Research Communication

Strict Reaction and Substrate Specificity of AGXT2L1, the Human *O*-Phosphoethanolamine Phospho-Lyase

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Abstract

Dysregulated expression of the *AGXT2L1* gene has been associated to neuropsychiatric disorders. Recently the gene product was shown to possess *O*-phosphoethanolamine phospholyase activity. We here analyze the specificity of AGXT2L1 in terms of both reaction and substrate. We show that the enzyme, despite having evolved from a transaminase ancestor, is at least 500-fold more active as a lyase than as an aminotransferase. Furthermore, the lyase reaction is very selective for *O*-phosphoethanolamine, strongly discriminating against closely related compounds, and we dissect the factors that contribute to such narrow substrate specificity. Overall, AGXT2L1 function appears to be rigidly confined to phospholipid metabolism, which is altered in neuropsychiatric disturbances. © 2013 IUBMB Life, 65(7):645–650, 2013

Keywords: *pyridoxal-phosphate; class-II aminotransferases; catalytic promiscuity; schizophrenia; bipolar disorder; phospholipid metabolism*

Introduction

Although enzymes are often described as exquisitely specific, both in terms of reaction catalyzed and of substrates recognized, this is not the rule. Today it is well appreciated that a substantial fraction of metabolic enzymes are promiscuous, meaning that they can catalyze reactions of different types and with different substrates (1,2). In particular, enzymes dependent on the cofactor pyridoxal 5′-phosphate (PLP) are considered prototypical promiscuous catalysts, due to the intrinsic versatility of PLP (3,4). One pertinent example is alanine-glyoxylate aminotransferase 2 (AGXT2), a mitochondrial PLP-dependent enzyme that transaminates a variety of substrates – such as L-alanine, 5-aminolevulinate (5-ALA), β-alanine and D-3-aminoisobutyrate (5) – and also acts as a lyase on L-cysteine conjugates (6) – a secondary activity possessed by several aminotransferases (7).

AGXT2L1 is a brain enzyme, so-named after its similarity to AGXT2 (36% sequence identity), that may be involved in the pathogenesis of severe neuropsychiatric disturbances. For example, in a study designed to pinpoint common gene expression profiles between schizophrenia and bipolar disorder, the *AGXT2L1* gene was the most consistently upregulated in the brains of deceased patients with either condition, as compared to control subjects (8). Such an increased expression might contribute to the diseases or represent a compensatory response to some prior neurochemical imbalance. Potentially consistent with this latter possibility was the finding that, in mouse brain, *AGXT2L1* was the most upregulated gene following treatment with the mood-stabilizer lithium carbonate (9). All these findings elicited a considerable interest on *AGXT2L1*, but for a long time the function of the encoded protein remained obscure.

Recently, Veiga-da-Cunha and coworkers (10) demonstrated in an elegant study that AGXT2L1 is a lyase acting on

Abbreviations: *PLP, pyridoxal-5′ phosphate; PMP, pyridoxamine-5′ phosphate; AGXT2, alanine-glyoxylate transaminase 2; AGXT2L1, alanine-glyoxylate transaminase 2-like 1; 5-ALA, 5-aminolevulinate; PEA, O-phosphoethanolamine; SEA, O-sulfoethanolamine; 3-APP, 3-aminopropylphosphonate; GABA-AT, γ-aminobutyrate aminotransferase.*

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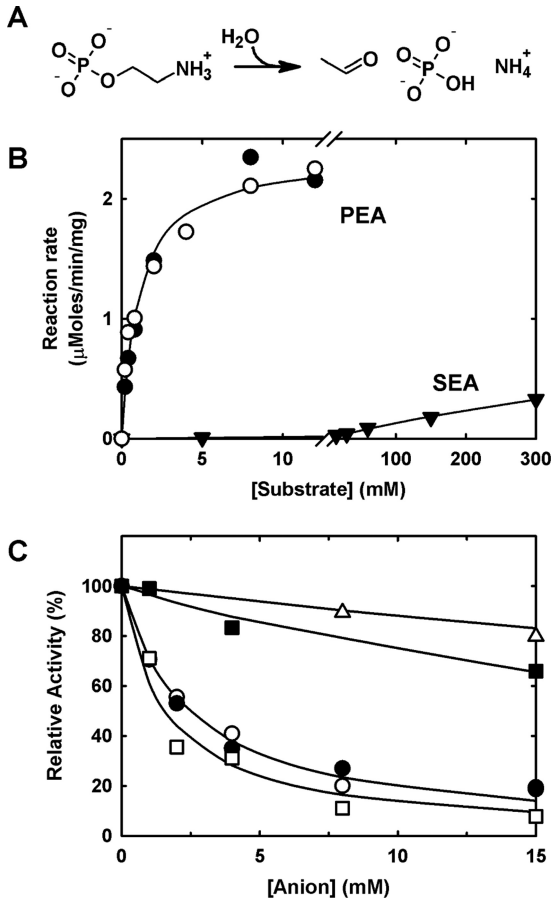

FIG 1

(A) The PEA phospho-lyase reaction (12,13) catalyzed by recombinant AGXT2L1 (10,11). (B) Rate of the elimination reaction as a function of the concentration of PEA (○, ●) or SEA (▼) at 30° C, pH 8.0. Fitting of the PEA data points to the Michaelis–Menten equation yielded $K_M^{PEA} = 0.9 \pm 0.2$ *mM and* $V_{max} = 2.4 \pm 0.2\,\mu$Moles/min per mg *of protein (corresponding to* $k_{cat}^{SEA} = 2.3\,sec^{-1}$*). As the SEA elimination rate did not reach saturation even at the highest substrate concentration tested, reliable values of* k_{cat}^{SEA} *and* K_M^{SEA} *could not be determined by data fitting. However,* $(k_{cat}/K_M)^{SEA}$*, representing the initial slope of the Michaelis–Menten hyperbola, was 1.4 ± 0.3* M^{-1} sec^{-1}*. (C) Inhibitory effect of various anions on the rate of the PEA elimination reaction, pH 8. PEA concentration was 1 mM. The data points refer to phosphate (○), sulfate (●), glyoxylate (□), methyl sulfate (■) and SEA (△). Calculated* K_i *values are given in Supporting Information Table 2.*

O-phosphoethanolamine (PEA). In other words, they showed that the enzyme irreversibly degrades PEA to acetaldehyde, phosphate and ammonia (Fig. 1A) (10–13), also providing a limited characterization of this activity. While this result was remarkable, it left several questions unanswered.

First, the reported catalytic efficiency of the PEA phospho-lyase reaction was modest, raising doubts that perhaps other metabolites could be better substrates of AGXT2L1. Furthermore, it remained unclear whether the enzyme showed reaction promiscuity (like its relative AGXT2) and could for example act as a transaminase towards some substrate, contributing to different metabolic pathways. More generally, it was not known whether AGXT2L1 would bind other common amino compounds, which might function as modulators, as reported for other brain-specific PLP-dependent enzymes (14). Addressing these and similar questions seemed important to better understand AGXT2L1 as a catalyst and to shed more light on its actual biological role.

Herein, we explore the reaction and substrate selectivity of AGXT2L1, showing that the enzyme is strikingly specific and non-promiscuous. We discuss the implications of these findings for the evolution, mechanism and metabolic function of AGXT2L1.

Experimental Procedures

Materials and methods used in this work are provided as Supporting Information.

Results

Efficiency and Specificity of the Lyase Reaction Catalyzed by AGXT2L1

We characterized the activity of recombinant AGXT2L1 towards PEA. At the optimal pH 8.0, the observed kinetic parameters were: $K_M^{PEA} = 0.9$ mM, $k_{cat}^{PEA} = 2.3\ sec^{-1}$ and $(k_{cat}/K_M)^{PEA} = 2,600\,M^{-1}\,sec^{-1}$ (Fig. 1B). These values are close to those reported by Veiga-da-Cunha et al (10), who performed their experiments at pH 7.4. The K_M^{PEA} value was also very similar to that reported over 40 years ago by Fleshood and Pitot for a partially purified PEA phospho-lyase from rabbit (13).

Tests on a dozen commercially available PEA analogs showed that the lyase activity of AGXT2L1 is extremely substrate-specific (Supporting Information Table 1). When the phosphate moiety of PEA was replaced by other good leaving groups, such as bromine or a thiol, the elimination reaction was virtually undetectable. A carboxylate adjacent to the amino carbon (as in *O*-phosphoserine) or a glycerol moiety attached to the phosphate (as in glycerophosphoethanolamine) also drastically prevented activity.

We studied in detail the reaction of AGXT2L1 with *O*-sulfoethanolamine (SEA), a PEA analog containing a sulfate (rather than phosphate) leaving group. Despite its close structural similarity to PEA, SEA reacted very poorly. Its specificity constant, $(k_{cat}/K_M)^{SEA}$, was ~1.4 M^{-1} sec^{-1}, or 1,800-fold lower than $(k_{cat}/K_M)^{PEA}$. While the SEA elimination rate did not reach saturation even at 300 mM substrate (Fig. 1B), preventing determination of accurate individual values for k_{cat}^{SEA} and K_M^{SEA}, most of the discrimination against SEA clearly mirrored a very high K_M for this compound. The k_{cat}/K_M gap between PEA and SEA did not change much in the pH 6–9 range (Supporting Information Fig. 1).

Relatively Unspecific Inhibition of AGXT2L1 by Anions

The strict substrate specificity of AGXT2L1 contrasted with its facile inhibition by a variety of anionic compounds. In addition to the reported competitive inhibition of AGXT2L1 by phosphate (10), we observed a very similar inhibition by other inorganic anions such phosphite and sulfate (Fig. 1C; Supporting Information Table 2). Methyl sulfate was one order of magnitude less effective than sulfate, while SEA inhibited very weakly. Glyoxylate and to a lesser extent pyruvate were also inhibitors (Fig. 1C; Supporting Information Table 2).

Spectroscopic Screening of AGXT2L1 Binding Selectivity

In PLP-dependent enzymes, cofactor absorption is often exploited to monitor the binding of ligands and the formation of catalytic intermediates (*e.g.*, *15*, *16*). For example in aminotransferases, reaction with an amino group donor substrate in the absence of amino group acceptors leads to a half-transamination, that is, the conversion of PLP ($\lambda_{max} \approx 410$ nm) to pyridoxamine 5′-phosphate (PMP; $\lambda_{max} \approx 330$ nm).

The reaction of AGXT2L1 with PEA was accompanied by significant changes in the cofactor spectrum, which we tentatively attributed to the formation of a Schiff base with the amine (Supporting Information Fig. 2). We also screened spectroscopically the interaction of AGXT2L1 with ~70 other amino compounds (Supporting Information Table 2), to identify possible additional ligands. Our test set tried to include the most common amino metabolites (especially amino acids and brain amines) as well as compounds sufficiently diverse in terms of chemical properties (anionic, cationic, aromatic etc.), to gather information on the binding selectivity of AGXT2L1. The results of this screening can be summarized as follows.

i. The vast majority of compounds caused only negligible spectral changes, implying their inability (at least at the 5 mM concentration used) to bind and react at the AGXT2L1 active site. Most of the compounds that did react were ω-amines, that is, carried a terminal amino group not adjacent to a carboxylate.
ii. L-cysteine and related aminothiols gave rise to bands at ~340 nm (Fig. 2A), attributable to thiazolidine adducts formed with PLP (14). Aminothiols in fact behaved as time-dependent inhibitors of AGXT2L1 (not shown), although inhibition was negligible at the concentrations of L-cysteine typically found in the cytosol ($\leq$0.1 mM; (17)).
iii. Reaction of AGXT2L1 with 5-ALA, a substrate of AGXT2 (5), produced a sharp band at 507 nm (Fig. 2A). This band strongly resembled the peaks that, in other enzymes, are attributed to stabilized carbanionic intermediates (quinonoids), formed upon cleavage of one of the bonds connecting the amino carbon to its substituents (18). Quinonoid bands were formed by other compounds that, like 5-ALA, bear a keto function adjacent to the amino group (*e.g.*, 2-aminoacetophenone).

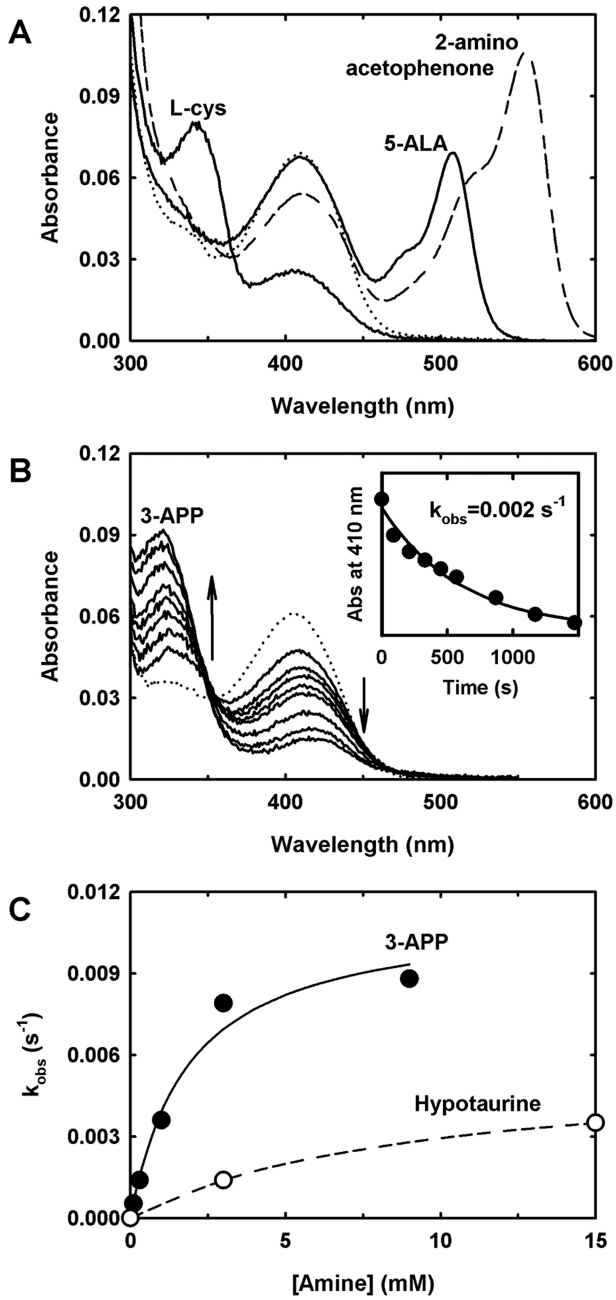

FIG 2

(A) Absorption spectra of recombinant AGXT2L1 (9 μM) in 25 mM Bis-Tris propane, pH 8, 20°C, were recorded in the absence of ligands (dotted line) and upon reaction with 5 mM L-cysteine, or 5 mM 5-ALA or 0.5 mM 2-aminoacetophenone. (B) Progressive spectral changes observed in the presence of 5 mM 3-APP (pH 8, 20°C). Inset: time-course of the half-transamination. (C) Dependence of k_{obs} for the half-transamination (PMP formation) on the concentration of 3-APP (25 mM triethanolamine buffer, pH 8, 30°C). The solid line is a fit of the data to a hyperbolic function, yielding $K_{0.5} = 1.9$ mM and an extrapolated maximum k_{obs} of 1.1×10^{-2} sec^{-1}. For comparison, the half-transamination rates obtained at two concentrations of hypotaurine are also shown.

iv. The only evidences for substantial PMP formation, and hence for half-transamination, were obtained upon incubating the enzyme with small, anionic ω-amines such as taurine, hypotaurine and 3-APP. Even in these cases, accumulation of a 330-nm band took minutes to occur (Fig. 2B). Formation of this species could be partially reversed by the addition of pyruvate, glyoxylate or α-ketoglutarate (not shown).

Aminotransferase Activity of AGXT2L1

We studied in more detail the reaction of 3-APP, which differs from PEA only for having the ester oxygen atom replaced by a methylene group. This difference is expected to interfere with the lyase reaction, which in fact was undetectable (Supporting Information Table 1), but not necessarily with the transamination. Indeed, 3-APP formed PMP faster than any other amino compound tested.

We monitored the reaction of 3-APP (0.1–10 mM) with AGXT2L1 at 30°C. At each 3-APP concentration, the data fit well to a monoexponential process and the observed pseudo-first-order rate constants increased hyperbolically as a function of the amine concentration (Fig. 2C). The initial slope of the hyperbola (*i.e.*, the apparent second-order rate constant for the half-transamination) was $5.3 \pm 1.3\ M^{-1}\ sec^{-1}$. This value represents an upper limit of k_{cat}/K_M for a full transamination reaction, which cannot proceed faster than its first half. Stated another way, our data imply that the specificity constant for 3-APP transamination (whatever the amino group acceptor) is going to be $\geq$500-fold lower than $(k_{cat}/K_M)^{PEA}$ for the lyase reaction.

To buttress this conclusion, we assessed the actual amine-pyruvate aminotransferase activity of AGXT2L1, by measuring formation of the product L-alanine (19). Pyruvate was kept constant at 20 mM, while 3-APP was used at concentrations up to 50 mM. In all these assays, the observed rate of 3-APP transamination remained well below the limit expected based on the half-transamination data (Supporting Information Fig. 3).

Discussion

Evolution of AGXT2L1: of an Aminotransferase a Phospho-lyase Made

AGXT2L1 belongs to a subfamily of proteins termed "class II aminotransferases" (20) that, as the name suggests, is composed almost entirely by transaminases (Supporting Information Fig. 4). This subfamily is not known to include any *bona fide* lyase apart from AGXT2L1 (and the related protein AGXT2L2 (10)), strongly suggesting that the enzyme has evolved from an aminotransferase ancestor. However, AGXT2L1 is at least 500-fold more efficient as a phospho-lyase than as an aminotransferase, implying that evolution has led to both a new reaction specificity and to a strongly limited catalytic promiscuity.

What modifications converted an ancestral aminotransferase into a (specific) phospho-lyase? Some insights may be obtained by analyzing conservation at key active site positions between AGXT2L1, AGXT2 and other related transaminases of known structure, such as the bacterial GABA aminotransferase (GABA-AT; Supporting Information Table 3). A striking change involves Ile50, which in GABA-AT provides a roof for the substrate binding site and may help dictate reaction specificity. This aliphatic residue corresponds to Val110 in AGXT2 but has no obvious counterpart in AGXT2L1, suggesting substantial differences in the local structure. Another significant change involves Arg398, which in GABA-AT binds the α-carboxylate of the ketoacid substrate: this residue is conserved in AGXT2, but mutated to a shorter Lys in AGXT2L1.

Determinants of Binding Selectivity in AGXT2L1

The specificity of AGXT2L1 for PEA as compared to structural analogs can be mostly explained as binding selectivity. Our data suggest that at least three factors may be enforcing such selectivity. First, the enzyme clearly favors binding ω-amines, suggesting that the active site structure discriminates against amino compounds that contain an α-carboxylate (*e.g.*, *O*-phospho-L-serine). Second, the size of the ligand is certainly a factor and it is reasonable to assume that binding of bulky PEA analogs such as glycerophosphoethanolamine (Supporting Information Table 1) or *O*-phospho-5 hydroxy-L-lysine (10) may be hampered due to steric hindrance. Finally, some analogs that are smaller than PEA and potentially very reactive (*e.g.*, 2-bromoethylamine) may nevertheless bind poorly because they lack an anionic function.

Indeed, our data imply the occurrence, in the AGXT2L1 active site, of a subsite responsible for binding anionic groups. By itself this subsite does not seem very selective, as a variety of anions, in addition to phosphate, can act as competitive inhibitors. On the other hand, the requirements for interacting with the subsite may be stricter when it comes to binding anionic amines.

Consider in particular SEA. This compound is nearly isosteric with PEA, while free sulfate shows a K_i essentially identical to phosphate. Nonetheless, $(k_{cat}/K_M)^{SEA}$ is 1,800-fold lower than $(k_{cat}/K_M)^{PEA}$ and such a dramatic difference is mostly attributable to weaker binding of SEA (Fig. 1B). The sulfate group in SEA bears a single negative charge (rather than two like the phosphate group in PEA) but this explains only in part the observed affinity gap; in fact, K_i for monoanionic methylsulfate is just 12-fold higher than K_i for plain sulfate (Supporting Information Table 2). Thus, an exceedingly large discrimination between phosphate and sulfate appears to occur when the two groups are part of an anionic amine. One possibility is that binding of the amine is accompanied by a conformational change that imparts an increased selectivity to the anion subsite.

Support for a conformational change upon substrate binding comes from an analysis of K_M^{PEA}. There are indications that this parameter can be considered an overall dissociation constant of the substrate from AGXT2L1. If this is the case, K_M^{PEA} should depend both on the interactions of PEA with the anion subsite and on the formation of covalent intermediates with PLP. However, the value of K_M^{PEA} is nearly identical to K_i for inorganic phosphate, suggesting that the binding energy

gained from covalently linking the amino group to PLP is almost entirely used up to pay for some other unfavorable process, possibly a rearrangement of the active site structure (21).

Considerations on the Biological Role of AGXT2L1

AGXT2L1 has been apparently shaped by evolution to maximize specificity and limit promiscuity, rather than to optimize absolute catalytic performance (even though its catalytic parameters are comparable to those reported for other mammalian phospho-lyases (10,22)). A recent study (1) has highlighted three major conditions under which specific enzymes are metabolically preferred to promiscuous ones, namely i) when the catalyzed reaction is essential, ii) when a high metabolic flux is needed, and iii) when the enzyme activity must be tightly regulated. The need for a strict regulation may be especially relevant for AGXT2L1, whose reaction is irreversible and generates potentially toxic products.

When is AGXT2L1 activity effectively required? PEA is both a product of sphingolipid degradation and a precursor of glycerophospholipid biosynthesis (Supporting Information Fig. 5) and it can accumulate when membrane remodeling or degradation increases. Such an accumulation may be undesirable for several reasons – for example, it has been shown that PEA and the parent compound ethanolamine interfere with mitochondrial function (23) – and under these circumstances AGXT2L1 could function as a 'relief valve', to counteract the rise in PEA concentration. PEA can be also degraded by specific phosphatases, but this reaction generates ethanolamine and is easily reversed by ethanolamine kinase (Supporting Information Fig. 5).

There is a vast literature associating neuropsychiatric disorders to alterations in the brain levels of PEA and related phosphomonoesters and in the membrane phospholipid metabolism (*24* and references therein). In keeping with this association, our findings confine the role of AGXT2L1 to phospholipid metabolism. An increased expression of *AGXT2L1* during disease (8) might be primarily a compensatory mechanism to alleviate the effects of, for example, excessive membrane degradation. On the other hand, drawbacks of a prolonged overproduction of AGXT2L1 may include a chronic exposition of the cells to acetaldehyde and ammonia and an altered homeostasis of sphingolipids and glycerophospholipids.

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