

According to the Pfam database [24], mammalian AGPHD1 and the homologous bacterial proteins belong to the APH (aminoglycoside phosphotransferase) family, which comprises enzymes that phosphorylate aminoglycosides on their 3rd carbon. Yet, the low degree of identity between human AGPHD1 and its bacterial homologues with authentic aminoglycoside phosphotransferases, suggested that AGPHD1 was likely to catalyze the phosphorylation of a substrate different from antibiotics. A common feature of the substrates of the enzymes that belong to the APH family and of the related homoserine kinase and fructosamine kinase families [see Pfam database - Family: APH (PF01636)] is that they comprise an amino group close to the phosphorylated carbon. Furthermore, our hypothesis that AGPHD1 was functionally linked to enzymes using PLP as a cofactor (i.e. AGXT2L1 or AGXT2L2), independently suggested that it catalyzed the phosphorylation of an aminated substrate. Consequently, a
literature and database (Exasy-Enzymes; Brenda) search for mammalian enzymes that catalyze the phosphorylation of an aminated substrate that had not yet been molecularly identified, led us to consider that AGPHD1 could encode 5Hyl-kinase. Interestingly, the BioGPS database shows that the expression of the AGHD1 mRNA in mouse tissues is restricted to liver and kidneys, which is consistent with the known tissue distribution of the 5Hyl-kinase activity [25] in support of our hypothesis that AGPHD1 encoded 5Hyl-kinase.

If this hypothesis was correct, we expected that either AGXT2L1 or AGXT2L2 would correspond to the PLP-dependent enzyme that catalyzes the elimination reaction converting 5PHyl to 2-AASA, ammonia and Pi [13]. The other of this pair of enzymes would catalyze a similar reaction on PEtN, a close structural analogue of C5-C6 of 5PHyl [11]. This role of AGXT2L1 and AGXT2L2 is consistent with these proteins being predicted to be cytosolic, which is the localization of both ammoniophospholyases. In the following sections we provide evidence for these assignments.

Characterization of recombinant human AGPHD1 as a 5Hyl-kinase - Using a spectrophotometric assay monitoring the formation of ADP or GDP, we found that homogeneous recombinant human AGPHD1 with a C-terminal His6-tag catalyzed the phosphorylation of 5Hyl using either ATP-Mg or GDP-Mg as phosphoryl donors. The substrate saturation curves for 5Hyl were hyperbolic as determined with double reciprocal plots (not shown). AGPDH1 displayed a much higher affinity for GTP-Mg than for ATP-Mg, the $K_m$ being more than 200-fold lower for the former than for the latter nucleotide (Table 2). The $V_{max}$ was, however, about 10-fold higher with ATP-Mg than with GTP-Mg, resulting in a catalytic efficiency that was $\approx$ 22-fold higher with GTP-Mg than with ATP-Mg. The $K_m$ for 5Hyl was about 35-fold lower when a near-physiological concentration of GTP-Mg (0.2 mM) was used instead of ATP-Mg (2 mM). No evidence was observed for phosphorylation of ethanolamine, hydroxyproline, homoserine, serine or choline (all tested at 1 or 5 mM, in the presence of 2 mM ATP-Mg). To check whether the kinase was specific for the physiological form of hydroxylysine, we compared the rate of phosphorylation of 5Hyl with that of a 4-fold higher concentration of a mixture containing all four 5-hydroxy-DL-lysine isomers. In the presence of 1 mM ATP-Mg, the activity of AGPHD1 measured with 50 and 100 µM 5-hydroxy-DL-lysine was $\approx$1.3 and 1.15-fold higher than that measured with 12.5 and 25 µM 5Hyl, respectively. These findings suggested that the best substrate is the physiological form but that human AGPHD1 can also phosphorylate at a lower rate at least one other isomer.

The availability of purified 5Hyl-kinase allowed us to prepare radioactive and non-radioactive 5PHyl as described in Experimental Procedures. When chromatographed on an anion-exchange resin (DOWEX-AG1X8) in the presence of 10 mM Hepes at pH 7.5, the reaction product behaved as expected for a compound with a partial negative charge. The purified 5PHyl was used to characterize the 5Hyl-phospholyase activity of AGXT2L2 (see below).

PEtN-phospholyase activity of AGXT2L1 - We began by determining the PEtN-phospholyase activity of purified recombinant human AGXT2L1 and AGXT2L2 by measuring spectrophotometrically the production of acetaldehyde in the presence of 0.5 mM PEtN and 10 µM PLP. AGXT2L1 catalyzed this reaction while AGXT2L2 was inactive. At pH 7.4, the $K_m$ of AGXT2L1 for PEtN was 680 ± 85 µM and its $V_{max}$ was 1.46 ± 0.06 µmol.min⁻¹.mg⁻¹ protein. We also assessed the formation of the other two reaction products, ammonia and Pi. Figure 3 shows that AGXT2L1 indeed catalyzed the formation of stoichiometric amounts of acetaldehyde, ammonia and Pi. In these experiments, the reaction was not linear with time, but this was most likely due to inhibition by Pi, a known inhibitor of rabbit PEtN-phospholyase. Inhibition was confirmed in the case of human AGXT2L1: Pi increased the apparent $K_m$, but did not change the $V_{max}$, consistent with a competitive inhibition. The calculated $K_i$ was 1.2 mM (results not shown), which probably explains the lack of linearity with time observed in the reaction shown in Figure 3. When PLP was omitted from the reaction mixture, the activity of AGXT2L1 measured with 0.5 mM substrate was reduced to less than 10 % of the activity observed in the presence of 10 µM PLP, consistent with PLP being a cofactor of this enzyme. Neither
ethanol (20 or 80 mM), nor phosphoserine (0.2, 1 or 5 mM) affected AGXT2L1 activity in the presence of 1 or 5 mM PEn.

**5PHyl-phospholyase activity of AGXT2L2** - The 5PHyl-phospholyase activity of recombinant human AGXT2L2 and AGXT2L1 was tested through the production of \[^{32}P\]Pi from \[^{32}P\]5PHyl. AGXT2L2 catalyzed this reaction while AGXT2L1 was inactive, consistent with AGXT2L1 being the PEn-phospholyase. Fig. 4A shows that after 30 min incubation at 30°C in the presence of 1 mM 5PHyl, AGXT2L2 had formed stoichiometric amounts (≈ 0.5 mM) of Pi, ammonia and 2-AASA. The saturation curve of AGXT2L2 for 5PHyl (Fig. 4B) suggested that the enzyme was inhibited by excess substrate. A \(K_m\) for 5PHyl of 16.9 ± 4.5 \(\mu\)M and a \(V_{max} = 256 ± 15\) nmol.min\(^{-1}\).mg\(^{-1}\) protein were computed from the linear portion of double reciprocal plots. In the presence of 0.5 mM 5PHyl, 2, 5 and 10 mM Pi increasingly inhibited the production of ammonia by AGXT2L2. At the highest concentration of Pi, the activity was reduced by 50% (not shown).

**Absence of aminotransferase activity of AGXT2L1 and AGXT2L2** - We also tested the potential aminotransferase activity of purified recombinant AGXT2L1 and AGXT2L2 (with either a N- or a C-terminal His\(_6\)-tag) by measuring glutamate formation in incubations containing \(\alpha\)-ketoglutarate (0.1 or 2 mM) as amine acceptor together with \(\beta\)-alanine, \(\beta\)-aminoisobutyrate (0.1 or 1 mM), L-lysine, L-ornithine or \(\gamma\)-aminobutyrate (1 or 25 mM) as amine donor. We similarly measured pyruvate disappearance in incubations containing pyruvate (0.02, 0.1 or 0.5 mM) as amine acceptor together with \(\beta\)-alanine or \(\beta\)-aminoisobutyrate (0.1 or 1 mM). No aminotransferase activity of AGXT2L1 or AGXT2L2 could be detected in these assays.

**DISCUSSION**

5Hyl-kinase is AGPHD1 – This identification is supported by the finding that the catalytic activity and the kinetic properties of human recombinant AGPHD1 perfectly agree with the known properties of rat liver 5Hyl-kinase [14]. Furthermore, the data on the tissue distribution of the AGPHD1 mRNA (BioGPS database) fit with the knowledge that 5Hyl-kinase activity is primarily present in liver and kidneys. This distribution suggests that 5Hyl-kinase plays a major role in degrading 5Hyl derived from food. Its presence at lower levels in other tissues indicates that it participates also in the catabolism of 5Hyl derived from local collagen breakdown.

The identification of the function of AGPHD1 will allow one to determine if hydroxylysinuria and hydroxylysinemia [26, 27, 28, 29] are caused by mutations in its gene. Children and adults with this metabolic abnormality have mental and/or neurological defects, but the clinical picture is not consistent, making it unclear whether the clinical symptoms are really caused by the defect in 5Hyl metabolism. Knowledge of the identity of the 5Hyl-kinase gene will help resolve this question, e.g. by identifying additional human subjects with a defect in this enzyme through high-throughput genome sequencing approaches.

**AGXT2L2 is 5PHyl-phospholyase** - Here also the identification rests on the demonstration that recombinant AGXT2L2 catalyses the same, PLP-dependent reaction as 5PHyl-phospholyase [13], with similar kinetic properties and tissue distribution [25]. 5PHyl-phospholyase is a cytosolic enzyme, which agrees with the predictions made from the sequence of AGXT2L2 both with TargetP [30] and Psort II [31]. By contrast, 2-AASA synthase, an enzyme involved in the breakdown of lysine via saccharopine (see Fig. 5), is mitochondrial. Thus, 2-AASA is formed both in the cytosol and in mitochondria. Consistent with this, ALDH7A1 [32, 33], the aldehyde dehydrogenase that oxidizes 2-AASA, exists as two isoforms, one mitochondrial and the other cytosolic, which result from the use of alternative in-frame translation initiation codons.

Mutations in the gene encoding AGXT2L2 should give rise to increased urinary excretion of 5PHyl. This condition has only been described in two unrelated patients, both with neurological abnormalities [34]. The observation that 5PHyl is a normal constituent of adult brain [35] suggests that this compound may accumulate in this tissue and possibly have detrimental effects on the nervous system if its metabolizing enzyme is deficient. The molecular identification of 5PHyl-phospholyase will help expand the number of observations of a defect in this enzyme and improve our understanding of the pathophysiological mechanisms.
AGXT2L1 is PEtN-phospholyase – This identification is based on the same arguments as for the two other enzymes. PEtN-phospholyase activity is mainly present in the liver and at lower levels in the kidneys of mammals [36], which is in agreement with the data for the expression of mouse AGXT2L1 (BioGPS database). Unlike AGXT2, which is a mitochondrial enzyme, AGXT2L1 is predicted to be in the cytosol, which is also consistent with the subcellular localization observed for PEtN-phospholyase.

PEtN- and 5PHyL-phospholyases catalyze identical reactions on substrates with similar structures (Figure 5) and it is therefore not surprising that their sequences are closely related. Remarkably AGXT2L1 has a C-terminal extension of ∼50 residues compared to AGXT2L2. This extension is possibly involved in the regulation of AGXT2L1, either by undergoing a posttranslational modification or by forming a (hypothetical) allosteric site. PEtN-phospholyase catalyses indeed a critical reaction, which should be regulated in such a way that PEtN, a precursor for phosphatidylethanolamine [37], is maintained at a constant level, about 1 mM in brain [38, 39]. Changing this concentration may lead to perturbations in the metabolism of phosphatidylethanolamine, as indicated by the finding that overexpression of mammalian ethanolamine kinase (EKI1) in COS-7 cells increased the intracellular levels of both PEtN and phosphatidylethanolamine [6].

AGXT2L1 and disease - The high level of expression of AGXT2L1 in human brain (BioGPS and the NCBI/GEO profile databases) suggests that this enzyme plays an important role in the metabolism of PEtN and in the control of its concentration in the human CNS. It is therefore likely that defects in this enzyme will lead to neurological perturbations.

Phosphoethanolaminuria is often associated with mutations in the gene encoding tissue-non-specific alkaline phosphatase (TNSAP) [40]. This enzyme is bound to the cell membrane and cleaves extracellular substrates such as PLP, inorganic pyrophosphate and PEtN. Thus, AGXT2L1 deficiency is likely to lead to ethanolaminuria rather than phosphoethanolaminuria. Ethanolaminuria is likely to be caused also by ethanolamine kinase deficiency. There are few reports of ethanolaminuria [41, 42, 43, 44] and its significance is not known. The variability in the associated symptoms (often including neurological symptoms) and in the amount of ethanolamine excreted in urine suggests that different genetic defects may be involved.

Besides this, several studies suggest that AGXT2L1 may be involved in psychiatric disorders. AGXT2L1 was reported to be among the most upregulated brain-specific genes in bipolar disorder and schizophrenia [45]. AGXT2L1 was downregulated in suicide completers [46] and in 72% of Major Depressive Disorder (MDD) - suicide cases compared with age-matched controls [47]. A genome-wide association analysis indicated the association of schizophrenia with SNPs in the region of the COL25A1 gene, near the AGXT2L1 gene, on chromosome 4q25 [48]. AGXT2L1 was the most upregulated gene in a genome-wide expression study following two weeks of lithium administration to mice [49]. With the identification of AGXT2L1 as the PEtN catabolic enzyme, these findings suggest that the pathophysiology of schizophrenia and bipolar disorder may involve a perturbation in PEtN metabolism. Although it is difficult to formulate a coherent hypothesis at this stage, this conclusion is consistent with the finding that the PEtN and glycerophosphorylethanolamine levels, as determined by 31P- Magnetic Resonance Spectroscopy, are increased in the brain of chronic, medicated schizophrenic compared to matched controls [39].

Function of the bacterial homologues of vertebrate AGPHD1 and AGXT2L1/2 - The role of the bacterial homologues of AGXT2L1/2 and AGPHD1 is unclear at this stage. Erwinia carotovora (Pectobacterium atrosepticum in Figure 2) has two different loci comprising each an AGXT2L1/2 homologue and an AGPHD1 homologue. It is likely that one or both of these pairs of enzymes are involved in the conversion of ethanolamine to acetaldehyde that has been reported previously [12]. However, the fact that ethanolamine metabolism did not proceed further than its conversion to acetaldehyde due to the lack of acetaldehyde dehydrogenase suggests that the physiological function of these enzymes in this bacterium must be in the metabolism of a compound different from ethanolamine. The absence of an aminoadipate semialdehyde dehydrogenase orthologue from the E. carotovora genome (unpublished data).
observations) argues against 5Hyl being the physiological substrate.

A candidate that needs to be taken into consideration, at least in some bacteria, is 2-hydroxyputrescine, a major polyamine in some prokaryotes [51]. As this compound has a hydroxyl group that neighbors an amine, it may be metabolized by phosphorylation followed by ammoniophospholysis, which would lead to the formation of aminobutyraldehyde. Interestingly, the genome of *Conexibacter woesei* DSM 14684 comprises a gene encoding a ‘trifunctional protein’ next to a gene encoding a putative 4-aminobutyraldehyde dehydrogenase (unpublished observations). Furthermore, in two of the genomes shown in Figure 2, the AGXT2L1/2 and AGPHD1 bacterial homologues are encoded by genes that are close to operons involved in polyamine transport. These enzymes could therefore be involved in the metabolism of 2-hydroxyputrescine in prokaryotes.

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FOOTNOTES

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4The abbreviations used are: 2-AASA, 2-aminoacidopropyl semialdehyde; APH, aminoglycoside phosphotransferase; 5Hyl, (5R)-5-hydroxy-L-lysine; PEtN, phosphoethanolamine; 5PHyl, (5R)-5-phosphohydroxy-L-lysine; Pi, inorganic phosphate; PLP, Pyridoxal-phosphate.
FIGURE LEGENDS

FIGURE 1. Evolutionary tree of AGXT2L1 and AGXT2L2 and related sequences
The phylogenetic tree was inferred from the aligned protein sequences using Bayesian analysis (MrBayes) methods. The horizontal bar represents 50 substitutions per 100 amino-acid residues. The following sequences were used to construct the tree: AGXT2 (Alanine-glyoxylate aminotransferase 2) from Homo sapiens (NP_114106), Mus musculus (Q3UEG6) and Monodelphis domestica (XP_001373137); AGXT2L1 (AGXT2 - like 1) from Homo sapiens (NP_112569), Mus musculus (NP_082183) and Monodelphis domestica (XP_001364959); AGXT2L2 (AGXT2 - L2) from Homo sapiens (NP_699204), Mus musculus (EDL33664) and Ornithorhynchus anatinus (XP_001518367); GABAT (γ-aminobutyrate aminotransferase) from Homo sapiens (NP_065737), Mus musculus (NP_766549) and Monodelphis domestica (XP_001373137); OAT (ornithine aminotransferase) from Homo sapiens (NP_000265), Mus musculus (BAE26718) and Monodelphis domestica (XP_001363028); bacterial proteins from Flavobacterium psychrophilum JIP02/86 (YP_001295781), Pectobacterium atrosepticum SCRI1043 (YP_050155), Polaromonas sp. JS666 (YP_547276) and two proteins from Sinorhizobium meliloti 1021 (NP_437638, protein 1 and NP_436219, protein 2).

FIGURE 2. Association of bacterial homologues of AGXT2L1/L2 with putative aminoglycoside phosphotransferases in bacterial genomes.
The figure shows representative examples of the genomic region comprising the bacterial homologues of AGXT2L1/L2 shown in Figure 1. In some cases, the homologue is a bifunctional protein (YP_001295781) fused with a putative phosphotransferase (as seen in Flavobacterium psychrophilum JIP02/86 contig NC_009613.1) or a trifunctional protein (NP_437638) with an additional putative peptidase of the M23/M37 family (‘region 1’ of the genome of Sinorhizobium meliloti 1021 contig NC_003078). In other cases, it is encoded by a gene neighboring another gene encoding a protein of the aminoglycoside phosphotransferase family (YP_050155 and YP_050154 in Pectobacterium atrosepticum SCRI1043, contig NC_004547.2; YP_547276 and YP_547277 in Polaromonas sp. JS666, contig NC_007948.1; NP_436219 and NP_436218 in region 2 of the genome of Sinorhizobium meliloti 1021 contig NC_003037.1). Note in some cases the proximity to genes putatively involved in polyamine metabolism (PotA, PotB, PotC and PotD, which are different components of the spermidine/putrescine transporter as well as acetyl-polyamine deacetylase).

FIGURE 3. P*EtN phospholyase activity of recombinant human AGXT2L1.
AGXT2L1 was incubated with 5 mM P*EtN for 10 and 20 min at 30°C, before stopping the reaction. Acetaldehyde (CH₃CHO), ammonia (NH₃) and inorganic phosphate (Pi) were measured in the incubation medium.

FIGURE 4. 5*Hyl phospholyase activity of recombinant human AGXT2L2.
(A) AGXT2L2 was incubated with 1 mM 5*Hyl for 30 min at 30°C, as described in the experimental procedures. After stopping the reaction, 2-aminoadipate semialdehyde (2-AASA), ammonia (NH₃) and inorganic phosphate (Pi) were measured in the incubation medium. (B) The saturation curve of AGXT2L2 was obtained by measuring the production of [³²P]Pi from [³²P]5*Hyl in the presence of variable concentrations of unlabelled 5*Hyl.

FIGURE 5. Contribution of AGPHD1, AGXT2L2 and AGXT2L1 to the metabolism of 5*Hyl and ethanolamine in vertebrates.
Table 1: Sequence of the primers used for the amplification and cloning of human AGXT2L1, AGXT2L2 and AGPHD1 in its expression plasmid.

| Primer            | Sequence                     | Restriction site | Plasmid         | Protein expressed |
|-------------------|------------------------------|------------------|-----------------|-------------------|
| hAGXT2L1-NdeI     | 5’ cccatATGTGCGAG-CTGTCAGTAAG 3’ | NdeI             | pET24a or pET28a | N- and C-tagged 6xHisAGXT2L1 |
| bis28hAGXT2L1-NotI| 5’ ggggggggcTCTAGTCTTGGCCCTTACTG 3’ | NotI             | pET28a         | N-tagged 6xHisAGXT2L1 |
| bis24hAGXT2L1-NotI| 5’ aaggggggcTCTAGTGAC-CTCTTACTGAGC 3’ | NotI             | pET24a         | C-tagged 6xHisAGXT2L1 |
| S-AGXT2L1 (1)     | 5’ GCAGACTCATCCA-GTGCTTA 3’ |                  |                |                   |
| AS-AGXT2L1 (1)    | 5’ GCTGGAGGAATTA-TTTGTCGG 3’ |                  |                |                   |
| hAGXT2L2-NdeI     | 5’ cccatATGGCGCGCA-GACCAGCGC 3’ | NdeI             | pET24a or pET28a | N- and C-tagged 6xHisAGXT2L2 |
| 28hAGXT2L2-NotI   | 5’ ggggggggcTTAGGCGTGGGCCTCAGC 3’ | NotI             | pET28a         | N-tagged 6xHisAGXT2L2 |
| 24hAGXT2L2-NotI   | 5’ ggggggggcGGGCTGGAGCCTCAGCGT 3’ | NotI             | pET24a         | C-tagged 6xHisAGXT2L2 |
| hAGPHD1-NdeI      | 5’ gacccatATGCAAGTCGGAAACTCATACGC 3’ | NdeI             | pET28a         | N-tagged 6xHisAGPHD1 |
| bishAGPHD1-NotI   | 5’ ggggggggcTCACATGGAGATCCCCAGATTCC 3’ | NotI             |                |                   |
| hAGPHD1-NcoI (2)  | 5’ gacccATGGCAAGTGGAAACTCATACGCAG 3’ | NcoI             | pET28a         | C-tagged 6xHisAGPHD1 |
| bishAGPHD1-NotI (without stop) | 5’ ggggggggcCATGGAGACTTCAGATTGAC 3’ | NotI             |                |                   |

The nucleotides that correspond to the coding sequences are in capital letters and the restriction sites added are in small letters and underlined.

(1) Primers corresponding to internal sequences of the AGXT2L1 cDNA that were combined with external ones to amplify the 5’ and the 3’ regions of the AGXT2L1 coding sequence. The overlapping PCR products were then used to obtain the full length coding sequence using the corresponding external primers.

(2) In order to create a NcoI restriction site we replaced a T in the second codon by a G (shown in bold). In addition to the C-terminal His$_6$-tag, this results in Ser2Ala replacement in the expressed recombinant protein.
Table 2: Kinetic properties of recombinant human AGPHD1.

The results are the means of three determinations ± S.E.

| Substrates                        | $V_{max}$ µmol.min$^{-1}$.mg$^{-1}$ | $K_m$ µM | $k_{cat}/K_m$ sec$^{-1}$.M$^{-1}$ |
|-----------------------------------|-----------------------------------|--------|---------------------------------|
| 5R-5-hydroxy-L-lysine [0 – 1 mM]  |                                   |        |                                 |
| + 2 mM ATP                        | 4.95 ± 0.34                       | 280 ± 40 | 12 x 10$^3$                     |
| + 0.2 mM GTP                      | 0.49 ± 0.01                       | 7.6 ± 0.1 | 45 x 10$^3$                     |
| ATP [0 – 2.5 mM]                  |                                   |        |                                 |
| + 0.5 mM 5R-5-hydroxy-L-lysine    | 5.28 ± 0.20                       | 810 ± 70 | 5 x 10$^3$                      |
| GTP [0 – 25 µM]                   |                                   |        |                                 |
| + 0.5 mM 5R-5-hydroxy-L-lysine    | 0.57 ± 0.03                       | 3.5 ± 1.1 | 110 x 10$^3$                   |
Figure 1 - Veiga-da-Cunha et al.
Figure 2 - Veiga-da-Cunha et al.

**Human homologue:**

**AGPHD1**

- M23-domain peptidase

**AGXT2L1/L2**

- Identity: 29%
- Identity: 26% 49/51% (AGXT2L2/L1)
- Identity: 27% 39/43% (AGXT2L2/L1)
- Identity: 23% 44% (AGXT2L2/L1)
- Identity: 26% 40/41% (AGXT2L1/L2)

**PotC**  PotB  PotA  PotD

**Acetylpolyamine deacetylase**

**Sinorhizobium meliloti**

- genome region 1

**Flavobacterium psychrophilum**

**Sinorhizobium meliloti**

- genome region 2

**Polaromonas sp.**

**Pectobacterium atrosepticum**
Figure 3 - Veiga-da-Cunha et al.

Product formed by AGXT2L1 (mM)

- Product formation over time (10 min vs. 20 min)
- Products: CH₃CHO, NH₃, Pi

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Figure 4 - Veiga-da-Cunha et al.

**A**

Reaction products formed by AGXT2L2 (mM)

- 2-AASA
- NH$_3$
- Pi

**B**

(5R)-5-phosphohydroxy-L-lysine phospholylase activity of AGXT2L2 (nmol/min/mg)

$K_m = 16.9 \pm 4.5 \mu$M
Molecular identification of hydroxylysine kinase and of the ammoniophospholyases acting on 5-phosphohydroxy-L-lysine and phosphoethanolamine
Maria Veiga-da-Cunha, Farah Hadi, Thomas Balligand, Vincent Stroobant and Emile Van Schaftingen

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