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Molecular insights into primary hyperoxaluria type i pathogenesis

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1. ABSTRACT

Primary hyperoxaluria type 1 (PH1) is a rare autosomal recessive disorder of glyoxylate metabolism caused by the deficiency of liver peroxisomal alanine:glyoxylate aminotransferase (AGT), a pyridoxal 5'-phosphate (PLP)–dependent enzyme. The PH1 pathogenesis is mostly due to single point mutations (more than 150 so far identified) on the *AGXT* gene, and is characterized by a marked heterogeneity in terms of genotype, enzymatic and clinical phenotypes. This article presents an up to date review of selected aspects of the biochemical properties of the two allelic forms of AGT and of some PH1-causing variants. These recent discoveries highlight the effects at the protein level of the pathogenic mutations, and, together with previous cell biology and clinical data, (i) improve the understanding of the molecular basis of PH1 pathogenesis, and (ii) help to delineate perspectives for predicting the response to pyridoxine treatment or for suggesting new strategies for PH1 patients bearing the analyzed mutations.

2. INTRODUCTION

Pyridoxal 5-phosphate (PLP), a vitamer of vitamin B6, is a very versatile coenzyme acting on more than 140 enzyme-catalyzed human reactions involved in the metabolism of amino acids, biogenic amines, amino sugars and tetrapyrrolic compounds (1). In PLP-catalyzed reactions the cofactor is covalently bound to the apoenzyme by a Schiff base linkage with a lysine residue and functions as an electronic sink able to stabilize negatively-charged reaction intermediates thanks to the positive charge of its pyridine nitrogen. The outstanding involvement of PLP in physiological processes is also proven by the variety of inborn errors of metabolism affecting PLP-enzymes among which homocystinuria, caused by the deficiency of cystathionine-β-synthase, and sideroblastic anemia, caused by the deficiency of δ-aminolevulinic acid synthase, are well known examples (2).

3. PRIMARY HYPEROXALURIA TYPE I

Primary Hyperoxaluria Type I (PH1) is a rare autosomal recessive disorder of glyoxylate metabolism

whose prevalence in European population is estimated as 1-3 per million. The disease is caused by the deficiency of liver peroxisomal alanine:glyoxylate aminotransferase (AGT), a PLP-dependent enzyme that catalyzes the conversion of L-alanine and glyoxylate to pyruvate and glycine, respectively. The absence of functional AGT allows glyoxylate to be oxidized to oxalate in liver cytosol. Oxalate is an end-product of metabolism and is removed from the body mainly by renal excretion. In PH1, the increased oxalate concentration in urine leads to the formation and deposition of insoluble calcium oxalate crystals, resulting in nephrocalcinosis and urolithiasis. This could lead to renal failure that in turn allows the progressive calcium oxalate deposition in various body sites, a potentially fatal condition named systemic oxalosis (3) .

AGT is encoded by the *AGXT* gene, located on chromosome 2q37.3, and is present in human populations as two polymorphic variants, the "major allele" (AGT-Ma) and the less common "minor allele" (AGT-Mi) (4-5). The frequency of the minor allele can vary in different populations ranging from a 28% in the Sami North Sweden population to about 2-3% in Japanese people; the average frequency in European and North American populations is about 20%. AGT-Mi differs from AGT-Ma by a 74-bp duplication in intron 1 and by the presence of the mutations 32C→T and 1020A→G leading to the Pro11Leu and Ile340Met amino acid substitutions, respectively (6-7). More than 150 different pathogenic mutations on the *AGXT* gene leading to PH1 have been identified so far, that encompass nonsense, frameshift and missense mutations. While nonsense and frameshifts are null mutations that lead to the complete loss of the gene product, the most common type of *AGXT* mutations are single amino acid substitutions that lead to the synthesis of an aberrant gene product (8). Many PH1-causing mutations, co-segregate and functionally interact with the minor allele polymorphism. In fact, although the presence of the AGT-Mi polymorphism is not pathogenic *per se*, it makes AGT more susceptible to the effect of several missense mutations, which are predicted to be not pathogenic in the absence of the polymorphism (9) (see below).

PH1 is a life-threatening and difficult to treat disease. Classical treatments, aimed to decrease the amounts of oxalate in the body and to prevent kidney failure or to restore kidney functionality, by dialysis or kidney transplantation, are not curative being only directed to the symptoms of the disease . Available treatments for PH1 addressed to the cause of the disease are pyridoxine (Vitamin B6) therapy and liver transplantation (3). Pyridoxine is converted in the body to PLP, the essential cofactor of AGT, but the molecular mechanism of action of this molecule in PH1 patients is nowadays unknown. Moreover, only a minority (10-30%) of patients respond to pyridoxine therapy, and clinical studies seem to indicate that responsiveness is confined to mutations that result in AGT mistargeting (10-11). The only curative approach available for PH1 patients unresponsive to pyridoxine is liver transplantation, which reintroduces most of the body's requirements of AGT but represents a very wasteful intervention because an entire organ is employed to replace only one defective gene.

Notwithstanding the progress made during the last three decades, which has greatly improved the clinical management of PH1 patients, several issues still need to be addressed. One of the most important is the definition of a clear relationship between the genotype, the enzymatic phenotype and the clinical phenotype. In fact PH1 is a disease endowed with a great heterogeneity at both the clinical and the enzymatic phenotype level. The course of the disease can be very different in patients that share the same genotype, thus suggesting a role of genetic or environmental factors, and a large clinical variation can be observed even within the same family (12). Moreover, a great variety of enzymatic phenotypes leading to AGT deficiency can be noticed. On the basis of cell biology analyses, they have been classified in three categories: mutations leading to the loss of both AGT catalytic activity and immunoreactivity, mutations leading to the loss of AGT catalytic activity but not immunoreactivity, mutations leading to the loss of neither AGT catalytic activity nor immunoreactivity (3). It has been demonstrated that in the latter case the disease is due to the aberrant localization of AGT to mitochondria, where the protein is catalytically active but is unable to detoxify the glyoxylate formed inside peroxisomes (13-15). However, the limited number of patients makes difficult to establish any clear correlation between a particular genotype and the effects of the mutation at the enzyme and clinical level (12).

Recently, several efforts have been made to improve the knowledge of the PH1 molecular pathogenesis starting from the detailed study of the biochemical features of AGT as the base to define how a particular amino acid substitution can alter protein's functional and structural properties (Table 1) (16-19). The results obtained have not only shed light on some molecular mechanisms leading to AGT deficiency but have also provided a worthwhile tool to predict the response to pyridoxine therapy and to suggest new treatment strategies for PH1 patients bearing the analyzed mutation.

4. BIOCHEMICAL PROPERTIES OF NORMAL AGT

AGT-Ma is a homodimeric protein each subunit of which, consisting of 392 residues, has a molecular mass of 43 KDa. As shown by the crystal structure of the enzyme in a complex with the competitive inhibitor amino-oxyacetic acid (20), AGT-Ma belongs to the Fold Type I class of PLP-dependent enzymes. Each subunit comprises three structural domains (Figure 1A): an N-terminal extension (residues 1-21) that wraps over the opposite subunit; a large domain (residues 22-282) that has a three layer βαβ architecture and forms a great part of the active site; a C-terminal small domain (residues 283-392) that has a two layer αβ structure and contains the signals for the peroxisomal localization of the protein. Like in all PLP-enzymes, the coenzyme is covalently bound to the apoprotein by a Schiff base linkage with Lys209 (internal aldimine), and its binding at the active site is stabilized by the ring-stacking interaction with the indole ring of Trp108,

Table 1. Summary of AGT variants described in this review

Construct	Sequence mutation	Amino acid sostitution	Major (Ma) or Minor (Mi) haplotipe
AGT-Ma			
AGT-Mi	c.32C>T c.1020A>G	Pro11Leu Ile340Met	
G41R-Mi	c.121G>A	Gly41Arg	Ma/Mi
G41V-Mi	c.122G>T	Gly41Val	Ma
G82E-Ma	c.245G>A	Gly82Glu	Ma
F152I-Mi	c.454T>A	Phe152Ile	Mi
G170R-Mi	c.508G>A	Gly170Arg	Mi

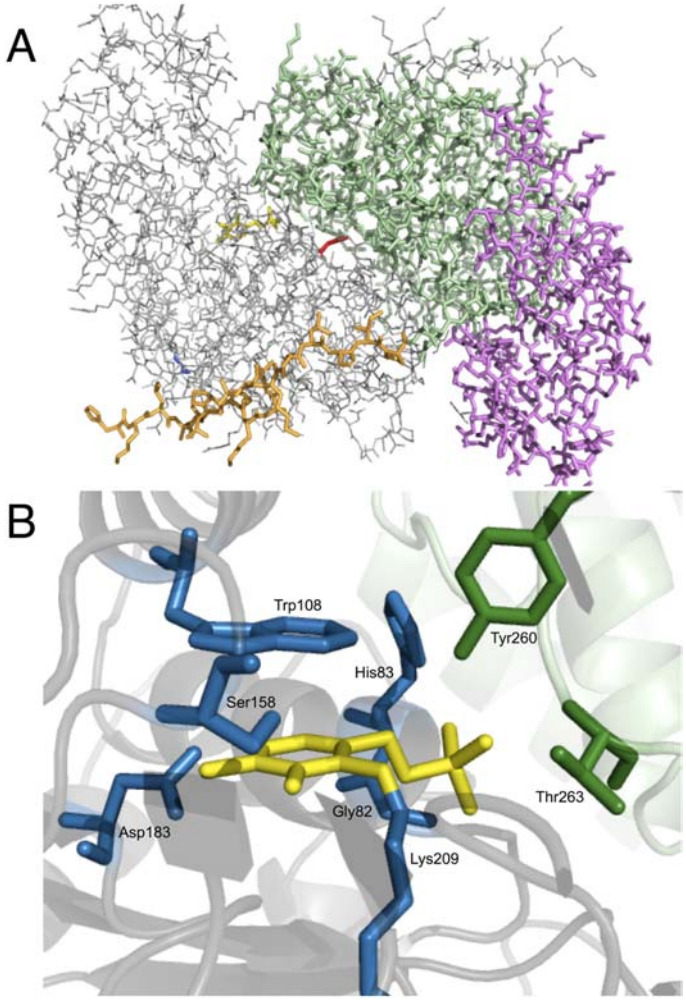

Figure 1. Crystal structure of human AGT-Ma (PDB code 1H0C (20)). A) Stick representation of the AGT dimer. One subunit is coloured white while in the other subunit the N-terminus (residues 1-21) is orange, the large domain (residues 22-282) is coloured green, and the small domain (residues 283-392) is coloured violet. PLP, Gly41 and Gly170 are coloured yellow, red and blue, respectively. B) Ribbon representation of the AGT-Ma active site with one subunit coloured gray and the opposite subunit coloured green. PLP is represented as yellow sticks. Active site residues directly interacting with the coenzyme are labelled and shown as sticks. Blue residues belong to the PLP-binding subunit while green residues belong to the opposite subunit. The figure was rendered using PyMol.

the salt bridge between the protonated pyridine nitrogen and Asp183 and the hydrogen bonds of the hydroxyl O3 of PLP with Ser158 and of the phosphate group of PLP with His83, Gly82, Tyr260* and Thr263* (the * stands for residues from the neighboring subunit) (Figure 1B). AGT-Ma shows a large dimerization interface (~23% of the solvent accessible area) that comprises both interactions between the two large domains and contacts between the N-terminal extension of one subunit and the large domain of the other (20).

A detailed picture of the mechanism of action of AGT-Ma has been given by means of spectroscopic, kinetic and computational methods (16). Figure 2 reports the proposed mechanism of the overall transamination reaction catalyzed by the enzyme. In the first half-reaction, upon L-

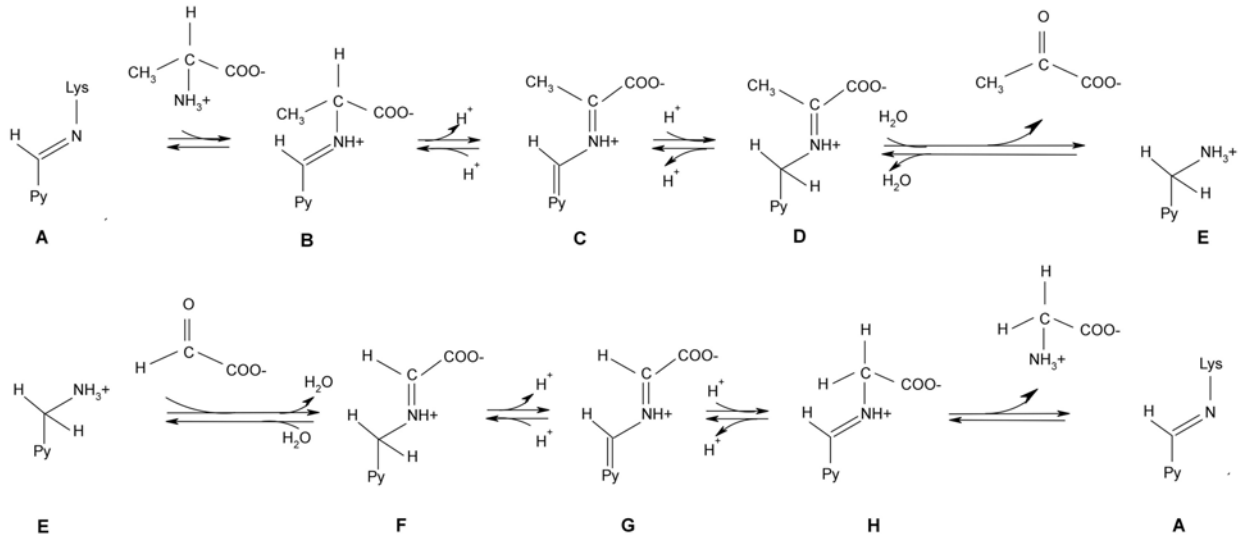

Figure 2. Kinetic mechanism of the overall transamination catalyzed by AGT (16). A: AGT-PLP; B: L-alanine external aldimine; C: alanine-PLP quinonoid intermediate; D: pyruvate ketimine intermediate; E: AGT-PMP; F: glyoxylate ketimine intermediate; G: glycine-PLP quinonoid intermediate; H: glycine external aldimine.

alanine binding, the AGT-PLP internal aldimine (A) is converted into the external aldimine (B). Then, the L-alanine α-proton extraction by a base catalyst generates the resonance-stabilized quinonoid intermediate (C), which is reprotonated at the C4' of the coenzyme to give the ketimine intermediate (D). The latter is then hydrolyzed to give pyruvate and the pyridoxamine 5'-phosphate (PMP) form of the coenzyme (E). In the second half-reaction, the AGT-PMP complex (E) binds glyoxylate and, by the same steps of the first half-reaction but in reverse direction (E→F→G→H→A), it is converted to AGT-PLP and generates glycine. Steady-state and presteady-state kinetic studies indicated that, in analogy with other aminotransferases, the overall transamination catalyzed by AGT-Ma follows a ping-pong kinetic mechanism whose rate-limiting step is the formation of ketimine or its hydrolysis. Moreover, the equilibrium constant of the overall transamination was found to be ~9400, and it was suggested that this value is mainly driven by the glyoxylate↔glycine equilibrium largely shifted toward glycine. These data, along with the finding that the k_{cat} for the pair alanine/glyoxylate (45 s^{-1}) is about 100-fold higher than that for the pair glycine/pyruvate (0.3 s^{-1}) led to the conclusion that AGT is highly specific for the glyoxylate-to-glycine conversion, in agreement with the proposed physiological role of the enzyme in glyoxylate detoxification (16). Like in many PLP-enzymes, the binding of the coenzyme at the AGT-Ma active site gives rise to specific absorbance and dichroic signals. In particular the AGT-PLP complex is characterized by an absorbance band at 420 nm associated with a positive dichroic signal centered at 429 nm, while the AGT-PMP complex is characterized by an absorbance band at 335 nm associated with a positive dichroic signal centered at 320 nm (Figure 3). Fluorescence and circular dichroism studies indicated that AGT has an higher affinity for PMP ($K_{D(PMP)}$ << 100 nM) than for PLP ($K_{D(PLP)}$ = 270 nM) (Table 2) and that PMP remains tightly bound to the enzyme during the transamination reaction. Moreover, basing on bioinformatic analyses, it has been suggested that subtle rearrangements of active site aromatic residues and a tilting of the coenzyme moiety could occur during the AGT-PLP↔AGT-PMP interconversion. These structural changes could be very important to prevent PMP release and allow a more efficient glyoxylate-to-glycine conversion (16).

AGT-Ma is characterized by a wide substrate and reaction specificity. Indeed, besides L-alanine, the enzyme is able to utilize as amino donors several amino acids like L-serine, L-phenylalanine, L-arginine, L-glutamate and L-aspartate, although with different catalytic efficiencies. Moreover, it catalyzes the α,β-elimination of β-chloro-L-alanine, with turnover times measured in seconds, and the β-elimination and half-transamination of L-cysteine, with turnover times measured in minutes (21).

5. EFFECTS OF THE POLYMORPHIC MUTATIONS P11L AND I340M ON THE AGT FUNCTIONAL AND STRUCTURAL PROPERTIES

The product of the minor allelic form of the *AGXT* gene, AGT-Mi, is characterized by the two point mutations P11L and I340M (Table 1). Although these mutations are not known to cause any clinical phenotype, the presence of the minor allele makes AGT more susceptible to the effects of many PH1-associated mutations that are pathogenic only when associated with the minor allele (9). This explains why many investigations have been carried out to understand the differences between the two allelic forms of AGT at a cellular and molecular level.

Studies performed on human hepatocytes (9, 15) expressing the major and the minor allele of AGT indicate

Table2, Steady-state kinetic parameters and equilibrium coenzyme binding constants of AGT-Ma, AGT-Mi and variants

	k_{cat} (s^{-1})	$K_{M\ L\text{-}Ala}$ (mM)	$k_{cat}/K_{m\ L\text{-}alanine}$ ($mM^{-1}s^{-1}$)	$K_{M\ Glyoxylate}$ (mM)	$k_{cat}/K_{m\ Glyoxylate}$ ($mM^{-1}s^{-1}$)	$K_{D\ PLP}$ (μM)	$K_{D\ PMP}$ (μM)
AGT-Ma	45 ± 2[1]	31 ± 4[1]	1.4 ± 0.2	0.23 ± 0.05[1]	196 ± 43	0.27 ± 0.03[1]	< 0.1[1]
AGT-Mi	35 ± 2[2]	28 ± 2[2]	1.2 ± 0.1	0.22 ± 0.01[2]	159 ± 12	0.26 ± 0.02[2]	< 0.1[2]
G41R-Ma	19.5 ±0.5[3]	22 ± 2 [3]	0.89 ± 0.08	0.41 ± 0.04 [3]	48 ± 5	1.5 ± 0,4[3]	> 5mM[3]
G41R-Mi	10.8 ± 0.4[3]	30 ±5 [3]	0.36 ± 0.06	0.32 ± 0.02 [3]	34 ± 2	6.1 ± 0.5[3]	> 5mM[3]
G41V-Ma	17.7 ± 0.6[3]	42 ± 4[3]	0.42 ± 0.04	0.13 ±0.02[3]	136 ± 21	0.55 ± 0.01[3]	> 5mM[3]
G82E-Ma	0.07 ± 0.03	15 ± 2[1]	0.0047 ± 0.0021	0.15 ± 0.06[1]	0.47 ± 0.27	198 ± 50[1]	> 5mM[1]
F152I-Mi	37 ± 1	41 ± 1[2]	0.90 ± 0.03	0.25 ± 0.03[2]	148 ± 18	0.085 ± 0.001[2]	19 ± 4[2]
G170R-Mi	34 ± 1	36 ± 2[4]	0.94 ± 0.06	0.4 ± 0.1[4]	85 ± 21	0.4 ± 0.1[4]	N.D.

N.D., not determined, [1]from ref 16, [2]from ref. 19, [3]from ref. 18

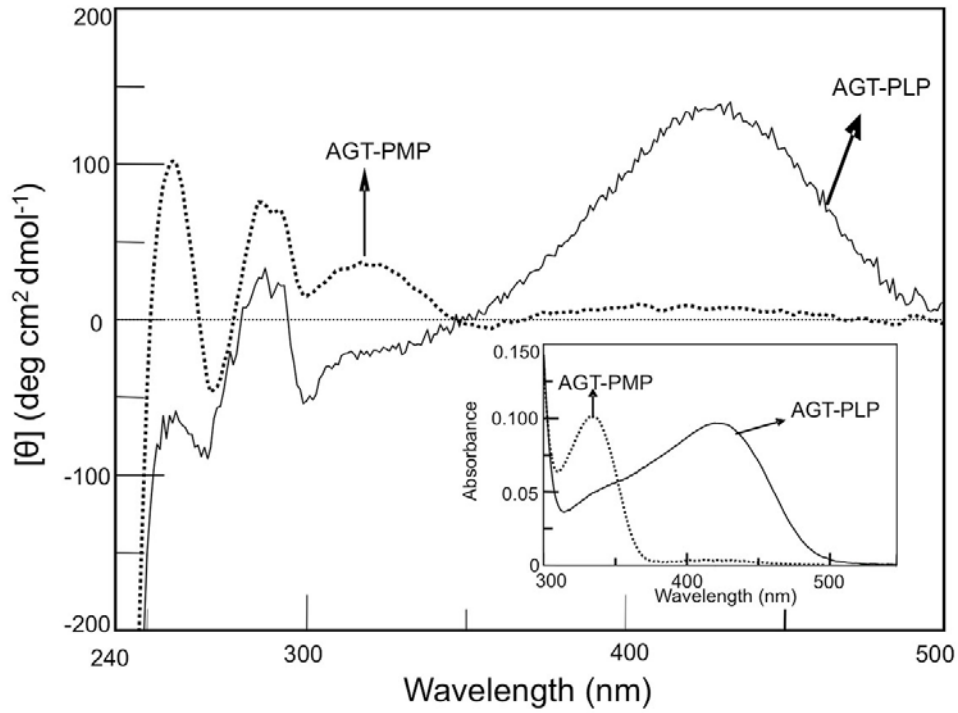

Figure 3. CD and absorbance spectra of AGT-Ma in the PLP and PMP form. UV-visible CD spectra of AGT-PLP (—) and AGT-PMP (▪▪▪▪) in 100 mM potassium phosphate buffer, pH 7.4 (16). Inset: UV-visible absorbance spectra of AGT-PLP (—) and AGT-PMP (▪▪▪▪) in 100 mM potassium phosphate buffer, pH 7.4. Enzyme concentration was 10 μM.

that AGT-Mi is characterized by a catalytic activity slightly lower (about 50-70%) than that of AGT-Ma as well as by an about 5% mistargeting of the protein from peroxisomes to mitochondria, which is due to the P11L substitution that generates a mitochondrial targeting sequence at the N-terminus of AGT. Moreover, it has been reported that AGT-Mi is less stable *in vivo* than AGT-Ma, as revealed by yeast complementation assays (22), and is more susceptible to proteolytic degradation and to aggregation, as revealed by pulse-chase and cross-linking experiments performed on cell-free transcription/translation products (23).

Recently, the biochemical properties distinguishing the two allelic forms of AGT have been thoroughly analyzed *in vitro* with purified recombinant AGT-Ma and AGT-Mi (19). These studies have indicated that the P11L/I340M mutations, typical of the minor allele, i) do not affect either the UV-visible absorbance, dichroic and fluorescence features of AGT or the $K_{D(PLP)}$ value, thus suggesting that no gross conformational changes have occurred and that the two species share a similar active site architecture and ii) these mutations induce a slight decrease (about 30%) of the k_{cat} value for the overall transamination of alanine and glyoxylate (Table 2). This is in agreement with the decreased enzymatic activity observed *in vivo*. Nevertheless, under conditions of chemical and thermal stress, a decreased stability of AGT-Mi with respect to AGT-Ma has been demonstrated. When the urea-induced equilibrium unfolding process of the two proteins was analyzed, both the holo and the apo-form of AGT-Mi were found to undergo dimer dissociation at lower urea concentrations with respect to the corresponding form of AGT-Ma, and that this destabilization is ascribable to the P11L mutation (17). It has been suggested that the substitution of Pro11 with a leucine residue would loosen the interaction of the N-terminal arm of one subunit of AGT with the large domain of the opposite subunit, thus facilitating dimer dissociation. This perturbation could also be transmitted to the AGT active site through a loop (residues 24-32) that contributes to the PLP binding site.

Table 3, Transition midpoint of thermal denaturation (Tm) and inactivation (Ti) of AGT-Ma, AGT-Mi and pathogenic variants

	Tm (°C)				Ti (°C)	
	DSC		CD (222nm)			
	Holo form	Apo form	Holo form	Apo form	Holo form	Apo form
AGT-Ma	77.3[2]	62.4[2]	N.D.	N.D.	77.4[1]	59.1[1]
AGT-Mi	73.2[2]	55.6[2]	73.6[3]	53.1[3] 66[3]	72.6[1]	52.2[1]
G41R-Ma	60.3[2]	57.6[2]	N.D.	N.D.	57.7[2]	53[2]
G41R-Mi	53.7[2]	N.D.	N.D.	N.D.	51.8[2]	46[2]
G41V-Ma	61.0[2]	58.3[2]	N.D.	N.D.	62.3[2]	54.5[2]
F152I-Mi	N.D.	N.D.	N.D.	N.D.	69.9[1]	52.4[1]
G170R-Mi	N.D.	N.D.	72.5[3]	48.2[3] 64[3]	N.D.	N.D.

N.D., not determined, [1]from ref 19, [2]from ref. 18, [3]from ref. 17

This could explain why P11L mutation induces PLP release from holoAGT-Mi at urea concentrations lower than those necessary for holoAGT-Ma. In addition, thermal denaturation of AGT-Ma and AGT-Mi has been performed using different procedures, including differential scanning calorimetry, CD-monitored thermal unfolding and thermal inactivation (Table 3). These experiments provided evidence that the P11L/I340M mutations decrease the thermal stability of both the holo- and the apo-form of AGT and that the decrease is driven by the Pro11-to-Leu substitution, in agreement with the chemical denaturation results (17-19).

Overall these analyses, besides elucidating the differences between AGT-Ma and AGT-Mi, represented the starting point to investigate the effect of pathogenic mutations associated with PH1 and to unravel the defects of the corresponding variants at molecular level.

6. LOSS OF AGT CATALYTIC ACTIVITY: THE G82E VARIANT

A 245G→A mutation on the major allele of the *AGXT* gene leading to the G82E substitution is one of the rarest PH1-causing mutations. Clinical data show that normal levels of correctly targeted AGT and the complete absence of transaminase activity represent the enzymatic phenotype typical of PH1 patients bearing G82E mutation (24). About ten years ago, Lumb et al. (9) reported that the purified G82E variant expressed in *E.coli* is devoid of catalytic activity and is unable to bind PLP. Gly82 is an active site residue whose peptide NH, along with the side-chain of His83, contributes to the binding of the coenzyme through hydrogen-bonding interactions with the phosphate group of PLP (Figure 1B) (20). Thus, it has been argued that the absence of catalytic activity in the variant is strictly related to the inability of the protein to bind PLP as a consequence of steric hindrance by the glutamate side chain. On 2007 (16), the detailed inspection of the biochemical properties of the purified recombinant G82E variant highlighted a more complex scenario. In fact, evidence has been provided that the G82E mutation greatly affects the binding affinity for both coenzymatic forms, as shown by the decrease of the equilibrium dissociation constant value for PLP and PMP in the variant of about 700 and 50000-fold, respectively (Table 2). Moreover, the absorbance and CD (Figure 4) spectral properties of G82E in the presence of exogenous coenzyme show that Gly82 to glutamate mutation significantly changes the microenvironment of the internal aldimine of AGT. These data have been explained by considering that Gly82 is part of a glycine loop that stabilizes the phosphate of PLP (20) and that the side chain of Glu82 in the variant could partially distort the active site thus altering coenzyme binding mode and affinity. Interestingly, the catalytic efficiency of the G82E variant for the alanine/glyoxylate pair measured in the presence of saturating exogenous PLP has been found to be about 0.1% that of AGT-Ma (Table 2). Kinetic analyses of the two half-transamination reactions allowed to infer that i) in contrast with what was observed with AGT-Ma, the rate-limiting step for the L-alanine half-transamination catalyzed by the G82E variant is the external aldimine formation (A→B in Figure 2); ii) the addition of a pre-formed PLP-L-alanine Schiff base could partially improve the half-transamination rate and iii) the glyoxylate half-transamination catalyzed by the apomutant in the presence of PMP occurs with a rate 1800-fold lower than that of AGT-Ma (16).

On the basis of all these data, it was concluded that the molecular defect of the G82E pathogenic variant does not lie in the inability to bind PLP, but rather in a perturbed active site microenvironment that causes a strong reduction of the coenzyme binding affinity and a decrease in the catalytic efficiency due to the slowing down of the amino acids transaldimination step and of the AGT-PMP to AGT-PLP conversion. Thus, the administration of pyridoxine does not seem to represent an ideal therapy to treat PH1 patients bearing G82E mutation, while the administration of a preformed external aldimine PLP-L-alanine could possibly be a better approach by bypassing the transaldimination step.

7. LOSS OF BOTH AGT CATALYTIC ACTIVITY AND IMMUNOREACTIVITY: THE GLY41 VARIANTS

Gly41 variants are three pathogenic forms of AGT characterized by the presence of the G41R point mutation, associated with either the major or the minor allele (G41R-Ma and G41R-Mi), or the G41V mutation associated with the major allele (G41V-Ma) (25-26). While no information are currently available about the *in vivo* enzymatic phenotype of patients bearing the G41R or the G41V mutation associated with the major allele, the analysis of liver biopsies of PH1 patients bearing the G41R mutation associated with the minor allele revealed the nearly complete absence of both AGT catalytic activity and immunoreactivity and the presence of amorphous core-like AGT aggregates inside patient's peroxisomes (25). As shown in Figure 1A, Gly41 is located at the end of the helix 34-42 running at the dimer interface, and the peptide group

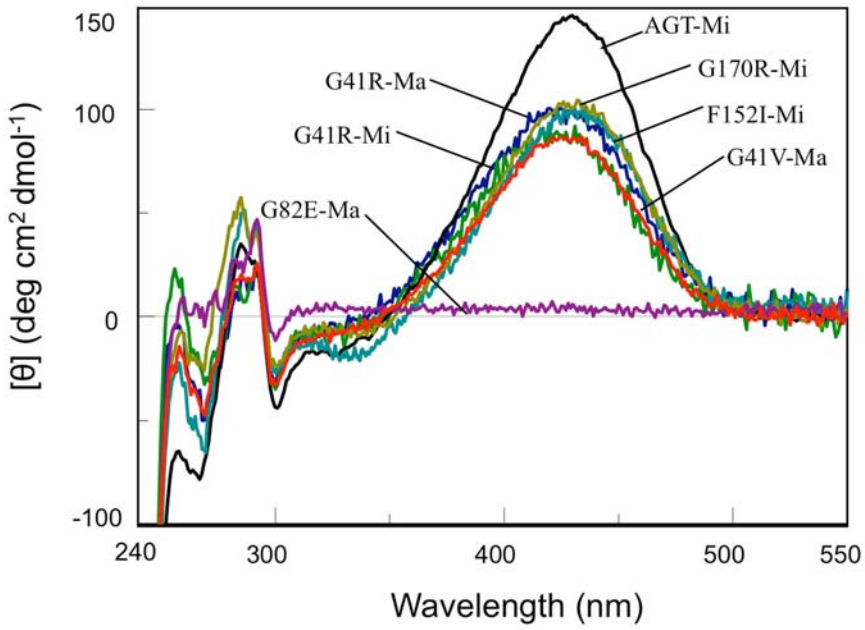

Figure 4. CD spectra of polymorphic and pathogenic variants of AGT in the holo-form (16, 18-19). UV-visible CD spectra of AGT-Mi (black), G82E-Ma (violet), G41R-Ma (blue), G41R-Mi (green), G41V-Ma (orange), G170R-Mi (yellow), F152I-Mi (cyan) in 100 mM potassium phosphate buffer, pH 7.4 at 10 μM enzyme concentration.

connecting Gly41 and Gly42 of one monomer is in van der Waals contact with the equivalent group of the other monomer. On these bases it has been proposed, but not proved, that the pathogenicity of Gly41 variants was linked to a dimer destabilization caused by the arginine or valine side-chains that would lead to a non-functional monomeric protein prone to degradation and aggregation (20). Coulter-Mackie et al. (23) reported that unlike AGT-Ma and AGT-Mi, Gly41 variants are subjected to proteasomal degradation as well as to an ATP-independent intracellular proteolytic activity when expressed by a cell-free transcription/translation system. Moreover, dimerization impairment for G41R-Ma and G41R-Mi was established by these authors on the basis of cross-linking and pulse-chase experiments. Recently, the molecular effect of Gly41 mutation on AGT has been investigated by studying the biochemical properties of purified recombinant Gly41 variants (18). Size-exclusion chromatography experiments did not allow us to detect any effect of the mutations on the dimerization of G41R-Mi, G41R-Ma and G41V-Ma variants in the holo-form, while they revealed an increase from 3 to about 20 fold in the monomer-dimer equilibrium dissociation constant value of the variants in the apo-form with respect to that of apoAGT-Ma or apoAGT-Mi. These results well agree with the proposed effect of the valine or arginine side chains introduced at the AGT interface on dimer stability. However, they also prove that, unlike what expected, Gly41 variants are able to form a dimer. Interestingly, the biochemical analyses showed that G41R-Ma, G41R-Mi and G41V-Ma in the dimeric form differ from AGT-Ma and AGT-Mi under several respects. In fact they display, even to a different extent, a reduced affinity for PLP and PMP, a reduced catalytic activity (Table 2), altered UV-visible absorbance, dichroic (Figure 4) and fluorescence features as well as a reduced thermostability (Table 3). Another striking feature that distinguish Gly41 variants from AGT-Ma and AGT-Mi comes from limited proteolysis experiments. In fact, the three variants are susceptible to both trypsin and proteinase K digestion. It has been shown that trypsin is able to cleave the Arg41-Gly42 or the Arg122-Val123 peptide bonds of G41R-Mi and G41R-Ma and the Arg36-Ile37 peptide bond of G41V-Ma (27). However, being the trypsin digestion performed on a cell-free transcription/translation product, it is not possible to understand whether the proteolytic degradation occurs on the monomeric or the dimeric form of each variant. On the other hand, proteinase K digestion, performed at protein concentrations in which Gly41 variants are in the dimeric form, results in the cleavage of the Met53-Tyr54 peptide bond (18). This suggested that the N-terminal region in Gly41 variants could be flexible and/or exposed to the solvent. In agreement with these results, the comparison between the conformational space sampled by the putative structures of AGT-Mi and G41R-Mi, carried out by high-temperature molecular dynamics simulations, suggested that the Gly41-to-Arg substitution in AGT could cause a marked increase in the fluctuation of the first 44 N-terminal residues (Figure 5) and their exposure to the solvent as well as a partial unwinding of the helix 34-42. On the other hand, no fluctuation of the N-terminal region can be seen for AGT-Mi. Overall, the above reported spectroscopic, kinetic and limited proteolysis data, along with the bioinformatic analysis, led to the conclusion that Gly41 mutation not only destabilizes the dimeric structure of AGT, but also induces some structural changes possibly related to the N-terminus of the protein in the dimeric form.

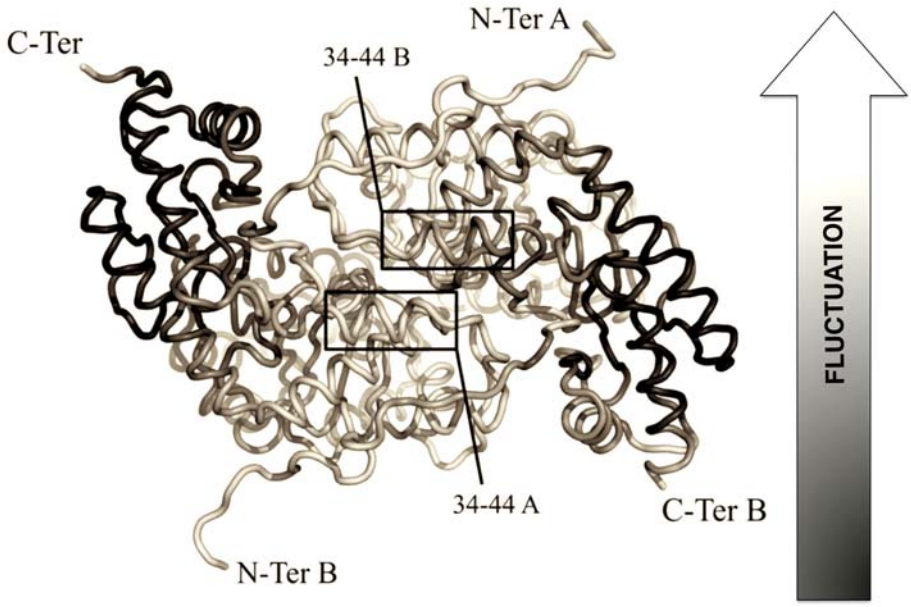

Figure 5. Average putative structure of G41R-Mi (18). The figure shows the protein backbone shaded in tones of gray, black meaning less movement and white meaning more movement, on the basis of the fluctuation observed by molecular dynamics simulations. The position of the helix 34-42 containing the mutated residue is highlighted.

Liver biopsies of patients bearing G41R mutation on the background of the minor allele are characterized by the presence of intra-peroxisomal amorphous core-like structures that stain for AGT but are unreactive against other six peroxisomal enzymes, as shown by immunoelectron microscopy. These data led to the conclusion that G41R mutation is associated with an AGT intra-peroxisomal aggregation (25). Moreover, the authors found that the presence of these cores correlated with the presence of high-molecular weight bands detected by the anti-AGT antibody on western-blot analyses. In order to shed light on the aggregative behaviour of Gly41 variants, the molecular dimensions of the mutants were studied by dynamic light scattering (DLS) under physiological conditions of temperature, ionic strength and pH (18). These studies showed that G41R-Ma, G41R-Mi and G41V-Ma, both in their holo- and apo-forms, are prone to aggregation and that the associative process is driven by inter-molecular electrostatic interactions. In fact, although DLS does not allow to estimate the absolute percentage of high molecular weight aggregates (~5000 Da) in the sample, the rate and the extent of the time-dependent changes in the total count rate increase as the ionic strength decreases.

A model to explain this propensity to aggregate of Gly41 variants has been proposed. Figure 6 compares the possible dimerization pathway of AGT-Ma with that of Gly41 variants. In both cases, the folded monomer M could either bind PLP and then dimerize (route M→ M_{PLP}→ D_{PLP}) or dimerize and then bind PLP (route M→ D → D_{PLP}). In the case of Gly41 variants, electrostatic potential surface calculations have revealed that the fluctuation of the N-terminus caused by Gly41 mutation would uncover several negatively charged residues on both the holo and the apo-form of the AGT dimer. This would convert the positively charged surface of AGT into a surface that displays a dipole segregation of charges. The negative patches could possibly interact with positive patches of neighbouring dimers, thus inducing the electrostatically-driven aggregation of the protein according to the model depicted on panel B of Figure 6 (18). However, the physiological meaning of this proposed mechanism of aggregation is currently unknown. Moreover, it remains to be established if the *in vivo* aggregation is due to electrostatic forces and if the aggregates have a structure similar to those formed *in vitro*.

Altogether, the above described results allow to propose a plausible model that could explain how the Gly41 mutation leads to AGT deficiency. Indeed, they clearly indicate that the enzymatic defect of the Gly41 variants does not only rely on the destabilization of the AGT dimeric structure, but also on a structural change probably related to the N-terminus causing the susceptibility of the variants to proteolytic degradation and their propensity to aggregate under physiological conditions. Although data from Coulter-Mackie et al. (27) suggest that the presence of PLP could decrease the sensitivity of Gly41 variants to trypsin, pyridoxine therapy alone does not seem to be sufficient to treat PH1 patients bearing mutation at Gly41 because the molecular defects of the variants are related to both their holo- and apo-forms. Rather, a promising approach could be the administration of small molecules able to stabilize the native state of the protein. Indeed, some preliminary data indicate that osmolytes, like trimethylamine-N-oxide and betaine, are able to partially reduce the aggregation extent of Gly41 variants (18). Although one should bear in mind that both these molecules are not suitable for clinical use because of

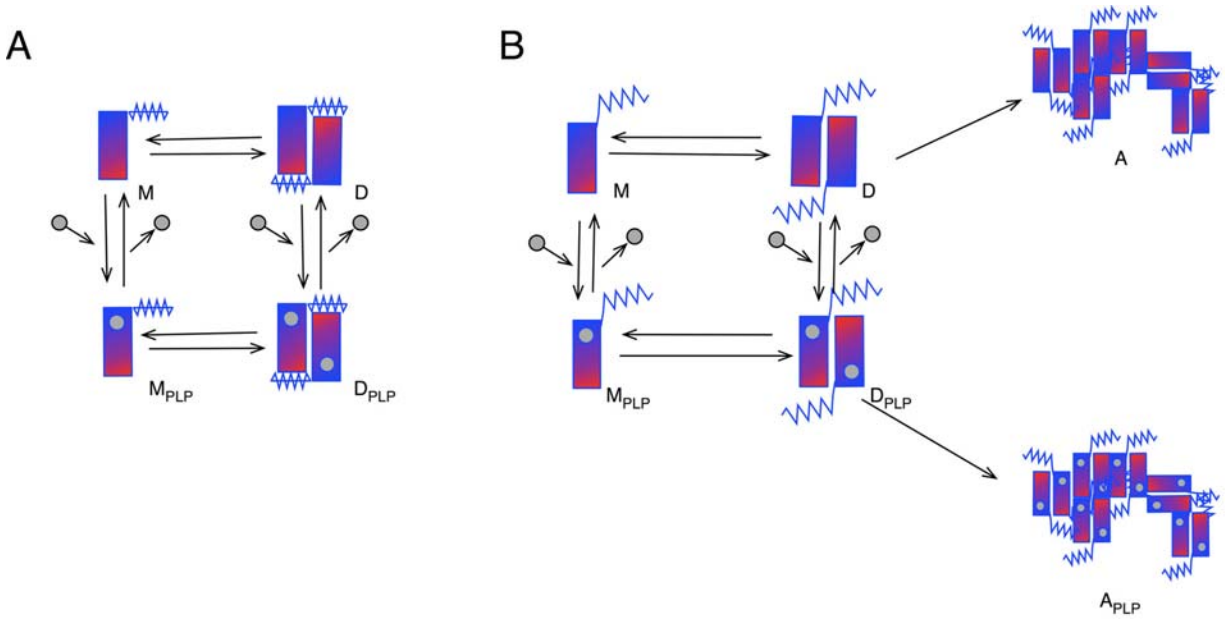

Figure 6. Proposed dimerization pathways of AGT-Ma (A) and of Gly41 variants (B). The blue and red colour in the folded protein subunits represent positively and negatively charged surfaces, respectively. M: folded monomer; M_{PLP}: PLP-bound monomer; D: apodimer; D_{PLP}, holodimer; A: insoluble aggregates; A_{PLP}: insoluble PLP-bound aggregates; ●: PLP

their poor specificity, they provide a good proof-of-principle for the development of a new strategy to counteract the disease. In this regard, it should be mentioned that the second most common mutation causing PH1, the 731T→C nucleotide substitution leading to the I244T amino acid replacement on the minor allele, causes AGT misfolding and aggregate formation that can be at least partially reversed by the use of small osmolytes, particularly betaine (28). However, these studies have been performed in different eukaryotic cell lines stably expressing the I244T-Mi variant and the detailed analysis of the *in vitro* aggregation propensity of the variant in the recombinant purified form is still lacking. Thus, it is not possible to define if the aggregation mechanism is similar to that observed on Gly41 variants.

8. MISTARGETING OF AGT: THE F152I-MI AND THE G170R-MI VARIANTS.

A large subclass of PH1 patients, corresponding to about one third of the total, displays an enzymatic phenotype in which neither the loss of AGT catalytic activity, nor the loss of immunoreactivity can be noticed. The point mutations on the *AGXT* gene associated with this phenotype are G170R, the most common PH1-causing mutation, and F152I. Both mutations cosegregate with the minor allele polymorphism and are supposed to be innocuous when associated with the major allele (15, 25, 29-31).

The enzymatic phenotype of the G170R-Mi variant has been widely studied during the last twenty years. The analysis of AGT subcellular localization on liver biopsies of patients bearing G170R mutation on the minor allele as well as on COS cells transiently expressing the G170R-Mi variant led to the conclusion that the molecular defect associated with this mutation is the mislocalization of the protein (32). The G170R-Mi variant, instead to be correctly targeted to peroxisomes, localizes to mitochondria where it is unable to fulfill its physiological role of glyoxylate detoxification. Cell biology investigations have proved the combined effect of the P11L polymorphic mutation and the G170R pathogenic mutation on the abnormal mitochondrial targeting of the protein. The P11L substitution allows the N-terminus of AGT to adopt a conformation of an amphiphilic α-helix able to act as a mitochondrial targeting sequence (4), while the G170R substitution is supposed to delay AGT folding and dimerization enough to make the protein compatible with the mitochondrial import machinery. In fact, while peroxisomes import fully-folded proteins, mitochondria can only import partly folded monomeric proteins (33). Lumb et al. described how non-specific factors known to increase protein stability are able to correct the enzyme trafficking defect of the G170R-Mi variant, while treatments known to decrease protein stability exacerbate the mistargeting (34). In order to shed light on the mechanistic bases of AGT mistargeting, the crystal structure of the G170R-Ma variant has been solved at a resolution of 2.6 Å (35). It showed that the pathogenic mutation does not significantly change the overall structure of AGT, but induces local perturbations leading to the loss of the hydrogen-bonding interaction between Arg197 of one monomer and Lys5 of the other (Figure 7). Although these results did not allow these scientists to evaluate the combined effect of the P11L and G170R mutations, they provided a possible explanation to the suggested reduction in dimer stability induced by the pathogenic mutation. Recently, the effect of the G170R mutation on AGT-Mi has been studied by elucidating both the biochemical features and the stability under urea stress

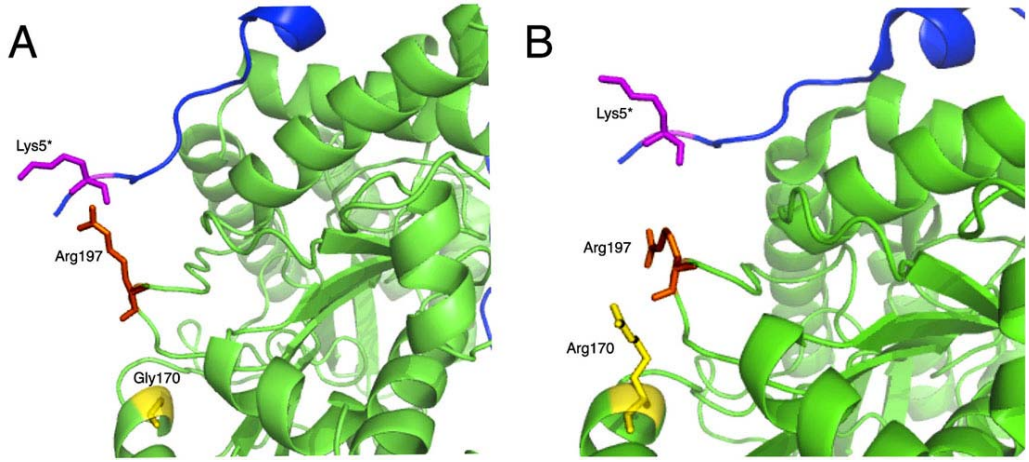

Figure 7. Localized effect of the G170R mutation on AGT (35). (A) Ribbon diagram of the AGT structure (PDB code 1H0C) showing the region in which Gly170 is located; (B) Ribbon diagram of the structure of the G170R-Ma variant (PDB code 1j04) showing the position of Arg170. The subunit comprising residue 170 is coloured green while the opposite subunit is coloured blue. Residues at position 5, 170 and 197 are labelled and coloured as purple, yellow and orange sticks, respectively. The * means that the residue comes from the other subunit.

of the two species (17). The results obtained indicated that the variant in the holo-form displays spectroscopic and kinetic features as well as PLP binding affinity analogous to those of AGT-Mi, thus confirming previous studies showing that the molecular defect of the variant does not consist in a loss of AGT functional activity. However, when the sensitivity to both urea and thermal stress of the variant was investigated, it was found that while holoG170R-Mi is indistinguishable from holoAGT-Mi, apoG170R-Mi shows a stability of the dimeric structure lower than that of apoAGT-Mi. In fact, monomerization occurs with a Cm of ~1.1 M urea for apoAGT-Mi and of ~1.4 M urea for apoG170R-Mi. Moreover, while the Tm of holoG170R-Mi is equal, within the limits of experimental error, to that of holoAGT-Mi, the Tm of the first transition of apoG170R-Mi is about 5°C lower than the corresponding value of apoAGT-Mi (Table 3). Thus, the enzymatic defect of the G170R-Mi variant is related to a lower stability of the dimeric structure of the apoenzymatic form of the protein. In agreement with these results, Pey et al. (36) reported that although holoG170R-Mi does not display gross conformational changes as compared with AGT-Mi, the apo-form of the variant shows an about 1000-fold increase in the thermal unfolding rate with respect to apoAGT-Mi. Moreover, when expressed in a mammalian cell-free system at neutral pH, the variant shows an higher tendency to interact with the Hsp70 and Hsp90 chaperones, thus suggesting that the reduced stability could be associated with a kinetic trapping of the variant in a partly-folded state. On these bases, it has been also suggested that this interaction with molecular chaperones in the cytosol could be responsible for the mistargeting of the G170R-Mi variant by facilitating the delivery of the protein to the mitochondrial import machinery.

Only few reports have been published describing the enzymatic phenotype associated with F152I mutation on the minor allele. A mistargeting defect similar to that observed for the G170R-Mi variant has been suggested for the F152I-Mi variant by studying liver biopsies of patients heterozygous for the F152I and G41R mutations on the minor allele background as well as patients homozygous for the F152I mutation (25). *In vitro* studies upon expression in *E.coli* have shown that the F152I-Mi mutant: (i) is prone to aggregation (9), thus suggesting that in the patients the variant could be unstable and rapidly degraded; (ii) has a catalytic activity of about 14% compared to that of AGT-Mi (27), and (iii) shows a low sensitivity to trypsin cleavage (27), thus suggesting that the protein should be largely in the folded state. In order to provide insights into the molecular defect of the F152I-Mi variant, as well as into the molecular synergism between the F152I mutation and the minor allele polymorphism, the effect of the mutation on the biochemical properties of the major and minor AGT allele has been investigated (19). The data obtained provided evidence that the Phe152 mutation does not significantly change neither the spectroscopic (Figure 4) and kinetic features of AGT, nor the PLP binding affinity (Table 2). It causes instead a strong reduction of the PMP binding affinity (Table 2), which leads to the progressive conversion of the variant from the holo- to the apo-form during the overall transamination reaction in the absence of exogenous PLP. In addition, the determination of the half-inactivation temperatures (Ti) of F152I-Mi and AGT-Mi, in both the holo and the apo-forms (Table 3) showed that the F152I-Mi variant in the apo-form has a 6°C reduction in the Ti with respect to apoAGT-Mi. Moreover, unlike apoAGT-Mi, apoF152I-Mi undergoes a time-dependent loss of enzymatic activity when incubated at 37°C, which is associated with a loss of the peak area on size-exclusion chromatography and is indicative of an aggregation event (Figure 8). The finding that the loss of activity and the aggregation extent increase at low protein concentrations led to the conclusion that both phenomena are due to protein monomerization. From these results it appears that the enzymatic defect of the F152I-Mi variant

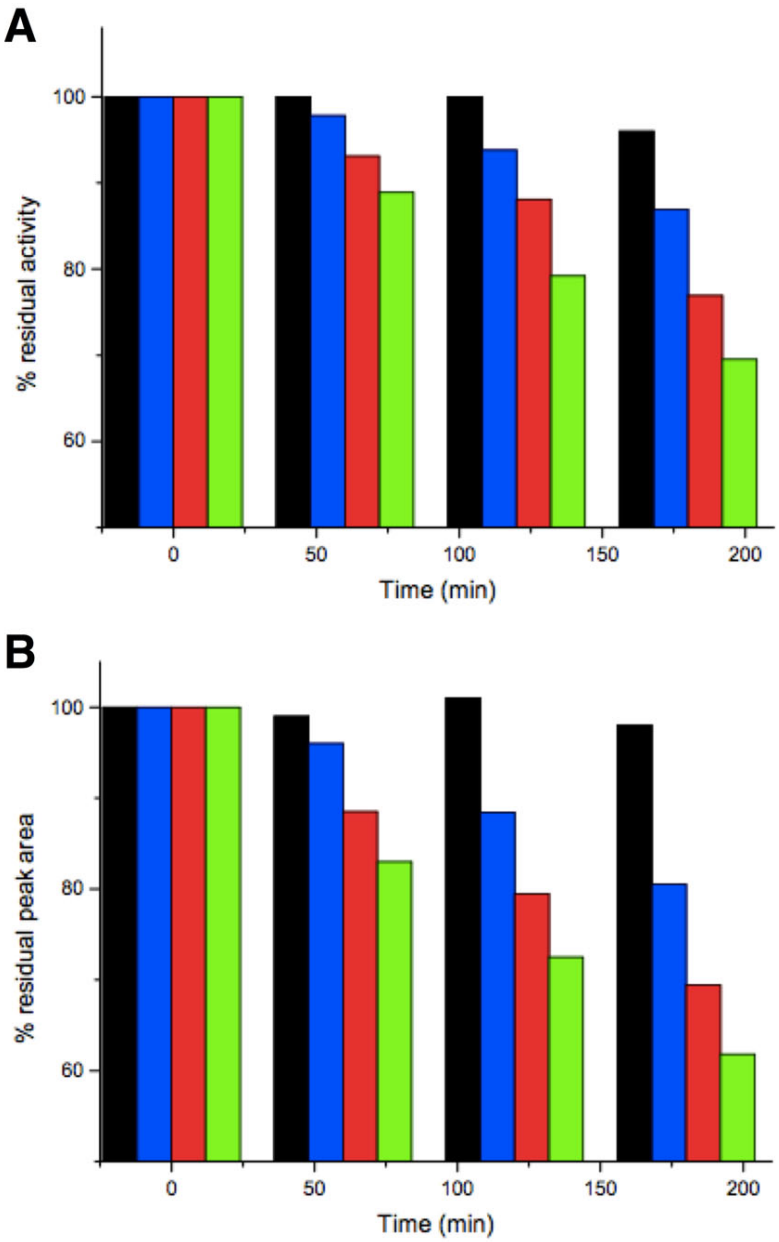

Figure 8. Histograms showing the time course of the loss of peak area on size-exclusion chromatography (A) and loss of transaminase activity (B) of apoF152I-Mi upon incubation at 37°C (19). ApoF152I-Mi was incubated at 37°C in 100 mM potassium phosphate buffer pH 7.4 at 1 μM (blue bars), 0.5 μM (red bars) or 0.25 μM (green bars) enzyme concentration. Aliquots were withdrawn at the indicated times and they were either assayed for transaminase activity or subjected to size-exclusion chromatography. Black bars represent the time course of transaminase activity and peak area for apoAGT-Mi at 0.25 μM enzyme concentration upon incubation at 37°C in 100 mM potassium phosphate buffer pH 7.4.

not only consists in the propensity of the variant to be converted into the apo-form during the overall transamination in the absence of added coenzyme, but also in the intrinsic instability of the apo-form at physiological temperature, due to protein monomerization.

Overall, the characterization of the G170R-Mi and F152I-Mi variants at a molecular level, along with previous evidence coming from cell biology and biochemical analyses, allows to propose a model that explains the pathogenic mechanism of the mutations and their synergism with the minor allele polymorphism. Figure 9 represents the possible routes that AGT could follow during its folding in the cytosol. Considering that vitamin B6 concentration in the whole blood is about 2 μM (23) and that monomeric species have probably a lower affinity for the coenzyme with respect to the dimeric ones, it can be postulated that the route M*→M→D→D_{PLP} should be

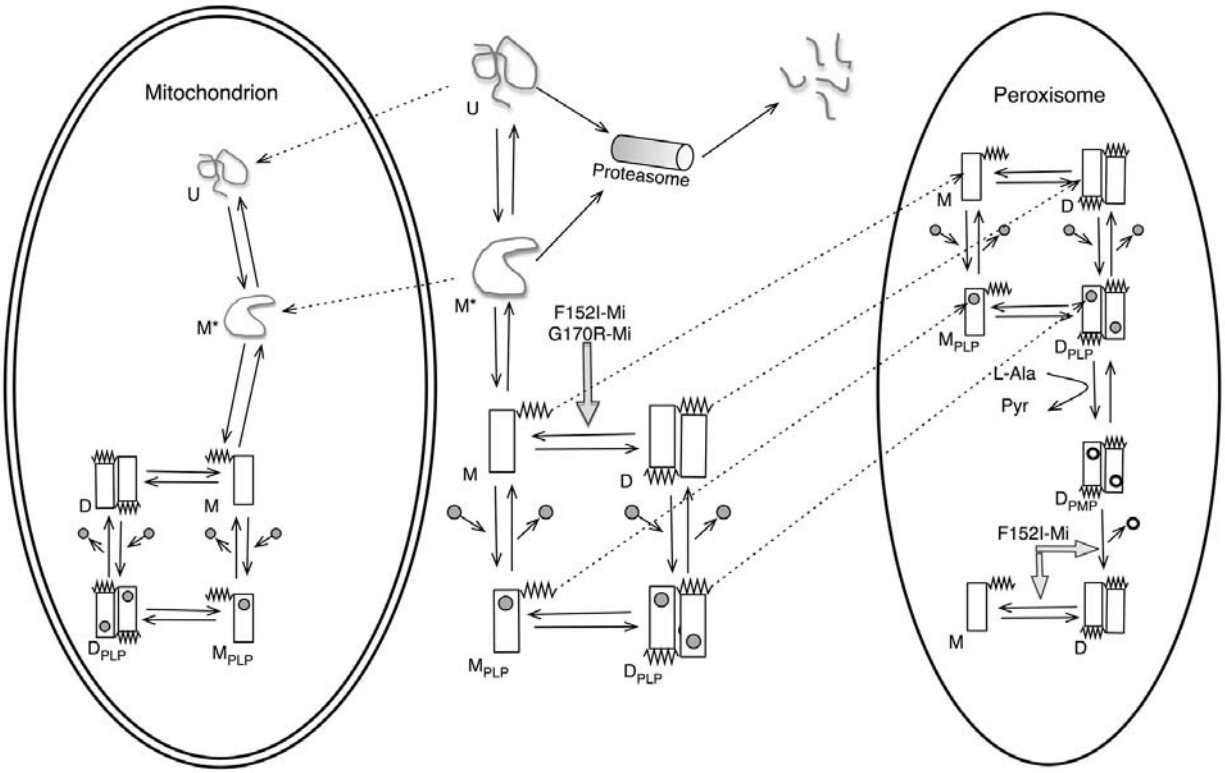

Figure 9. Proposed folding and targeting pathway of AGT. U: unfolded monomer; M*: partly folded monomer: M: folded monomer; M_{PLP}: PLP-bound monomer; D: apodimer; D_{PLP}, holodimer in the PLP form; D_{PMP}, holodimer in the PMP form; ●: PLP; ○: PMP. Gray arrows indicate the step (s) possibly affected by the G170R and/or F152I mutation.

preferred by AGT with respect to the M*→M→M_{PLP}→D_{PLP} route. U and M* are compatible with the mitochondrial import machinery, which recognizes N-terminal targeting sequences and acts on partly-folded monomeric species. On the other hand, M, M_{PLP}, D, and D_{PLP} are only compatible with the peroxisomal import machinery, that recognizes C-terminal targeting sequences and acts on folded species (32), even in the dimeric form. Nevertheless, it cannot be excluded that M* could be imported into peroxisomes. In the case of G170R-Mi, data indicate that the holoenzyme of the variant does not significantly differ from AGT-Mi, while the apoenzyme displays a lower stability of the dimeric structure. This implies that the step affected by the mutation should be the conversion of M* or M to D. The consequent accumulation of the M* or M species would synergize with the presence of the N-terminal putative MTS of the minor allele thus allowing more protein to be imported to mitochondria. For F152I-Mi, two defective aspects can be pointed out: i) the propensity of the apovariant to monomerize would lead to the accumulation of M* or M, in analogy to what proposed for the G170R-Mi variant, and ii) the low affinity of the variant for PMP would allow the protein to be converted into the apo-form inside peroxisomes, thus leading to inactivation.

On the basis of clinical reports published to date, the responsiveness to pyridoxine therapy has been demonstrated for patients bearing the G170R mutation on the minor allele, and suggested for patients bearing the F152I mutation on the minor allele (12, 31). Although it is known that pyridoxine can be converted to PLP in the human body, the molecular mechanism underlining the effectiveness of the treatment is unknown. Based on the proposed model for the pathogenicity of the G170R-Mi and F152I-Mi variants, it is possible to speculate that the increased levels of PLP in the cytosol could have: (i) a prosthetic role, by shifting the equilibrium from the apo- to the holo-form of the variants. For the F152I-Mi variant, this would also prevent apoenzyme formation during the overall transamination and (ii) a conformational role, by possibly binding to the monomeric species M* and M, and promoting the formation of D_{PLP} through the route M*→M→M_{PLP}→D_{PLP}. In each case, the final result would be the stabilization of the dimeric form of the protein, which is incompatible with the mitochondrial import but compatible with the peroxisomal import.

9. ACKNOWLEDGEMENTS

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10. REFERENCES

1. R. Percudani and A. Peracchi: A genomic overview of pyridoxal-phosphate-dependent enzymes. *EMBO Rep*, 4(9), 850-4 (2003)

2. A. C. Eliot and J. F. Kirsch: Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. *Annu Rev Biochem*, 73, 383-415 (2004)

3. C. J. Danpure: Molecular etiology of primary hyperoxaluria type 1: new directions for treatment. *Am J Nephrol*, 25(3), 303-10 (2005)

4. P. E. Purdue, J. Allsop, G. Isaya, L. E. Rosenberg and C. J. Danpure: Mistargeting of peroxisomal L-alanine:glyoxylate aminotransferase to mitochondria in primary hyperoxaluria patients depends upon activation of a cryptic mitochondrial targeting sequence by a point mutation. *Proc Natl Acad Sci U S A*, 88(23), 10900-4 (1991)

5. P. E. Purdue, M. J. Lumb, M. Fox, G. Griffo, C. Hamon-Benais, S. Povey and C. J. Danpure: Characterization and chromosomal mapping of a genomic clone encoding human alanine:glyoxylate aminotransferase. *Genomics*, 10(1), 34-42 (1991)

6. E. F. Caldwell, L. R. Mayor, M. G. Thomas and C. J. Danpure: Diet and the frequency of the alanine:glyoxylate aminotransferase Pro11Leu polymorphism in different human populations. *Hum Genet*, 115(6), 504-9 (2004)

7. C. J. Danpure, G. M. Birdsey, G. Rumsby, M. J. Lumb, P. E. Purdue and J. Allsop: Molecular characterization and clinical use of a polymorphic tandem repeat in an intron of the human alanine:glyoxylate aminotransferase gene. *Hum Genet*, 94(1), 55-64 (1994)

8. E. L. Williams, C. Acquaviva, A. Amoroso, F. Chevalier, M. Coulter-Mackie, C. G. Monico, D. Giachino, T. Owen, A. Robbiano, E. Salido, H. Waterham and G. Rumsby: Primary hyperoxaluria type 1: update and additional mutation analysis of the AGXT gene. *Hum Mutat*, 30(6), 910-7 (2009)

9. M. J. Lumb and C. J. Danpure: Functional synergism between the most common polymorphism in human alanine:glyoxylate aminotransferase and four of the most common disease-causing mutations. *J Biol Chem*, 275(46), 36415-22 (2000)

10. C. G. Monico, J. B. Olson and D. S. Milliner: Implications of genotype and enzyme phenotype in pyridoxine response of patients with type I primary hyperoxaluria. *Am J Nephrol*, 25(2), 183-8 (2005)

11. C. G. Monico, S. Rossetti, J. B. Olson and D. S. Milliner: Pyridoxine effect in type I primary hyperoxaluria is associated with the most common mutant allele. *Kidney Int*, 67(5), 1704-9 (2005)

12. J. Harambat, S. Fargue, C. Acquaviva, M. F. Gagnadoux, F. Janssen, A. Liutkus, C. Mourani, M. A. Macher, D. Abramowicz, C. Legendre, A. Durrbach, M. Tsimaratos, H. Nivet, E. Girardin, A. M. Schott, M. O. Rolland and P. Cochat: Genotype-phenotype correlation in primary hyperoxaluria type 1: the p.Gly170Arg AGXT mutation is associated with a better outcome. *Kidney Int*, 77(5), 443-9 (2010)

13. C. J. Danpure, P. J. Cooper, P. J. Wise and P. R. Jennings: An enzyme trafficking defect in two patients with primary hyperoxaluria type 1: peroxisomal alanine/glyoxylate aminotransferase rerouted to mitochondria. *J Cell Biol*, 108(4), 1345-52 (1989)

14. P. E. Purdue, M. J. Lumb, J. Allsop and C. J. Danpure: An intronic duplication in the alanine: glyoxylate aminotransferase gene facilitates identification of mutations in compound heterozygote patients with primary hyperoxaluria type 1. *Hum Genet*, 87(4), 394-6 (1991)

15. P. E. Purdue, Y. Takada and C. J. Danpure: Identification of mutations associated with peroxisome-to-mitochondrion mistargeting of alanine/glyoxylate aminotransferase in primary hyperoxaluria type 1. *J Cell Biol*, 111(6 Pt 1), 2341-51 (1990)

16. B. Cellini, M. Bertoldi, R. Montioli, A. Paiardini and C. Borri Voltattorni: Human wild-type alanine:glyoxylate aminotransferase and its naturally occurring G82E variant: functional properties and physiological implications. *Biochem J*, 408(1), 39-50 (2007)

17. B. Cellini, A. Lorenzetto, R. Montioli, E. Oppici and C. B. Voltattorni: Human liver peroxisomal alanine:glyoxylate aminotransferase: Different stability under chemical stress of the major allele, the minor allele, and its pathogenic G170R variant. *Biochimie*, 92(12), 1801-11 (2010)

18. B. Cellini, R. Montioli, A. Paiardini, A. Lorenzetto, F. Maset, T. Bellini, E. Oppici and C. B. Voltattorni: Molecular defects of the glycine 41 variants of alanine glyoxylate aminotransferase associated with primary hyperoxaluria type I. *Proc Natl Acad Sci U S A*, 107(7), 2896-901 (2010)

19. B. Cellini, R. Montioli, A. Paiardini, A. Lorenzetto and C. B. Voltattorni: Molecular Insight into the Synergism between the Minor Allele of Human Liver Peroxisomal Alanine:Glyoxylate Aminotransferase and the F152I Mutation. *J Biol Chem*, 284(13), 8349-58 (2009)

20. X. Zhang, S. M. Roe, Y. Hou, M. Bartlam, Z. Rao, L. H. Pearl and C. J. Danpure: Crystal structure of alanine:glyoxylate aminotransferase and the relationship between genotype and enzymatic phenotype in primary hyperoxaluria type 1. *J Mol Biol*, 331(3), 643-52 (2003)

21. M. Bertoldi, B. Cellini, A. Paiardini, R. Montioli and C. Borri Voltattorni: Reactions of human liver peroxisomal alanine:glyoxylate aminotransferase with beta-chloro-L-alanine and L-cysteine: spectroscopic and kinetic analysis. *Biochim Biophys Acta*, 1784(9), 1356-62 (2008)

22. E. D. Hopper, A. M. Pittman, M. C. Fitzgerald and C. L. Tucker: In Vivo and in Vitro Examination of Stability of

Primary Hyperoxaluria-associated Human Alanine:Glyoxylate Aminotransferase. *J Biol Chem*, 283(45), 30493-502 (2008)

23. M. B. Coulter-Mackie and Q. Lian: Consequences of missense mutations for dimerization and turnover of alanine:glyoxylate aminotransferase: study of a spectrum of mutations. *Mol Genet Metab*, 89(4), 349-59 (2006)

24. P. E. Purdue, M. J. Lumb, J. Allsop, Y. Minatogawa and C. J. Danpure: A glycine-to-glutamate substitution abolishes alanine:glyoxylate aminotransferase catalytic activity in a subset of patients with primary hyperoxaluria type 1. *Genomics*, 13(1), 215-8 (1992)

25. C. J. Danpure, P. E. Purdue, P. Fryer, S. Griffiths, J. Allsop, M. J. Lumb, K. M. Guttridge, P. R. Jennings, J. I. Scheinman, S. M. Mauer and et al.: Enzymological and mutational analysis of a complex primary hyperoxaluria type 1 phenotype involving alanine:glyoxylate aminotransferase peroxisome-to-mitochondrion mistargeting and intraperoxisomal aggregation. *Am J Hum Genet*, 53(2), 417-32 (1993)

26. D. Pirulli, D. Puzzer, L. Ferri, S. Crovella, A. Amoroso, C. Ferrettini, M. Marangella, G. Mazzola and F. Florian: Molecular analysis of hyperoxaluria type 1 in Italian patients reveals eight new mutations in the alanine: glyoxylate aminotransferase gene. *Hum Genet*, 104(6), 523-5 (1999)

27. M. B. Coulter-Mackie and Q. Lian: Partial trypsin digestion as an indicator of mis-folding of mutant alanine:glyoxylate aminotransferase and chaperone effects of specific ligands. Study of a spectrum of missense mutants. *Mol Genet Metab*, 94(3), 368-74 (2008)

28. A. Santana, E. Salido, A. Torres and L. J. Shapiro: Primary hyperoxaluria type 1 in the Canary Islands: a conformational disease due to I244T mutation in the P11L-containing alanine:glyoxylate aminotransferase. *Proc Natl Acad Sci U S A*, 100(12), 7277-82 (2003)

29. A. Amoroso, D. Pirulli, F. Florian, D. Puzzer, M. Boniotto, S. Crovella, S. Zezlina, A. Spano, G. Mazzola, S. Savoldi, C. Ferrettini, S. Berutti, M. Petrarulo and M. Marangella: AGXT gene mutations and their influence on clinical heterogeneity of type 1 primary hyperoxaluria. *J Am Soc Nephrol*, 12(10), 2072-9 (2001)

30. M. B. Coulter-Mackie, G. Rumsby, D. A. Applegarth and J. R. Toone: Three novel deletions in the alanine:glyoxylate aminotransferase gene of three patients with type 1 hyperoxaluria. *Mol Genet Metab*, 74(3), 314-21 (2001)

31. C. S. van Woerden, J. W. Groothoff, F. A. Wijburg, C. Annink, R. J. Wanders and H. R. Waterham: Clinical implications of mutation analysis in primary hyperoxaluria type 1. *Kidney Int*, 66(2), 746-52 (2004)

32. C. J. Danpure: Primary hyperoxaluria type 1: AGT mistargeting highlights the fundamental differences between the peroxisomal and mitochondrial protein import pathways. *Biochim Biophys Acta*, 1763(12), 1776-84 (2006)

33. J. M. Leiper, P. B. Oatey and C. J. Danpure: Inhibition of alanine:glyoxylate aminotransferase 1 dimerization is a prerequisite for its peroxisome-to-mitochondrion mistargeting in primary hyperoxaluria type 1. *J Cell Biol*, 135(4), 939-51 (1996)

34. M. J. Lumb, G. M. Birdsey and C. J. Danpure: Correction of an enzyme trafficking defect in hereditary kidney stone disease in vitro. *Biochem J*, 374(Pt 1), 79-87 (2003)

35. S. Djordjevic, X. Zhang, M. Bartlam, S. Ye, Z. Rao and C. J. Danpure: Structural implications of a G170R mutation of alanine:glyoxylate aminotransferase that is associated with peroxisome-to-mitochondrion mistargeting. *Acta Crystallogr Sect F Struct Biol Cryst Commun*, 66(Pt 3), 233-6 (2010)

36. A. L. Pey, E. Salido and J. M. Sanchez-Ruiz: Role of low native state kinetic stability and interaction of partially unfolded states with molecular chaperones in the mitochondrial protein mistargeting associated with primary hyperoxaluria. *Amino Acids* (2010)

Abbreviations: AGT, alanine:glyoxylate aminotransferase; PH1, Primary Hyperoxaluria Type I; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate.

Key words: Alanine, Glyoxylate Aminotransferase, Pyridoxal 5'-Phosphate, Pathogenic Variant, Molecular Defect,Review

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