***Caenorhabditis elegans* AGXT-1 is a mitochondrial and temperature-adapted ortholog of peroxisomal human AGT1: New insights into between-species divergence in glyoxylate metabolism.**

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Abstract

In humans, glyoxylate is an intermediary product of metabolism, whose concentration is finely balanced. Mutations in peroxisomal alanine:glyoxylate aminotransferase (hAGT1) cause primary hyperoxaluria type 1, which results in glyoxylate accumulation that is converted to toxic oxalate. In contrast, glyoxylate is used by the nematode *C. elegans* through a glyoxylate cycle to by-pass the decarboxylation steps of the tricarboxylic acid cycle and thus contributing to energy production and gluconeogenesis from stored lipids. To investigate the differences in glyoxylate metabolism between humans and *C. elegans* and to determine whether the nematode might be a suitable model for PH1, we have characterized here the predicted nematode ortholog of hAGT1 (AGXT-1) and compared its molecular properties with those of the human enzyme. Both enzymes form active PLP-dependent dimers with high specificity towards alanine and glyoxylate, and display similar

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three-dimensional structures. Interestingly, AGXT-1 shows 5-fold higher activity towards the alanine/glyoxylate pair than hAGT1. Thermal and chemical stability of AGXT-1 is lower than that of hAGT1, suggesting temperature-adaptation of the nematode enzyme linked to the lower optimal growth temperature of *C. elegans*. Remarkably, *in vivo* experiments demonstrate the mitochondrial localization of AGXT-1 in contrast to the peroxisomal compartmentalization of hAGT1. Our results support the view that the different glyoxylate metabolism in the nematode is associated with the divergent molecular properties and subcellular localization of the alanine:glyoxylate aminotransferase activity.

Keywords. Primary hyperoxaluria; enzyme kinetics; substrate specificity; protein stability; conformational disease.

1. Introduction

Glyoxylate is an intermediary product of metabolism in humans, which is formed from precursors such as glycine, glycolate, hydroxypyruvate and hydroxyproline. Among mammals, alanine:glyoxylate aminotransferase (AGT) protein compartmentalization is linked to metabolism of glyoxylate precursors, varying from mainly mitochondrial in carnivorous, peroxisomal in herbivorous and located in both organelles in omnivorous [1]. The human *AGXT* gene encodes an alanine:glyoxylate aminotransferase enzyme (hAGT1; E.C. 2.6.1.44) that is responsible for glyoxylate detoxification in peroxisomes of hepatocytes [2] thus balancing the glyoxylate concentration. The human genome encodes a second protein with alanine:glyoxylate aminotransferase activity named hAGT2 [3] that catalyses multiple aminotransferase reactions [4,5] and might be involved in the metabolism of glyoxylate within the mitochondria. hAGT1 is a pyridoxal 5'-phosphate (PLP)- dependent enzyme that catalyses the amino transfer from L-alanine to glyoxylate

14 resulting into pyruvate and glycine and this reaction is largely shifted towards glycine
15 formation [6]. Mutations in *AGXT* gene cause primary hyperoxaluria type 1 (PH1), an error
16 of amino acid metabolism inherited in an autosomal recessive manner [2]. PH1 results in
17 the accumulation of glyoxylate in hepatocytes where it is oxidized to oxalate (a metabolic-
18 end product in humans) causing progressive renal failure and ultimately leading to a build-
19 up of oxalate and life-threatening oxalate precipitation [2,7]. Currently, the best method to
20 treat PH1 is a double liver and kidney transplantation, but this treatment often shows
21 significant rates of morbidity and mortality.

22 After the complete sequencing of *Caenorhabditis elegans* genome, early
23 estimations indicated that around 74% of human gene sequences had nematode
24 counterparts [8]. Therefore, a lot of effort was put into identifying potential nematode
25 orthologs to human genes. As a result, using an *in silico* search of genes associated with
26 inborn errors of metabolism in humans, an open-reading frame termed as *T14D7.1* was
27 predicted as an ortholog of human *AGXT* gene [9]. The *T14D7.1* gene (now renamed as
28 *agxt-1*) is located on chromosome 2 and it is organized into 11 exons
29 (WBGene00011767), whose conceptual translation results into a 405 amino acids protein
30 (AGXT-1). Unlike humans, the nematode *C. elegans* has an active glyoxylate cycle (GC)
31 [10] that allows to bypass the decarboxylation steps of the tricarboxylic acid (TCA) cycle
32 [11] , thus linking catabolic and biosynthetic capacities. In the nematode, the key enzyme
33 of this cycle is a single bi-functional enzyme (ICL-1), which has isocitrate lyase activity (N-
34 terminal domain) and malate synthase activity (C-terminal domain) [12] that are regulated
35 in a developmentally specific manner [13,14] .

36 We herein present data supporting that the divergent glyoxylate metabolism
37 between humans and the nematode *C. elegans* could involve different molecular
38 properties and subcellular localization of their respective AGT enzymes. Further, we

39 demonstrate that even though AGXT-1 and hAGT1 proteins have similar quaternary
40 structure and substrate specificities, AGXT-1 displays higher specific activity and lower
41 protein stability, possibly reflecting temperature adaptation of the nematode enzyme. *In*
42 *vivo* studies demonstrate the mitochondrial localization of AGXT-1 in contrast to the
43 peroxisomal functional environment of hAGT1. In this work, we provide novel insights into
44 the evolutionary changes in protein stability, roles of AGT proteins and the divergence of
45 glyoxylate metabolism between vertebrates and invertebrates.

46 **2. Materials and Methods**

47 ***C. elegans* AGXT-1 cloning, expression and purification.** The *agxt-1* ORF was
48 amplified from a *C. elegans* cDNA library and cloned into a pET-28 (Novagen) vector using
49 the primers ACA**GCTAGC**ATGCAGCCAACAGGGAATCAAATA and TAT**GTCTGAC**TTAA-
50 ACCAAATTAGGATCCGATGGACTT forward and reverse respectively and restriction
51 enzymes NheI-HF and SalI-HF (New England Biolabs). This construct incorporates a His-
52 tag sequence at the N-terminal domain of AGXT-1. *E. coli* BL21(DE3) competent cells
53 were transformed with the plasmid and were grown in LB medium supplemented with 30
54 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin. Overnight cultures were diluted 40-fold in fresh LB-kanamycin for 3 h
55 at 37°C and induced at 4°C by adding IPTG 0.5 mM for 8 h. Cells were harvested and
56 lysed by sonication in binding buffer (20 mM NaH_2PO_4 , 200 mM NaCl, 50 mM imidazole,
57 pH 7.4) supplemented with protease inhibitors (EDTA-free protease inhibitor cocktail,
58 Roche). Soluble extracts obtained after ultracentrifugation at 70,000 x g were loaded onto
59 an IMAC columns (GE Healthcare) and eluted with binding buffer supplemented with 500
60 mM imidazole. His-AGXT-1 was further purified by size exclusion chromatography using a
61 HiLoadTM 16/60 SuperdexTM 200 column running in 20 mM Hepes (2-[4-(2-
62 hydroxyethyl)piperazin-1-yl]ethanesulfonic acid), 200 mM NaCl, pH 7.4. The concentration
63 of His-AGXT-1 (hereafter AGXT-1) protein was evaluated using a sequence-based

64 extinction coefficient of $0.763 \text{ ml}\cdot\text{mg}^{-1}\cdot\text{cm}^{-1}$ at 280 nm [15]. The purification of hAGT1 was
65 performed as described previously [16]. Isolation of apo-forms of both enzymes was
66 attempted by using the protocol previously described [17] . PLP concentration was
67 calculated by using a molar extinction coefficient of $4,900 \text{ M}^{-1}\cdot\text{cm}^{-1}$ at 388 nm [18].

68 **Spectroscopic analyses.** All the spectroscopic assays were performed in a 20 mM
69 Hepes, 200 mM NaCl, pH 7.4 buffer at 25°C. UV-visible absorption spectroscopy was
70 performed in an Agilent 8453 diode-array spectrophotometer using cuvettes with a path
71 length of 3 mm and 20 μM protein (in subunit). Near-UV/visible circular dichroism
72 measurements were performed in a Jasco J-710 spectropolarimeter by using 5-mm path
73 length cuvettes with 20 μM protein (in subunit). Dynamic light scattering was carried out
74 using a protein concentration of 5 μM (in subunit) in the presence of 50 μM PLP in a
75 Zetasizer Nano ZS (Malvern Inc.) with 3-mm path length cuvettes and applying the Stokes-
76 Einstein equation assuming a spherical shape for the scattering particles.

77 **Enzyme activity measurements.** The overall transaminase activity was measured at
78 37°C with a protein concentration of $2.5 \mu\text{g}\cdot\text{ml}^{-1}$ in the presence of 150 μM PLP in 0.1 M
79 phosphate buffer pH 8. The time of the reaction was 2 minutes and the substrate
80 concentration was 0.25-2 mM glyoxylate and 0-100 mM L-alanine. Pyruvate formation was
81 evaluated following the oxidation of NADH at 340 nm by a coupled enzyme assay using
82 lactate dehydrogenase [19] during 3 minutes at 37°C. Global fittings were performed
83 according to a double-displacement mechanisms [6,16]. To determine the pH
84 dependence, activity measurements were performed using this coupled assay in the
85 presence of 2 mM glyoxylate and 100 mM L-alanine at 37°C in the following buffers: 100
86 mM Hepes (pH 7-8), MES (2-(N-morpholino)ethanesulfonic acid, pH 6-6.5), acetate (pH
87 4.5-5.5) or formiate (pH 3-4). The time of the transamination reaction was set at 1.5
88 minutes (pH 7-8), 4 minutes (pH 6-6.5) and 10 minutes (pH 3.5-5.5). The dependence of

specific activity on temperature was measured at 15, 20, 25, 30 and 37°C with reaction times from 2 to 10 minutes. To investigate the substrate specificity, the overall transamination reaction was measured in the presence of different amino acids (L-alanine, L-serine, L-arginine, L-glutamate, L-aspartate and L-phenylalanine) and ketoacids (glyoxylate and pyruvate) by incubating the enzymes (2.5-100 µg·ml⁻¹) with the amino acid at 100 mM, the ketoacid at 2 mM and in the presence of 200 µM PLP in 0.1 M phosphate buffer, pH 8 and 37°C. Aliquots of each reaction mixture were collected at various times and the reaction was stopped by adding 10% (w/v) trichloroacetic acid. The amount of ketoacid consumed was determined by HPLC after derivatization with 2,4-dinitrophenylhydrazine as previously described [20].

Differential scanning calorimetry. DSC experiments were performed and analysed as previously described for hAGT1 [17,21]. Briefly, the model considers the irreversible denaturation of the native protein to a final state that cannot fold back, and this kinetic conversion is characterized by a first-order rate constant k , which changes with temperature according to the Arrhenius equation. The half-life at any temperature can thus be obtained from extrapolation of k to a given temperature following the Arrhenius plot and determined as $t_{1/2} = \ln(2)/k$.

Urea denaturation. Urea denaturation of AGXT-1 and hAGT1 was performed by incubating the enzymes (5 µM in protein subunit) in 20 mM Hepes, 200 mM NaCl, 1mM TCEP (Tris(2-carboxyethyl)phosphine) pH 7.4 with urea at concentrations ranging from 0-8 M. Urea concentration was determined by refractive index measurements. Samples were incubated at 25°C for 16 h and then denaturation was monitored by Far-UV circular dichroism spectroscopy (200-260 nm; 1 mm quartz cuvettes). Refolding experiments were performed by dilution of protein samples denatured in 8 M urea solution with urea-free

113 buffer and allowed to equilibrate at 25°C for 4 h prior to Far-UV CD spectroscopic
114 analyses.

115 **Structural modelling.** The structural model of AGXT-1 protein was obtained using the
116 Modeler v9.13 software, the AGXT-1 amino acids sequence and the crystal structure of
117 human hAGT1 (pdb 1H0C) protein as a template. The model refinement was performed by
118 energy minimization applying the CHARM27 force field of the MOE software. The
119 adjustment of the protonation state, the coordinate based isoelectric point calculation, the
120 electrostatic surface map drawing, the structural alignment and the images construction
121 were performed using MOE 2013 software (CCG group).

122 **Strains.** *C. elegans* worms were culture and handle as described before [22]. The
123 following strains were used: wild type N2 Bristol, UGR1 *alpEx1 [Pmyo-3::tomm-20:: gfp,*
124 *Pmyo-3::agxt-1(50aa)::tagrfp, Punc-122::gfp]*, UGR3 *alpEx3 [Pmyo-3:: tomm -20:: gfp,*
125 *Pmyo-3::agxt-1(100aa):: tagrfp, Punc-122:: gfp]*, UGR7 *alpEx7 [Pmyo-3:: tomm -20:: gfp,*
126 *Pmyo-3:: tagrfp, Punc-122:: gfp]*, UGR9 *agxt-1 (tm6307)*, UGR10 *icl-1 (ok531)*, and
127 UGR11 *agxt-1(tm6307); icl-1 (ok531)*.

128 **Molecular biology and transgenic lines.** The nucleotide sequence corresponding to the
129 first 50 and 100 amino acids of the *agxt-1* cDNA sequence were amplified and cloned into
130 a gateway plasmid pDONR221 These clones were fused in frame with tagRFP
131 (monomeric RFP) and the *unc-54* gene 3-UTR and expressed under the *myo-3* promoter,
132 using the Multi Site Gateway Pro Plus Kit (ThermoFisher, catalogue number 11791-100
133 and 11789-013). These plasmids were injected at 5 ng·µl⁻¹ together with a plasmid
134 expressing the mitochondrial fusion construct TOMM-20::GFP under the control of the
135 same *myo-3* promoter (kindly provided by Dr. Marc Hammarlund, Yale University) at 5
136 ng·µl⁻¹ and the co-injection marker *Punc-122::gfp*. Transgenic animals were generated
137 using standard techniques [23].

138 **Fluorescence and confocal image acquisition and analysis.** Images of fluorescence
139 fusion proteins were taken in live adults *C. elegans* nematodes using a 60X CFI Plan Apo
140 VC, numerical aperture 1.4, oil-immersion objective on an UltraView VoX spinning-disc
141 confocal microscope (PerkinElmer Life and Analytical Sciences). Worms were
142 synchronized 3 days prior to experiment, and first day of adulthood animals were
143 analysed. Animals were immobilized during image acquisition using 10 μ M Levamisol
144 (Sigma). Images were analysed using Volocity software (Improvision).

145 **Glyoxylate and oxalate determination.** *C. elegans* strains N2, UGR9, UGR10 and
146 UGR11 were grown on NGM plates supplemented with 10 mM Sodium Glyoxylate
147 monohydrate (Sigma). When bacterial food was about to exhaust, mixed stage
148 populations of worms were washed out from the plates with M9 buffer several times to
149 clean the bacteria off and worm pellet was frozen at -80°C overnight. Thawed pellet was
150 resuspended in 5 volumes of cold 10 mM Hepes and 0.5% CHAPS buffer supplemented
151 with complete protease inhibitor cocktail (Roche) and sonicated. Lysates were centrifuged
152 at 14000 rpm for 10 min at 4° and supernatants were transferred into a new tube and
153 immediately frozen. To evaluate glyoxylate content, lysates were deproteinized by the
154 addition of 10% (w/v) trichloroacetic acid and centrifuged at 13200 g for 2 min. The amount
155 of glyoxylate present in supernatant was determined by HPLC analysis after derivatization
156 with 2,4.dinitrophenylhydrazine as previously described [20] . The total protein content of
157 each lysate was determined before deproteinization by using the Bradford assay. Oxalate
158 content was evaluated by using the Oxalate Quantitative Enzymatic Color Test Kit (Greiner
159 Diagnostic GmbH) following manufacturer's protocol.

160 **3. Results**

161 **AGXT-1 is a mitochondrial protein.** According to a protein sequence comparison, *C.*
162 *elegans* AGXT-1 shows much higher identity/similarity to peroxisomal hAGT1 protein than

163 to mitochondrial hAGT2, which has a different fold (Table 1). The sequence of AGXT-1 is
 164 13 residues longer than hAGT1 and all catalytic residues involved in the binding and
 165 stabilization of the coenzyme at the active site are conserved in both proteins (Figure 1,
 166 red highlighted residues) [24]. However, we also found two interesting differences between
 167 the two primary sequences (Figure 1, green highlighted residues). First, the 13 extra
 168 residues of AGXT-1 protein are located in the N-terminal domain. Mitochondrial targeting
 169 sequences (MTS) usually consist of 20-60 residues in the N-terminal domain that are
 170 prone to form an amphipathic alpha helix [25]. Bioinformatics tools, such as MitoProt [26]
 171 or TargetP 1.1 Server [27], predict probabilities of 75-85% for the N-terminal domain of
 172 AGXT-1 to form a cleavable amphipathic alpha helix consistent with a potential MTS.
 173 Second, the AGXT-1 protein lacks the C-terminal tripeptide that constitutes the
 174 peroxisomal targeting sequence type 1 (PTS1) required for hAGT1 peroxisomal import
 175 through Pex5p-dependent route [28,29], which is conserved between humans and
 176 nematodes [30]. *C. elegans* also lacks the alternative PTS2 pathway to target proteins to
 177 peroxisomes [31]. All together, these data suggest that AGXT-1 is not likely to be imported
 178 to peroxisomes, in contrast to hAGT1 [2], but more likely to be imported to mitochondria.

Table 1. Identity and similarity in pairwise sequence alignments of AGXT-1, hAGT1 and hAGT2 proteins. Alignments were carried out using Clustal Omega [32]. GI numbers of protein sequences are: hAGT1-126522481, hAGT2-119576316 and AGXT-1-5824614.

Residues	hAGT1 vs. hAGT2	AGXT-1 vs. hAGT1	AGXT-1 vs. hAGT2
Identical	75 (19%)	167 (41%)	77 (19%)
Similar	112 (28%)	121 (30%)	103 (25%)

192 TOMM-20::GFP fusion protein [37,38]. When tagRFP was expressed alone (Figure 2a), a
 193 diffused cytosolic pattern was seen (Figure 2c), compared with the mitochondrial
 194 localization of TOMM-20::GFP protein (Figure 2b). However, tagRFP fused to either the
 195 first 50 or 100 amino acids of AGXT-1 (Figure 2e-i), was targeted to mitochondria (Figure
 196 2g-k), as demonstrated by the colocalization with TOMM-20::GFP (Figure 2h-l). These
 197 data confirm that the N-terminus of AGXT-1 encodes a functional MTS, thus reinforcing
 198 the idea of AGXT-1 being a mitochondrial enzyme in *C. elegans*.

Figure 2. **Mitochondrial localization of AGXT-1.** Cartoons representing the DNA constructs used to express tagRFP (a), the first 50 amino acids (e) and the first 100 amino acids (i) of AGXT-1 fused to tagRFP under the control of the *myo-3* promoter along with the *unc-54* 3'-UTR. TOMM-20::GFP expression is found with the typical tubular mitochondrial pattern in muscle cells (b, f and j). When tagRFP is expressed alone, a diffused cytosolic pattern is seen (c) and no colocalization is found with TOMM-20::GFP (d, merge). Fusing the first N-terminal 50 (MTS₅₀) (g) or 100 (MTS₁₀₀) (k) amino acids of the AGXT-1 to tagRFP targets the protein to mitochondria. The GFP and tagRFP fluorescence now colocalize to the same organelle (h and l, merge).

199 **AGXT-1 and hAGT1 share overall structure.** Taking into account the considerable
200 sequence homology between AGXT-1 and hAGT1, we built a structural homology model
201 of AGXT-1 using the available crystal structure of hAGT1 as a template [24]. Due to the
202 high flexibility of the N-terminus of hAGT1 and substantial sequence differences between
203 the N-terminal domains of both proteins (Figure 1), we did not include in the structural
204 alignment the first 38 and 23 amino acids of AGXT-1 and hAGT1, respectively. The AGXT-
205 1 model was subsequently refined by energy minimization. The superposition of the
206 obtained AGXT-1 model and the hAGT1 structure predicts that the two proteins share a
207 similar overall conformation and secondary structure composition (Figure 3a). The binding
208 mode of PLP to AGXT-1 protein appears to be very similar to that of the human enzyme
209 and involves a Schiff base linkage with Lys226, a base stacking hydrophobic interaction
210 between the pyridine ring and the side chain of Trp125, a salt bridge between the N1 of
211 PLP and Asp200, an H-bond of the 3'OH group of PLP and Ser175 and several H-bonds
212 between the phosphate group of the coenzyme and Gln225, Gly99 and His100 (Figure
213 3b). Moreover, two interchain contacts of the phosphate group of PLP in hAGT1 with
214 Tyr260 and Thr263 of the neighbouring subunit, are probably held by Tyr277 and Thr280
215 in AGXT-1.

216 However, some differences are visible in the active site region and on the protein
217 surface. As for the active site cleft, Ser81 that in hAGT1 is critical for PLP binding [39] is

218 replaced by a threonine residue (Thr98) in AGXT-1. Moreover, Arg360, whose side chain
 219 binds the carboxylate group of the substrate in hAGT1 [24], is replaced by Ile377 in AGXT-
 220 1 (Figure 3c), thus leading to a different active site polarity and possibly a different
 221 substrate binding mode. In addition, Trp246 and Met259 of hAGT1 are replaced by Glu263
 222 and Arg276 in AGXT-1. As shown in Figure 3d, these two charged residues could interact
 223 by a salt bridge, thus probably stabilizing the interface loop 276-282 comprising the
 224 aforementioned Tyr277 and Thr280 residues involved in the binding of the coenzyme.
 225 Electrostatic surface map calculations revealed that AGXT-1 would exhibit a higher density
 226 and a different distribution of the surface charges with respect to hAGT1 (not shown).

Figure 3. **Structural alignment of the AGXT-1 model with the hAGT1 structure.** Structural over imposition of AGXT-1 (orange) and hAGT1 (grey). The two backbones are represented as ribbons and the single residues as sticks. The PLP molecule is represented as green sticks.

227 **AGXT-1 is a dimeric PLP-dependent alanine:glyoxylate aminotransferase.** To
228 compare the molecular properties of AGXT-1 and hAGT1, both recombinant proteins were
229 expressed in and purified from *E. coli*. Along protein purification, size-exclusion
230 chromatography analyses showed a single peak with similar retention volumes for both
231 proteins and attributable to a dimer (82.6 ml and 84.9 ml in a Superdex™ 200 16/60 for
232 hAGT1 and AGXT-1, respectively). Dynamic light scattering studies also revealed similar
233 hydrodynamic diameters for both proteins (8.1 ± 0.1 nm vs. 8.8 ± 0.4 nm, for hAGT1 and
234 AGXT-1, respectively), thus further confirming the dimeric assembly of the two proteins
235 [16,40]. As isolated, both proteins show spectroscopic features corresponding to PLP
236 bound to the active site by a Schiff base with Lys209 in hAGT1 and Lys226 in AGXT-1.
237 The UV-visible absorption and CD spectra of AGXT-1 display a band at 430 nm, which
238 likely reflects the ketoenamine tautomer of the internal Schiff base, and a band at 340 nm,
239 possibly corresponding to enolimine tautomer (Figure 4 and [6]). After incubation of AGXT-
240 1 with L-alanine, the spectra show almost no signal at 430 nm and a main absorption peak
241 and weak dichroic band at 340 nm (Figure 4). These signals indicate the formation of the
242 pyridoxamine-5'-phosphate (PMP) form of the coenzyme, thus supporting the proper
243 binding of PLP in the active site of AGXT-1 to take the amino group from the substrate L-
244 alanine. Attempts to remove the coenzyme from the active site of AGXT-1 (to obtain the
245 apo-form) by using the procedure applied for hAGT1 [16,17] yielded a form of AGXT-1
246 with PMP tightly bound, suggesting a very high affinity and/or very slow dissociation rate of
247 PMP. Further reduction of the pH (below 5.8) resulted in irreversible denaturation of
248 AGXT-1 before the release of the coenzyme, thus preventing a comparison between the

249 apo-form of AGXT-1 and hAGT1. Together, these results suggest that AGXT-1 binds PMP
250 with higher affinity than hAGT1 and/or that the pH value for efficient PMP release is lower
251 for AGXT-1.

252 Next, the ability of AGXT-1 to catalyse the amino transfer by using the natural
253 substrates of hAGT1 (L-alanine and glyoxylate) was tested (Figure 5). Activity data were
254 analysed by using a coupled enzyme assay as described for hAGT1 [6,16]. Kinetic
255 parameters for the overall transamination show that AGXT-1 has 5-fold higher activity
256 ($V_{\max}$) than hAGT1 (Table 2), while apparent affinities (K_M) for both substrates are kept in
257 similar ranges. Only the apparent affinity for L-alanine is slightly higher (1.6-fold higher) for
258 AGXT-1 than for hAGT1. These results support a higher catalytic efficiency towards
259 glyoxylate for the nematode enzyme than for the hAGT1. In addition, both proteins show
260 similar dependence of the overall transaminase activity on temperature and pH (Figure 6).
261 Arrhenius analyses of temperature dependence of transaminase activity reveal an
262 activation energy value of $4.0 \pm 0.9 \text{ kcal} \cdot \text{mol}^{-1}$ for AGXT-1, somewhat higher than for
263 hAGT1 ($2.2 \pm 1.8 \text{ kcal} \cdot \text{mol}^{-1}$) (Figure 6a). The activity of both enzymes decreases at mild
264 acidic pH (Figure 6b-c), suggesting similar pattern of protonation states of the active site of
265 both proteins, which could be a sign of similar reaction specificities [41] and environmental
266 pH in their intracellular localization [42].

Figure 4. **Near-UV/visible spectroscopic analyses of AGXT-1 and hAGT1 in their PLP-bound forms and in the presence of 0.5 M L-alanine (PMP-forms).** (a) Near-UV/visible absorption spectra; (b) Near UV/visible circular dichroism spectra; Protein concentration was 20 μ M in monomer.

Table 2. **Kinetic parameters of the overall transaminase activity for AGXT-1 and hAGT1.** Data are mean $\pm$ s.d. from global fits from a coupled enzyme assay.

Parameter	AGXT-1	hAGT1
V_{max} ($mmol \cdot h^{-1} \cdot mg^{-1}$)	11.3 $\pm$ 0.7	2.2 $\pm$ 0.1
$K_{M,alanine}$ (mM)	12 $\pm$ 2	20 $\pm$ 2
$K_{M,glyoxylate}$ (mM)	0.26 $\pm$ 0.05	0.26 $\pm$ 0.04

Figure 5. **Enzyme kinetic analyses of AGXT-1 (a) and hAGT1 (b) proteins in the presence of L-alanine and glyoxylate.** Experiments were performed at 0.25 mM (closed circles) and 2 mM (open circles) glyoxylate and varying L-alanine concentrations. Lines are global best-fits to an enzyme-substituted kinetic mechanism yielding the kinetic parameters shown in Table 2.

267 The large difference in activity between AGXT-1 and hAGT1 towards the
 268 alanine:glyoxylate pair prompted us to investigate whether these two enzymes may share
 269 similar substrate specificity [6] . To this aim, we determined the specific activity of these
 270 enzymes by using glyoxylate or pyruvate as amino acceptors and different natural L-amino
 271 acids as amino donors (Figure 7). When using glyoxylate as amino acceptor, AGXT-1
 272 protein is very specific towards L-alanine showing 300-fold and 130-fold higher activity
 273 than for L-serine and L-phenylalanine and no detectable activity towards L-arginine, L-
 274 glutamate and L-aspartate (Figure 7a). Under similar conditions, hAGT1 is somewhat less
 275 specific, with 23-fold and 52-fold lower activity towards L-serine and L-arginine and 100-
 276 fold and 200-fold lower activity towards L-phenylalanine and L-glutamate (Figure 7a).
 277 Using pyruvate as amino acceptor (Figure 7b), both enzymes display lower activity
 278 towards L-serine than those measured using glyoxylate. Their activities towards L-

279 phenylalanine are comparable, L-arginine is a better substrate for hAGT1 than AGXT-1
 280 and L-glutamate and L-aspartate are poor substrates for both enzymes. Even though
 281 some differences exist, these studies support similar substrate specificities for both
 282 proteins and demonstrate that AGXT-1 protein is an alanine:glyoxylate aminotransferase.

Figure 6. **Temperature- and pH-dependence of the overall transaminase activity for AGXT-1 and hAGT1 proteins.** (a) Arrhenius plots for the enzymatic activity of AGXT-1 and hAGT1; (b) and (c) pH-dependence of the enzymatic activity of hAGT1 and AGXT-1, respectively. Activity was measured in the presence of 100 mM L-alanine and 2 mM glyoxylate.

Figure 7. **Substrate specificity of AGXT-1 and hAGT1 towards different amino acids as amino donor using glyoxylate (a) or pyruvate (b) as amino acceptors.** The concentration of amino acids was 100 mM and of ketoacids was 2 mM. N.Det.- not detected.

283 **AGXT-1 shows lower resistance towards thermal and chemical denaturation.**
 284 Thermal denaturation of AGXT-1 was studied by differential scanning calorimetry (DSC).
 285 Both AGXT-1 and hAGT1 show a single denaturation transition (Figure 8a), which is well
 286 described by a simple two-state irreversible denaturation model with first-order kinetics
 287 (supported by protein concentration independent denaturation transitions [16,17] and data
 288 not shown). The denaturation temperature (T_m) of AGXT-1 is about 12°C lower than that of
 289 hAGT1 (Table 3). The lower denaturation enthalpy (ΔH) of AGXT-1 seems to be a
 290 consequence of the lower thermal stability of this protein and a strongly temperature
 291 dependent ΔH with a theoretical denaturation heat capacity of about 11 kcal·mol⁻¹·K⁻¹

(based on the correlations by [43]), thus suggesting that the amount of tertiary structure lost upon thermal denaturation in both enzymes is fairly similar. Due to the kinetic control of thermal denaturation for both enzymes, the DSC analyses can be used to extrapolate the denaturation rate constants to physiological temperatures (inset Figure 8a). The extrapolated kinetic stability for AGXT-1 towards thermal denaturation at 37°C is around 200-fold lower than hAGT1 at this temperature (Table 3). However, when we compare the kinetic stability of both proteins at the corresponding physiological temperature (37°C for hAGT1 and 20°C for AGXT-1) the nematode protein is 90-fold more stable than hAGT1.

Table 3. Thermal denaturation parameters for AGXT-1 and hAGT1. The parameters have been determined from DSC scans using a two-state irreversible denaturation model. ¹ Data from Mesa-Torres, PLoS One, 2013. ² Determined at 3°C·min⁻¹ scan rate. ³ mean±s.d. from three different scan rates. ⁴ kinetic constant rates for irreversible denaturation extrapolated to 37°C (20°C)

Parameter	AGXT-1	hAGT1 ¹
T _m (°C) ²	69.8	82.1
ΔH (kcal·mol ⁻¹) ³	366±11	548±5
E _a (kcal·mol ⁻¹) ³	112±15	109±5
k _{37°C} (k _{20°C}) (min ⁻¹) ⁴	1.2·10 ⁻⁷ (7.1·10 ⁻¹²)	6.4·10 ⁻¹⁰

Urea induced denaturation of AGXT-1 was investigated by Far-UV circular dichroism spectroscopy (Figure 8b). Denaturation profiles of AGXT-1 and hAGT1 show a single transition and once again, AGXT-1 displays a lower stability with half-denaturation urea concentration of ~3.8 M (AGXT-1) vs. ~6 M (hAGT1). The cooperativity of urea-induced unfolding is apparently higher for AGXT-1 than for hAGT1. In contrast to hAGT1 ([44] and data not shown), urea unfolding of AGXT-1 is highly reversible (Figure 8b squares). This reversibility suggests that, upon chemical denaturation, the refolding pathway of AGXT-1 populates less aggregation-prone intermediate states.

Figure 8. **Stability of AGXT-1 and hAGT1 proteins towards thermal and chemical denaturation.** (a) Thermal denaturation profiles of holo AGXT-1 and hAGT1 at $3^{\circ}\text{C}\cdot\text{min}^{-1}$ and $5\ \mu\text{M}$ protein subunit in $20\ \text{mM}$ Na-Hepes, $200\ \text{mM}$ NaCl pH 7.4. Lines are best-fits to a two-state irreversible model with first-order kinetics; Inset: Arrhenius plots for thermal denaturation kinetics; (b) Urea-induced unfolding of holo AGXT-1 and hAGT1 at $5\ \mu\text{M}$ protein subunit in $20\ \text{mM}$ Na-Hepes, $200\ \text{mM}$ NaCl pH 7.4, $1\ \text{mM}$ TCEP at 25°C . Circles show the results for unfolding, while squares correspond to refolding experiments.

4. Discussion

Glyoxylate is a metabolic intermediary in humans that has to be detoxified mainly by hAGT1 in peroxisomes [2]. Instead, glyoxylate is a key metabolite in *C. elegans* due to the presence of an active glyoxylate cycle [10,12]. The sequencing of *C. elegans* genome predicted a putative ortholog of human AGXT gene in the nematode (ORF *T14D7.1*, [9] now renamed to *agxt-1*). However, the molecular properties of the protein product of *agxt-1* gene have not been previously investigated. Here, we have performed a side-by-side comparative study on the molecular properties of AGXT-1 protein in comparison with human hAGT1, showing that AGXT-1 is a functional PLP-dependent enzyme with aminotransferase activity and a higher activity and specificity towards the alanine:glyoxylate pair than hAGT1. Our results also support that both enzymes are

319 structurally and functionally alike, but show different protein stability and subcellular
320 localization, where AGXT-1 is mitochondrial and hAGT1 is peroxisomal.

321 The mitochondrial localization of AGXT-1 provides important insights into the
322 evolutionary adaptation of AGTs subcellular compartmentalization, and possibly, into its
323 relation with dietary origins of glyoxylate and the molecular origin of mitochondrial
324 mistargeting in PH1. AGT subcellular localization has represented a remarkable
325 conundrum for cell and evolutionary biologists and molecular pathologists. The AGTs
326 peroxisomal localization is attributed to a PTS1 located at the C-terminal domain, while the
327 mitochondrial localization is mainly controlled by the activation of a cryptic MTS in the N-
328 terminal domain that overrides the PTS1 route [1]. Therefore, the *AGXT* gene seems to
329 have evolved to meet dietary requirements, with alternative translation and transcription
330 sites to allow the protein to contain this strong MTS sequence [45]. In most omnivorous
331 mammals the AGT enzyme is distributed in mitochondria or peroxisomes, based on the
332 presence or absence of the MTS, respectively. In carnivorous mammals, the AGT
333 localization is mainly mitochondrial while a selective loss of the MTS is found in
334 herbivorous animals [46]. These evolutionary changes in subcellular distribution of AGT
335 associated with dietary changes have been recently exemplified by sequencing and
336 evolutionary analyses on different bat species with unparalleled dietary diversification [47].
337 Importantly, hAGT1 contains a very weak MTS, which becomes stronger in the presence
338 of the destabilizing P11L polymorphism and certain pathogenic mutations, that result into
339 mitochondrial mistargeting [16,48]. Unlike the behaviour found along evolution, which
340 seems to define the subcellular targeting of AGT using relatively simple transcriptional and
341 translational mechanisms, mitochondrial mistargeting of hAGT1 seems to depend strongly
342 on the cellular context. For instance, a given single genotype may lead or not to
343 mitochondrial import depending on the cell type and culture conditions [16,49,50]

344 highlighting the important role of molecular chaperones and/or other factors of the protein
345 homeostasis network in the final fate of hAGT1 disease-causing variants [16,17].

346 Along evolution, enzyme properties are selected to provide appropriate metabolic
347 rates at different physiological temperatures by tuning some structure-function
348 relationships i.e., stability, enzyme activity and ligand affinity [51]. While the physiological
349 temperature of humans is kept constant at 37°C, the nematode *C. elegans* is an ectotherm
350 organism that can survive between 8-27°C and whose physiology is highly affected by the
351 environmental temperature. The lower denaturation temperature and high catalytic activity
352 of AGXT-1 likely reflects temperature-adaptation [52]. Moreover, the similar apparent
353 affinities for natural substrates of hAGT1 displayed by AGXT-1, suggest resemblance in
354 substrate concentration or K_M :[substrate] ratio, at their respective organelles [53] . It must
355 be noted that the activity and intracellular turnover of hAGT1 must be tuned to be
356 appropriate at a customarily constant temperature of 37°C, while the conformational
357 stability of AGXT-1 seems to be adapted to lower temperatures. These results may
358 indicate that the overall stability of different AGT orthologs is optimized to provide an
359 adequate intracellular turnover at optimal growth temperature, which in the case of hAGT1
360 is severely compromised by disease-associated mutations leading to protein misfolding
361 and mistargeting [2,16,40,54].

362 The main biological function of hAGT1 is to create a glyoxylate sink in peroxisomes
363 of hepatocytes. In addition, there is a set of proteins that contribute to this human
364 glyoxylate metabolism. According to KEGG (Kyoto Encyclopedia of Genes and Genomes)
365 and REACTOME Pathway Database, these proteins are D-amino acid oxidase (hDAO),
366 hydroxyacid oxidase (hHAO), glyoxylate reductase / hydroxypyruvate reductase
367 (hGRHPR), D-4-hydroxy-2-oxoglutarate aldolase (hHOGA1), alanine:glyoxylate
368 aminotransferase 2 (hAGT2) and lactate dehydrogenase (hLDH) (Figure 9). Despite the

369 key role of glyoxylate cycle in *C. elegans* [55] (Figure 9), surprisingly the genome of the
370 nematode encodes orthologous proteins to those already described for the human
371 glyoxylate metabolism, with the only exception of mitochondrial hHOGA1 protein (Table 4).
372 Therefore the main routes of glyoxylate metabolism in humans are expected to work in the
373 nematode, at least to some extent. Some phenotypes have been described by large-scale
374 gene downregulation in *C. elegans*, such as the association of C31C9.2 (ortholog of
375 hGRHPR) [9] with low embryonic lethality [56] and slow growing [57], or T09B4.8
376 (ortholog of hAGT2) linked to a reduction of fat content [58]. However, according to the
377 WormBase Consortium (www.wormbase.org - WS249) no altered lethality, fertility or
378 development has been described to the lack of function of AGXT-1, F41E6.5 (ortholog of
379 hDAO), DAAO1 and LDH-1 proteins. This suggests that the steps of glyoxylate
380 metabolism that are catalysed by these enzymes may not be essential for the normal
381 development and metabolism of the nematode. Nonetheless, the relative contributions of
382 these enzymes to different biochemical pathways requires knowledge on their enzymatic
383 properties and regulation, expression levels and metabolic fluxes, which can be
384 developmental and environmental dependent [59]. Alternatively, there are no orthologous
385 proteins in humans to the key *C. elegans* enzyme ICL-1, although a human malate
386 synthase activity has been detected with unknown biological function [60].

Figure 9. **Main routes of glyoxylate metabolism in human hepatocytes and nematode body wall muscle cells.** AGT: alanine:glyoxylate aminotransferase, DAO: D-amino acid oxidase, HAO1: hydroxyacid oxidase, GRHPR: glyoxylate reductase / hydroxypyruvate reductase, HOGA1: D-4-hydroxy-2-oxoglutarate aldolase, AGT2: alanine:glyoxylate aminotransferase 2, LDH: lactate dehydrogenase and ICL-1: bi-functional isocitrate lyase:malate synthase.

387 In *C. elegans*, GC bypasses the decarboxylation steps of TCA cycle supplying a net
 388 synthesis of 4-carbon compounds and thus supporting gluconeogenesis by incorporating
 389 acetyl-CoA (particularly from β -oxidation of fatty acids in peroxisome and mitochondria)
 390 and supplying succinate to TCA cycle and malate to the gluconeogenesis pathway [13,14].

391 The GC activity is increased during embryogenesis, L1 larvae and dauer diapause, while
392 TCA cycle is increased in the L2, L3 and L4 stages, when energy demands and food
393 intake are increased [14]. The GC and TCA cycles share some enzymatic activities
394 (malate dehydrogenase, citrate synthase and aconitase), while some enzymes, ICL-1
395 (GC) and isocitrate dehydrogenase (TCA), compete for the same substrate (isocitrate).
396 Therefore the relative activity of these enzymes controls the ratio carbon flux through both
397 cycles (GC and TCA) [55]. Historically, unlike other nematodes [61], key enzymes of GC in
398 *C. elegans* have been considered to be compartmentalized in glyoxysome-like
399 microbodies by being found in a heavier band (apart from mitochondrial markers) in
400 isopycnic centrifugation [62] . However, it has been recently demonstrated that C-terminal
401 GFP tagged ICL-1 protein co-localizes with mitochondrial dye MitoTracker [63]. This
402 preview notion of a physical separation between GC and TCA enzymes implied either the
403 existence of isoforms of those shared enzymes activities in glyoxysomes or the
404 transportation of metabolites (e.g., isocitrate and succinate) across organelles membranes
405 [64], which was hypothesized to function as a metabolic regulatory mechanism [65]. The
406 existence of a mitochondrial alanine:glyoxylate aminotransferase (AGXT-1) and ICL-1
407 activities may imply a direct competition of both proteins for glyoxylate as substrate that
408 may participate in carbon flux through TCA and GC cycle pathways. The transamination of
409 glyoxylate into glycine by AGXT-1 could provide an alternative pathway for glyoxylate
410 conversion and also an additional way to modulate levels of glyoxylate in mitochondria,
411 which would otherwise be toxic and may affect the regulation of the TCA cycle by inhibiting
412 enzymes such as ketoglutarate dehydrogenase [66]. In humans, the hAGT1 activity is
413 known to act in the detoxification of glyoxylate within peroxisomes of hepatocytes, while
414 the presence of an active glyoxylate cycle in the nematode opens the possibility of
415 alternative metabolic roles for the AGXT-1 protein. To further characterize the role of

416 AGXT-1 on different metabolic pathways and developmental conditions, a detailed
 417 analysis of metabolites in cultures of *icl-1* and *agxt-1* mutants should be approached.

Table 4. **Orthologous proteins in *C. elegans* found from a BLASTP of human proteins involved in glyoxylate metabolism.** GI numbers of protein sequence used are: hAGT- 126522481; hDAO- 148539837; hHAO- 11068137; hGRHPR- 6912396; hHOGA- 31543060; hAGT2- 119576316; hLDH- 32693754. n/a, not available.

<i>H. sapiens</i>			<i>C. elegans</i>			
Protein	Residues	Localization	Protein	Residues	Identity	E value
hAGT	392	Perox.	AGXT-1	405	44%	$5 \cdot 10^{-115}$
hDAO	347	Perox.	DAAO1	322	35%	$8 \cdot 10^{-61}$
hHAO	370	Perox.	F41E6.5b	371	46%	$6 \cdot 10^{-115}$
hGRHPR	328	Perox./Cyt.	C31C9.2	322	30%	$1 \cdot 10^{-29}$
hHOGA	327	Mito.	Not found	n/a	n/a	n/a
hAGT2	514	Mito.	T09B4.8	444	53%	$1 \cdot 10^{-165}$
hLDH	332	Cyt.	LDH-1	333	54%	$6 \cdot 10^{-119}$

418 Table 5. **Glyoxylate and oxalate levels lysates of wild-type and mutant strains grown**
 419 **with glyoxylate.**

Strain	nmol glyoxylate/mg total protein	nmol oxalate/mg total protein
N2 Bristol	2.48	21.8
<i>agxt-1</i>	2.88	35.8
<i>icl-1</i>	2.22	26.9
<i>agxt-1; icl-1</i>	3.35	30.2

420 *C. elegans* is a remarkably useful model system to understand protein folding
 421 diseases and their pharmacological correction [67–69]. Our studies advance that the
 422 nematode *C.elegans* could be a model for PH1. Although simultaneous inactivation of
 423 *agxt-1* and *icl-1* genes does not result in a clear oxalate accumulation (see Table 5), we
 424 cannot rule out the possibility of a hyperoxaluric phenotype under the appropriate stress..
 425 Alternatively, models expressing disease-associated variants of hAGT1 in *C. elegans* may
 426 also provide a convenient *in vivo* platform to explore and dissect the complex protein
 427 homeostasis defects associated with PH1-causing mutations.

Acknowledgments.

We thank Prof. Jose Manuel Sanchez-Ruiz for support. This work was supported by the Spanish Ministry of Science and Innovation (CSD2009-00088, BIO2012-34937 and SAF2011-23933), Junta de Andalucia (P11-CTS-7187), FEDER Funds and the Oxalosis and Hyperoxaluria Foundation (to B.C.). A. L. P. acknowledges a Ramon y Cajal research contract (RyC2009-04147) from the Spanish ministry of Science and Innovation and the University of Granada. N. M-T acknowledges a FPI predoctoral fellowship from the Spanish Ministry of Science and Innovation. A.C.C. and N.T. were supported by the grant IOS-1353845 from the National Science Foundation (NSF). N.T. acknowledges the Tetelman Fellowship for International Research on the Sciences awarded by Yale University.

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