Gly161 mutations associated with Primary Hyperoxaluria Type I induce the cytosolic aggregation and the intracellular degradation of the apo-form of alanine:glyoxylate aminotransferase

Elisa Oppici, Alessandro Roncador, Riccardo Montioli, Silvia Bianconi, Barbara Cellini *

Department of Life Sciences and Reproduction, Section of Biological Chemistry, University of Verona, Strada Le Grazie 8 37134 Verona, Italy

A R T I C L E   I N F O

Article history:
Received 25 July 2013
Received in revised form 5 September 2013
Accepted 6 September 2013
Available online 17 September 2013

Keywords:
Primary Hyperoxaluria
Alanine:glyoxylate aminotransferase
Pyridoxal phosphate
Hereditary metabolic disease
Protein aggregation
Protein misfolding

A B S T R A C T

Primary Hyperoxaluria Type I (PH1) is a severe rare disorder of metabolism due to inherited mutations on liver peroxisomal alanine:glyoxylate aminotransferase (AGT), a pyridoxal 5′-phosphate (PLP)-dependent enzyme whose deficiency causes the deposition of calcium oxalate crystals in the kidneys and urinary tract. PH1 is an extremely heterogeneous disease and there are more than 150 disease-causing mutations currently known, most of which are missense mutations. Moreover, the molecular mechanisms by which missense mutations lead to AGT deficiency span from structural, functional to subcellular localization defects. Gly161 is a highly conserved residue whose mutation to Arg, Cys or Ser is associated with PH1. Here we investigated the molecular bases of the AGT deficit caused by Gly161 mutations with expression studies in a mammalian cellular system paired with biochemical analyses on the purified recombinant proteins. Our results show that the mutations of Gly161 (i) strongly reduce the expression levels and the intracellular half-life of AGT, and (ii) make the protein in the apo-form prone to an electrostatically-driven aggregation in the cell cytosol. The coenzyme PLP, by shifting the equilibrium from the apo- to the holo-form, is able to reduce the aggregation propensity of the variants, thus partly decreasing the effect of the mutations. Altogether, these results shed light on the mechanistic details underlying the pathogenicity of Gly161 variants, thus expanding our knowledge of the enzymatic phenotypes leading to AGT deficiency.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Primary Hyperoxaluria Type I (PH1, MIM 259900) is an autosomal recessive metabolic disorder caused by inherited mutations in the AGXT gene encoding liver peroxisomal alanine:glyoxylate aminotransferase (AGT) [1]. AGT is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the transamination of l-alanine and glyoxylate to pyruvate and glycine, respectively. The protein is dimeric and has an overall structure similar to that of other human Fold Type I aminotransferases [23]. Each subunit is made up of a large domain, comprising most of the active site and the dimerization interface, an N-terminal extension that wraps over the surface of the neighboring subunit, and a small domain, comprising regions involved in the interaction with the

Abbreviations: PH1, Primary Hyperoxaluria Type I; AGT, alanine:glyoxylate aminotransferase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; CHO, Chinese hamster ovary; DLS, dynamic light scattering; SEC, size-exclusion chromatography; PBS, phosphate buffered saline; BS(PEG)5, bis-N-succinimidy1-(-pentethylen glycol)ester; IFM, immunofluorescence microscopy; IEM, immune electron microscopy; BSA, bovine serum albumin

© Corresponding author. Tel.: +39 0458027293; fax: +39 0458027170. E-mail address: barbara.cellini@univr.it (B. Cellini).

0925–4439/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbadis.2013.09.002
and sensitizes the protein to the untoward effects of many of the mutations found in PH1 [11].

The vast majority of the PH1-causing mutations identified on the AGXT gene are single base pair substitutions that lead to the synthesis of an aberrant gene product [12]. Until now, the molecular effect of only a few mutations has been analyzed by a biochemical and/or a cell biology approach. These studies have indicated that missense mutations can cause functional (loss of catalytic activity, reduced coenzyme binding affinity), structural (reduced stability, reduced dimerization, aggregation propensity in vitro and/or in the cell) and/or subcellular localization effects (aberrant mitochondrial localization) [10,13–24,11,25,26,9,27].

Gly161 is a highly conserved residue and represents a hot-spot mutation site within the AGT sequence. In fact, three pathogenic mutations that affect the GCC triplet in exon 4 of the AGXT gene have been reported: the G161R mutation, co-segregating with the major allele, and the G161S and G161C mutations, both co-segregating with the minor allele [17,28]. The AGT crystal structure reveals that Gly161 is far from the active site and is part of the loop 154–168 located in the large domain in close contact with the small domain (Fig. 1) [4]. The substitution of the small side chain of glycine with the larger side chains of arginine, serine or cysteine is predicted to perturb the folding of both domains of the protein [20]. Studies on recombinant purified proteins have recently shown that the G161S and G161C mutations do not significantly alter the functional properties of AGT, but reduce the thermal stability of the protein more in the apo- than in the holo-form, thus pointing to the mutation mainly inducing a structural defect [20]. In agreement with these results, crude Escherichia coli lysates expressing the G161C-Mi, G161S-Mi and G161R-Ma variants display very low agreement with these results, crude Escherichia coli lysates expressing the G161C-Mi, G161S-Mi and G161R-Ma variants display very low activity and expression level [28] and cell-free expression experiments indicate that the G161R-Ma variant is prone to proteasomal and tryptic degradation [17,18]. Although these data confirm that the mutation of Gly161 could induce structural perturbations in AGT, the study of the variants in a cellular environment and of the molecular mechanisms underlying their pathogenicity is still lacking.

In the present work, by exploring the behavior of the Gly161 variants in a cellular model system and by analyzing the proteins in their recombinant purified form, we show that (i) the molecular defect of the variants only concerns their apo dimeric forms, which form cytosolic aggregates and are susceptible to intracellular degradation and (ii) PLP binding is able to reduce the aggregation extent of the variants by shifting the equilibrium from the apo- to the non-aggregating holo-form. These results allow us to better define the molecular defect of Gly161 variants, adding to our knowledge of mechanisms underlying AGT deficiency in PH1 and providing directions for treatment in patients bearing mutations at Gly161.

2. Materials and methods

2.1. Materials

PLP, pyridoxine, l-alanine, sodium glyoxylate, rabbit muscle l-lactic dehydrogenase, isopropyl-β-D-thiogalactoside (IPTG), EDTA and imidazole were purchased from Sigma. Ham’s F12 Glutamax medium was purchased from Invitrogen. All other chemicals were of the highest purity available. The rabbit polyclonal anti-AGT human and guinea-pig anti-peroxisomal protein antibodies were kindly provided by Prof. C.J. Danpure, University College London, UK [11,29–33]; the anti-rabbit HRP antibody was purchased from GE Healthcare. Oligonucleotides for site directed mutagenesis were purchased from MWG Biotech AG (Anzinger, Germany).

2.2. Site directed mutagenesis

The cDNA encoding human AGT has been cloned in the mammalian expression vector pcDNA3.1/V5-His-TOPO, by means of the TOPO cloning kit (Invitrogen). The desired mutations were introduced on the vector by the QuikChange site-directed mutagenesis kit (Stratagene), by using the previously reported oligonucleotides [20]. The E. coli expression vectors of the polymorphic and pathogenic variants of AGT were constructed starting from the pAGT-His construct that contains the complete open reading frame of AGT cloned in a pTrcHis2A expression plasmid as previously reported [20]. All the mutations were confirmed by entire DNA sequence analysis.

2.3. Cell culture and lysis

CHO cells were cultured at 37 °C under O2/CO2 (19:1) in Ham’s F12 Glutamax medium (Invitrogen) supplemented with fetal bovine serum (10%, v/v), penicillin (100 units/ml) and streptomycin (100 μg/ml). The standard pyridoxine concentration in this medium is 0.3 μM. Cells were transfected with Turbofect™ Transfection Reagent (Fermentas) according to the manufacturer’s instructions. Four hours after transfection cells were washed in phosphate buffered saline (PBS) and the medium was changed with complete Ham’s F12. Where indicated, from two days before transfection to the end of the experiment, cells were grown in the presence of added 100 μM pyridoxine. Transfection experiments were designed to minimize the variability introduced by transfection efficiency, which was controlled either by registering the GFP signal by fluorescence microscopy after co-transfection with pmaxGFP (Lonza) and/or by quantifying the neomycin selection gene by RT-PCR (see below). Only

![Fig. 1. Position of the Gly161 residue in the ACT structure. Crystal structure of AGT (PDB code 1H0C) in which one monomer is colored gray, while in the other monomer the large domain, the small domain and the N-terminal extension are colored orange, green and yellow, respectively. On the colored monomer PLP is represented as red sticks, Gly161 is represented as magenta sticks, and the loop 154–168 is colored blue. The inset shows the position and the microenvironment of Gly161.](image-url)
transfections with intra-experiment variability ≤10% and inter-experiment variability ≤15% were used.

Cells were harvested after 24 h and lysed in PBS, pH 7.2, plus protease inhibitor cocktail (Complete Mini, Roche), by five freeze/thaw cycles followed by addition of DNase (100 units/ml) at room temperature for 45 min. The whole cell extract was separated by centrifugation (29,200 g, 10 min, 4 °C) to obtain the soluble fraction. The pellets were then resuspended in an equal volume of denaturing gel loading buffer to obtain the insoluble fraction. The protein concentration in the total cell lysate and in the soluble fraction was measured using the Bradford protein assay.

2.4. Transcript expression analysis

CHO cells were grown in a 6-well plate and transfected as described above. Cells were harvested, washed with PBS, and RNA was extracted using RNeasy mini kit (Qiagen). The cDNA was synthesized from 1 μg of mRNA using the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RT-PCR was performed using 3 μl of a 1:10 cDNA dilution in a 25 μl reaction volume using SYBR Premix Ex Taq master mix (TaKaRa) on a Corbett RotorGene6000 thermocycler. The reaction conditions were initial denaturation at 95 °C for 15 min followed by 45 cycles of PCR which included 15 s melting at 95 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C. The samples were examined for the expression of human AGT using the forward primer 5′-AGCCGAGATTGACCATCAGC-3′ and the reverse primer 5′-GGACCCATGGATCTCAATGTC-3′. The amount of mRNA was calculated in relation to glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) mRNA in the same sample. Quantification of individual transcripts was performed using the ‘Comparative Quantitation’ software supplied by Corbett Research for the RotorGene. The mean efficiency of a group of cycling curves is calculated at the point that the cycling curves take off and used to calculate a fold change according to the formula: fold change = efficiency Ct1/Ct2, where Ct1 and Ct2 are the take off values of the cycling curves being compared. All reactions were performed at least in duplicate.

2.5. Western-blot analysis and chemical cross-linking

Ten micrograms of cell lysate were loaded per lane on a Mini Protean TGX™ pre-cast gel (Biorad) along with the Precision plus protein Kaleidoscope™ (Bio-Rad) molecular mass markers. Following transfer on a nitrocellulose membrane by the iBlot device (Invitrogen) the membrane was blocked in 5% bovine serum albumin (BSA) for 1 h at 37 °C. For AGT detection the membrane was incubated with polyclonal rabbit anti AGT serum (dilution 1:2000), washed three times in TBST (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) and then incubated with peroxidase-conjugated anti rabbit IgG (dilution 1:1000). Blotted proteins were detected with ECL® (Millipore), using the ChemiDoc XRS Imaging System (Bio-Rad, Hercules, CA). For densitometry analysis of western-blots, the band volume (intensity × mm²) was quantified using the software Quantity One 4.6.3. Results were expressed relative to the densitometry of AGT-Ma.

Cross-linking was performed with bis-N-succinimidyl-(pentaethylene glycol) ester (BS[PEG]5) (Pierce). After cell lysis, 25 μg of the whole lysate was cross-linked with BS[PEG]5 at 125 mM concentration and quenched in 50 mM Tris–HCl after 30 min. Ten micrograms of each sample were analysed by western blot as described above.

2.6. Pulse-chase experiments

Two million cells were seeded in a 10 cm dish and transfected with the pcDNA3.1 vectors encoding AGT-Ma, AGT-Mi, G161R-Ma, G161S-Mi or G161C-Mi. Twenty-four hours after transfection cells were incubated for 1 h in Dulbecco’s Modified Eagle Medium (DMEM) without methionine and cysteine (Gibco) and pulse labeled with 50 μCi of 35S methionine/cysteine (EasyTag™ EXPRESS 35S Protein Labeling Mix, [35S], Perkin Elmer). The standard content of pyridoxine of the DMEM medium is 19.6 μM. After 30 min an excess of unlabelled amino acids was added and cells were split and incubated in Ham’s F12 medium for chase periods from 4 to 48 h. The soluble fraction of each cell lysate (50 μg) was incubated overnight with 2 μg of guinea-pig anti-AGT antibody on a rotator at 4 °C. Thereafter, the immunoprecipitation reaction was performed at room temperature by adding 30 μl of agarose-protein A (GE, Healthcare) to the mixture and by incubating the solution for 1 h on a rotator at room temperature. The immunoprecipitated complex was pelleted by centrifugation at 29,200 g at 4 °C for 5 min, washed three times with IP wash/lysis buffer (Thermo Scientific), and resuspended in 20 μl of denaturing gel loading buffer. Proteins were separated by SDS–PAGE and radiolabelled AGT was detected by autoradiography. Band intensities were quantified using the software ImageJ in accordance with the ImageJ user guide. For each sample, the relative intensity with respect to that at the start of the chase (pulse) was used to calculate the half-life. Autoradiography films with different exposition times were acquired in order to have data suitable for quantification analyses.

2.7. Size exclusion chromatography (SEC)

SEC analyses of lysates of CHO cells were performed according to the method of Siekierska A. et al. [34] by transfecting 9 × 10⁶ of CHO cells with the vector encoding AGT-Ma or the G161R-Ma variant. Twenty-four hours after transfection cells were harvested and lysed in 500 μl of PBS as previously described. Total lysates were then loaded onto a Superdex S200 10/30 column (GE Healthcare), equilibrated in PBS plus 1 mM EDTA, 1 mM EGTA and 1 mM DTT, using an AKTA FPLC system (Amersham Biosciences). The absorbance signal at 280 nm of the eluate, representing the total protein content, was registered. Trichloroacetic acid 20% (v/v) was added to each eluted fraction and the samples were incubated overnight at 4 °C. After centrifugation at 16,000 g for 30 min, the precipitated protein was washed twice in PBS and resuspended in 20 μl of denaturing gel loading buffer. Ten microliters of each sample were separated by SDS–PAGE and immunoblotted against anti-AGT.

2.8. Immunofluorescence microscopy (IFM)

CHO cells were grown and transfected in a 24-well plate on glass coverslips. Twenty-four hours after transfection cells were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.3% Triton X-100 in phosphate buffer saline (PBS), and blocked in 3% BSA. For the immunol labelling, rabbit polyclonal anti-human AGT and anti-peroxisomal protein form guinea-pig were used as primary antibodies [29–33], and Alexa Fluor conjugated antibodies (AF488 and AF555, Life technologies) were used as secondary antibodies. Mitochondria were stained with Mitotracker Red CMXRs version, Molecular Probes, Invitrogen) and nuclei with DAPI. The coverslips were mounted over slides in AFM medium (Dako). The images were captured using a confocal laser-scanning fluorescence microscope Leica SP5 (Leica Microsystems, Mannheim, Germany) at 63× magnification and processed using Adobe Photoshop and ImageJ softwares (Rasband, W.S., ImageJ, U.S. National Institute of Health, Bethesda, Maryland, USA [http://rsb.info.nih.gov/j/, 1997–2008].

2.9. Immunoelectron microscopy (IEM)

CHO cells transiently expressing AGT-Ma or G161R-Ma were fixed for 2 h in a mixture of 4% paraformaldehyde and 0.4% glutaraldehyde in 0.2 M PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂ pH 6) and washed with a PBS–glycine solution. After washing, cells were detached from the dishes by a cell scraper, pelleted
and embedded in 10% gelatin, cooled in ice and cut into 1-mm³ blocks. The blocks were infused with 2.3 M sucrose at 4 °C overnight and frozen in liquid nitrogen. Ultrathin cryosections were cut using a Leica EM FC7 cryoultramicrotome and picked up using a 1:1 mix of 2% methylcellulose and 2.3 M sucrose. Sections were mounted on formvar carbon-coated copper grids, and the localization of AGT in the cryosections was analyzed using the rabbit polyclonal anti-human AGT followed by protein A conjugated with 10 nm gold particles. After labeling, the cryosections were embedded in methylcellulose-uranyl acetate mixture. Cryosections were further investigated using a FEI Tecnai-12 electron microscope (FEI, Eindhoven, The Netherlands) equipped with a Veleta CCD camera for digital image acquisition.

2.10. Enzyme activity measurement

AGT enzymatic activity of cell lysates of CHO cells expressing AGT-Ma, AGT-Mi, G161R-Ma, G161S-Mi and G161C-Mi was determined by incubating 90 μg of the soluble fraction of the lysate with 0.5 M L-alanine and 10 mM glyoxylate at 25 °C in 100 mM potassium phosphate buffer pH 7.4 in the presence or in the absence of 200 μM PLP. In order to obtain a detectable signal of pyruvate in the linear phase of the kinetics, the reaction time was set to 30 min for AGT-Ma and AGT-Mi and to 150 min for Gly161 variants. The reactions were stopped by adding TCA 10% (v/v) and the pyruvate production was measured using a spectrophotometric assay coupled with lactate dehydrogenase as previously described [35].

2.11. Protein expression and purification

The Gly161 variants in the His-tagged form were expressed in E. coli and purified with the procedure already described [36]. PLP (100 μM) was added to the eluted protein to obtain the holo-form and the solution was concentrated and extensively washed with 100 mM potassium phosphate buffer, pH 7.4, using Amicon Ultra 10 concentrators (Amicon). As isolated, Gly161 variants contain 2 mol of PLP per dimer, as revealed by releasing the cofactor in 0.1 M NaOH and using the extinction coefficient of 6600 M⁻¹ cm⁻¹ at 388 nm. The apo form of each variant was prepared as previously described [36]. The protein concentration in the AGT samples was determined by absorbance spectroscopy using an extinction coefficient of 9.54 × 10⁴ M⁻¹ cm⁻¹ at 280 nm [13].

2.12. Spectroscopic measurements

Absorption measurements were made with a Jasco V-550 spectrophotometer with 1 cm path length quartz cuvettes at a protein concentration of 1–10 μM in 100 mM potassium phosphate buffer, pH 7.4. The increase in turbidity following the aggregation of Gly161 variants was monitored by measuring the absorbance at 600 nm as a function of time under physiological conditions (150 mM ionic strength, pH 7.4, 37 °C).

Intrinsic fluorescence emission spectra were recorded on a Jasco FP-750 spectrophotometer equipped with a thermostatically controlled cell holder by using 1 cm path length quartz cuvettes. Protein emission spectra were taken from 300 to 500 nm (excitation at 280 nm) with both the excitation and the emission slits set to 5 nm. The protein concentration was 1 μM. CD spectra were registered with a Jasco-710 spectropolarimeter at 25 °C, by using 1 cm path-length cuvettes at 10 μM enzyme concentration. Dynamic light scattering (DLS) measurements were performed on a Zetapher Nano S device (Malvern Instruments) equipped with a Peltier temperature controller by using disposable 12.5 × 45-mm cells with stopper. The aggregation kinetics of AGT-Ma, AGT-Mi, G161S-Mi and G161C-Mi was studied by incubating each enzymatic species in potassium phosphate buffer, pH 7.4 at different ionic strength at 37 °C. The buffer was filtered immediately before use to eliminate any impurities.

2.13. Statistical analysis

Experiments have been performed at least in triplicate. Statistical analysis was performed with Origin® 7.03 (Origin Lab) or GraphPad Prism Version 5.0 (GraphPad software, San Diego, CA, USA).

3. Results

3.1. Gly161 variants show a reduced expression level and enzymatic activity in mammalian cells

In order to investigate how the mutation of Gly161 could alter the expression and the activity of AGT in a cell environment, the G161R-Ma, G161S-Mi and G161C-Mi variants were expressed in CHO cells. We did not succeed in the generation of stable clones expressing the variants, as a consequence of the high mortality of the transfected cells probably caused by the accumulation of insoluble protein aggregates (see below). CHO cells transiently expressing AGT-Ma, AGT-Mi and the Gly161 variants were analyzed for AGT enzymatic activity and expression level in the soluble fraction of the cellular lysate after 24 h. As previously reported [24], the transaminase activity of AGT-Mi was reduced by only 40% with respect to AGT-Ma (288 ± 20 nmol pyruvate/min/mg protein for AGT-Ma and 176 ± 15 nmol pyruvate/min/mg protein for AGT-Mi). The activity of G161R-Ma, G161S-Mi and G161C-Mi was 0.9 ± 0.1, 2.1 ± 0.3 and 1.5 ± 0.3 nmol pyruvate/min/mg protein, respectively. Moreover, Gly161 variants had a reduced expression level, as shown by the intensity of the specific band of immunoreactive AGT. The expression of G161R-Ma was 4% compared with that of AGT-Ma, and that of G161S-Mi and G161C-Mi was 12% and 5%, respectively, compared with that of AGT-Mi (Fig. 2). Considering that the G161S and G161C mutations do not affect the kcat of the overall transamination reaction [20], the very low specific activity of Gly161 variants expressed in CHO cells can be attributed to a strongly reduced expression level and/or to an increased propensity to proteolytic degradation.

RT-PCR experiments allowed us to rule out any possible influence of the mutations at transcriptional level. In fact, no significant differences were observed in the levels of the mRNA transcripts for cells expressing
AGT-Ma, AGT-Mi, G161R-Ma, G161S-Mi or G161C-Mi (Supplementary Fig. 1). Therefore, the reduced expression of Gly161 variants originates at the protein level.

3.2. Gly161 variants form cytosolic aggregates and show a reduced half-life in mammalian cells

We compared the soluble and insoluble fractions of lysates of CHO cells expressing AGT-Ma, AGT-Mi and the Gly161 variants by means of western-blot. While in the case of AGT-Ma and AGT-Mi about 75–80% of the protein was present in the soluble fraction, in the case of G161R-Ma, G161S-Mi and G161C-Mi variants 93%, 84% and 91% of the protein, respectively, was present in the insoluble fraction (Fig. 2). Although it should be noted that the total amount of protein (soluble plus insoluble fraction) present in Gly161 variants was lower with respect to that of AGT-Ma or AGT-Mi, these data suggest that the low expression level of the variants could be at least partly due to protein aggregation. In agreement with these results, two strong bands at 80–90 kDa, corresponding to the two structural isoforms of dimeric AGT [24], could be seen after cross-linking of the total lysates of cells expressing AGT-Ma or AGT-Mi. On the other hand, the cross-linking of the total lysates of cells expressing Gly161 variants leads to the appearance of a faint band at a molecular weight higher than 200 kDa, and to the almost complete loss of immunoreactive monomeric and dimeric species of AGT, as a consequence of the formation of high-molecular weight aggregates that were unable to enter the gel (Supplementary Fig. 2) [35]. We confirmed that Gly161 mutation induced the intracellular aggregation of AGT by SEC experiments performed on AGT-Ma and the G161R-Ma variant. Lysates of CHO cells expressing AGT-Ma or the G161R-Ma variant were subjected to SEC and the absorbance signal at 280 nm (representing the total protein content) was registered (Fig. 3A). Then, in order to measure the AGT content in the eluate fraction, some insights into this issue, we investigated the intracellular levels of apo- and holo-AGT by determining the AGT catalytic activity in the soluble fraction of the cellular lysates both in the presence of added PLP in the assay mixture, in order to measure only the amount of holoAGT [24]. The difference between the two measurements represents the amount of apoAGT. As shown in Fig. 7A, the specific activity of cells expressing Gly161 variants was only detectable when PLP was present in the assay mixture, while the activity of cells expressing AGT-Ma and AGT-Mi showed an about 40% increase after PLP addition. This indicates that the majority of AGT-Ma and AGT-Mi is present in the holo-form, whereas Gly161 variants are almost entirely in the apo-form.

3.3. Gly161 variants are present in the cell in the apo-form

Although it is clear that cytosolic aggregation and intracellular degradation are the molecular defects shared by the three Gly161 variants, the specific form (apo and/or holo in the dimeric and/or monomeric form) responsible for the defects needs to be identified. To achieve some insights into this issue, we investigated the intracellular levels of apo- and holo-AGT by determining the AGT catalytic activity in the soluble fraction of the cellular lysates both in the presence of added PLP in the assay mixture, in order to measure only the amount of holoAGT [24]. The difference between the two measurements represents the amount of apoAGT. As shown in Fig. 7A, the specific activity of cells expressing Gly161 variants was only detectable when PLP was present in the assay mixture, while the activity of cells expressing AGT-Ma and AGT-Mi showed an about 40% increase after PLP addition. This indicates that the majority of AGT-Ma and AGT-Mi is present in the holo-form, whereas Gly161 variants are almost entirely in the apo-form.

3.4. The addition of pyridoxine to the culture medium partly reduces the aggregation extent of Gly161 variants

To gain insights into the possible effect of the addition of the enzyme to the culture medium of Gly161 variants, starting from two days before transfection to the end of the experiment cells were grown in the presence of 100 μM pyridoxine, a vitamer of vitamin B6 known to be converted to PLP inside the cell [37]. It should be mentioned that the basal pyridoxine content of the medium is 0.3 μM. We found that (1) the enzymatic activity of G161R-Ma, G161S-Mi and G161C-Mi grown in the presence of 100 μM pyridoxine was 3.5, 2.5 and 2.1 fold higher, respectively, with respect to that of each variant grown in the basal medium, and (2) when 100 μM pyridoxine was present in the culture medium, the enzymatic activity of AGT-Ma, AGT-Mi and Gly161 variants did not increase upon addition of PLP to
the assay mixture, thus indicating that they were entirely present in the holo-form inside the cell. It should be noted that the specific activity of AGT-Ma and AGT-Mi dropped by about 25% in the presence of pyridoxine in the culture medium, a result possibly due to a previously observed inhibitory effect of the excess B6 vitamers [38] currently under investigation. Western-blot analyses showed that the presence of added pyridoxine to the culture medium did not change the total amount of expressed AGT of any of the analyzed enzymatic species (data not shown). However, the amount of AGT present in the soluble fraction of lysates of G161R-Ma, G161S-Mi and G161C-Mi cultured in the presence of 100 μM pyridoxine was 2.5, 3 and 2.8 fold higher, respectively, as compared with that of each variant cultured in the basal medium (Fig. 7B). These results suggest that the addition of the coenzyme shifts the equilibrium from the apo to the holo-form and that this partly prevents the aggregation of Gly161 variants.

3.5. Recombinant purified G161S-Mi and G161C-Mi variants in the apo-form are unstable and prone to aggregation under physiological conditions

On the basis of the results reported in Sections 3.4 and 3.5, it is reasonable to hypothesize that the aggregates could derive from the apo-form of Gly161 variants. To test this hypothesis, the analysis of the behavior of the variants in the recombinant purified form under physiological conditions was undertaken. As previously reported [20], when G161S-Mi and G161C-Mi were overexpressed in E. coli, a large portion of the protein was present in the insoluble fraction and the purification yield was about 10% compared to that of AGT-Ma. Unfortunately, if the G161R-Ma variant was expressed using the same procedure, the expression level was less than 5% with respect to AGT-Ma and the mutant protein was insoluble in crude extracts. Any attempt to increase protein solubility by lowering the expression temperature, by adding...
exogenous PLP and small osmolytes to the culture, or by using E. coli strains overexpressing molecular chaperones like GroEL/GroES, was unsuccessful. Therefore, we could not characterize the purified G161R-Ma variant.

Turbidity changes of AGT-Mi, G161C-Mi and G161S-Mi incubated under physiological conditions (37 °C, pH 7.4 and 150 mM ionic strength) were monitored. While no changes in the absorbance at 600 nm could be detected for holo- and apo-AGT-Mi (data not shown) and for the variants in the holo-form, a significant change in turbidity indicating an ongoing aggregation process was observed for apoG161S-Mi and apoG161C-Mi (Fig. 8A and B). The aggregation extent is similar for the two proteins, even if in the case of the G161C-Mi variant a decrease in turbidity was observed after 40 min due to aggregates precipitation. Notably, the addition of excess exogenous PLP (100 μM) decreased, even if at different degrees, the extent of aggregation of both apoG161S-Mi and apoG161C-Mi (Fig. 8). This result indicates that the binding of the coenzyme competes with the aggregation process and is able to shift the equilibrium versus the non-aggregating holoenzymatic form. In this regard it should be noticed that the rate constants of association of 100 μM PLP to apoAGT-Mi, apoG161S-Mi and apoG161C-Mi were equal to 0.28 ± 0.02 min⁻¹, 0.18 ± 0.03 min⁻¹ and 0.14 ± 0.01 min⁻¹, respectively.

DLS studies were undertaken to characterize the species present in solution during the incubation of AGT-Mi, G161S-Mi and G161C-Mi under

---

Fig. 6. Measurement of the half-life of AGT-Ma, AGT-Mi, and Gly161 variants in CHO cells. Transiently transfected CHO cells were pulse labeled with [35S] Cys-Met mix containing DMEM and then incubated in complete Ham's F12 medium for a chase time from 0 to 48 h. The standard content of pyridoxine of the DMEM medium is 19.6 μM, while that of the Ham's F12 medium is 0.3 μM. The soluble fraction of the cell lysates was immunoprecipitated with the anti-AGT antibody and loaded on SDS-PAGE; the signal of radiolabelled AGT was detected by autoradiography. Band intensities were quantified using the software ImageJ in accordance with the ImageJ user guide. For each sample, the relative intensity with respect to that at the start of the chase (pulse) was used to calculate the half-life.

Fig. 7. Effect of pyridoxine on the specific activity and expression level of AGT-Ma, AGT-Mi and Gly161 variants. Where indicated, from two days before transfection to the end of the experiment, cells were grown in the presence of added 100 μM pyridoxine. The basal level of pyridoxine in the culture medium is 0.3 μM. Twenty-four hours after transfection to the end of the experiment, cells were harvested and lysed. (A) The soluble cell lysate (100 μg) was incubated with 0.5 M L-alanine and 10 mM glyoxylate both in the absence (white) and in the presence (gray) of 200 μM PLP. The amount of pyruvate produced was determined and expressed as specific activity (nmol pyruvate/min/mg protein). (B) The soluble cell lysate (15 μg) was subjected to SDS/PAGE, immunoblotted with anti-AGT from rabbit (1:2000) and detected by chemiluminescence. The band shown for all enzymatic species in (B) is those of a single gel. Data are representative of three different experiments. Bar graphs represent the mean ± SEM.

Fig. 8. Time-dependent turbidity changes of purified G161S-Mi and G161C-Mi variants. Absorbance changes at 600 nm as a function of time of G161S-Mi (A) and G161C-Mi (B) at 2 μM concentration in the apo-form (square), holo-form (triangle) and apo-forms + 100 μM PLP (circle). The buffer was 60 mM potassium phosphate, pH 7.4, at 37 °C.
physiological conditions (37 °C, 150 mM ionic strength, pH 7.4). While the increase in the count rate, a parameter that depends on both the size and the quantity of the particles, was very slow for the holoenzymes and for apoAGT-Mi, a remarkable and fast aggregation process could be seen for apoG161S-Mi and apoG161C-Mi, with the count rate leveling off after ~20 and ~30 min, respectively (Fig. 9). The plots of the particle size against time reported in Fig. 10 showed that, although small aggregates (200–600 nm) were formed upon incubation of holo- and apoAGT-Mi, holoG161S-Mi and holoG161C-Mi, the signal of the dimer was continuously present. This means that the dimer was the most abundant species in solution, since the contribution to scattering from larger particles dominates the DLS signal, as the scattering intensity is proportional to the sixth power of a particle diameter. On the contrary, the signal of the dimer of apoG161S-Mi and apoG161C-Mi disappeared after 5–10 min and aggregates of ~1500 nm were formed. Moreover, after ~40 min apoG161C-Mi formed a distinct population of high-order aggregates (~5000 nm) that could explain the precipitation phenomenon seen in turbidimetry experiments (Fig. 8A). It should also be pointed out that the aggregation extent of the apovariants increased as the protein concentration increases, thus indicating that the aggregation did not depend on protein monomerization. Moreover, when the associative behavior of the variants was monitored at different ionic strengths (from 0.15 to 1.25 M), it could be observed that the aggregation extent increased as the ionic strength decreased (Supplementary Fig. 5). This result indicates that electrostatic forces mediate the interaction between dimers of apoG161S-Mi and apoG161C-Mi, possibly due to the impact of Gly161 mutations on the tertiary structure of apoAGT. In this regard, we found that in 0.1 M potassium phosphate buffer, pH 7.4, at 25 °C (i.e. under experimental conditions in which no aggregation occurs) the spectroscopic features of holo G161S and G161C were similar to those of holoAGT-Mi [22]. On the other hand, in comparison with apo ACT-Mi, apoG161C-Mi and apoG161S-Mi were characterized by (i) a different near-UV dichroic spectrum, as shown by a decreased ellipticity at 288 nm and an increased ellipticity at 265 nm, and (ii) a 5 nm red-shift of the intrinsic fluorescence emission maximum. (data not shown).

Altogether, the results obtained allowed us to conclude that the mutation of Gly161 to Ser or Cys on the background of the minor allele (i) changes the tertiary structure of apoAGT and slows down PLP binding, and (ii) under physiological conditions, makes AGT in the apo-form prone to a time-dependent aggregation, which is prevented by the binding of PLP.

### 4. Discussion

#### 4.1. Gly161 mutations cause a folding defect of AGT

In this study we provide a detailed characterization of the molecular and cellular effects of three missense mutations of AGT at position 161 leading to PH1: the G161R mutation, associated to the major allele, and the G161S and G161C mutations, associated to the minor allele. We show that: (i) the mutations of Gly161 strongly reduce the AGT expression level, both in *E. coli* and in a mammalian cellular model, and the

---

Fig. 9. Time-dependent aggregation of AGT-Mi and Gly161 variants under physiological conditions. The total count rate (measured as kilo counts per second) of AGT-Mi and Gly161 variants is plotted as a function of time. Symbols: triangle: AGT-Mi, square: G161S-Mi, circle: G161C-Mi. Open and closed symbols represent the holo- and apo-forms, respectively. All measurements have been performed at 1 μM enzyme concentration, 37 °C, in 60 mM potassium phosphate buffer, pH 7.4.

Fig. 10. Time-dependent changes of the apparent diameter of AGT-Mi and Gly161 variants under physiological conditions. The diameter of the species present in solution as function of time is reported for the indicated species in the holo and apo-form. Symbols: black open square: holo dimer, red open circle: apo dimer, black closed square: holo aggregates, red closed circle: apo aggregates, red open triangle: high-molecular weight aggregates. All measurements have been performed at 1 μM enzyme concentration, 37 °C, in 60 mM potassium phosphate buffer, pH 7.4.
reduction is not produced by differences at the level of transcription; (ii) when expressed in CHO cells, Gly161 variants form insoluble aggregates that localize in the cell cytosol; (iii) the substitution of Gly161 strongly reduces the AGT intracellular half-life in the order (G161R > G161C > G161S), in agreement with previous cell-free expression system studies [17]; (iv) under physiological conditions, purified G161S-Mi and G161C-Mi variants in the apo-form are prone to an electrostatically-driven aggregation. All in all, these results indicate that Gly161 variants, although at different extents, are mainly characterized by a folding defect such that it is the inability of the mutated peptide chain to achieve or maintain a fully functional conformation, rather than the loss of protein functionality per se, that causes the disease. Therefore, the mutation is expected to induce a conformational change ultimately responsible for the propensity of the protein to both degradation and aggregation.

4.2. Proposed molecular mechanism of the pathogenicity of Gly161 variants

We found that Gly161 variants expressed in CHO cells were entirely present in the apo-form because no enzymatic activity was detectable in the absence of PLP in the assay mixture (Fig. 7A). This result can be partly explained by the slower PLP binding rate of purified Gly161 variants with respect to AGT-Mi and implies that the molecular defects of these variants rely in their apo forms, prone to intracellular aggregation and susceptible to degradation. Both these events are responsible for the reduced expression level of the proteins. Notably, DLS measurements revealed aggregation propensity of the recombinant apo-forms of Gly161 variants, and not of their corresponding holo-forms, thus reinforcing the view that cytosolic aggregates originate from the apo form of the variants.

Previous DLS studies on purified G161S-Mi and G161C-Mi [20] and SEC analyses of lysates of CHO cells expressing G161R-Ma (Fig. 3) did not reveal the presence of monomeric AGT. Moreover, we show here that the aggregation extent of the apoproteins is proportional to protein concentration and increases with decreasing ionic strength. These data indicate that the variants in the dimeric form are prone to a self-association process mediated by the electrostatic interaction between patches of opposite charge. AGT shows a highly positive charge distribution around its surface [14]. Thus, whatever the conformational change caused by Gly161 mutation is, it can be hypothesized that it could lead to the exposure of negatively charged surfaces. This would create a dipole segregation of charges that leads to the electrostatic aggregation of the protein, similarly to what previously found for pathogenic variants of AGT bearing mutations at Gly41 [14]. Following this view, it is reasonable to think that the conformational change induced by Gly161 mutation could also cause the exposure of stretches susceptible to proteolytic degradation. Although this would explain the enhanced intracellular degradation of the variants, a direct evidence for this interpretation is lacking.

On the basis of the results obtained, and taking into consideration previous data on the response of AGT to chemical stress [10], a plausible model that describes the molecular defect of Gly161 variants can be outlined (Fig. 11A). Previous studies performed under chemical stress conditions have suggested that in the AGT folding pathway the unfolded polypeptide chain (U) generates monomeric AGT (M) passing through a partly folded monomeric intermediate (M*). M then dimerizes (D) and binds PLP (D\text{PLP}) [39]. The conformational change affecting Gly161 variants probably alters the positive electrostatic surface of apoAGT leading to the exposure of negative patches. This makes the apodimeric form (D) susceptible to a self-assembly process that competes with PLP binding and leads to the formation of high-molecular weight aggregates (AGG\text{D}). Thus, the molecular defect of Gly161 variants seems to affect mostly the apo-form of the protein, as previously found for other pathogenic variants [10,15]. However, Gly161 variants show a peculiar behavior, because they form large aggregates that localize in the cell cytosol, as indicated by IFM and IEM analyses. In fact, the increased propensity to aggregation in both non-cellular and cellular systems is not a novelty in the array of enzymatic phenotypes leading to AGT deficiency [14,15,22,40], but only the formation of intraperoxisomal aggregates of AGT has been already reported in liver biopsies of PH1 patients and in CHO cells expressing the G41R-Mi variant [19,24]. Moreover, although we do not know if the ultrastructure of the aggregates formed by Gly161 variants is similar to that of the intraperoxisomal aggregates formed by Gly41 variants, some considerations on the cytosolic localization of Gly161 variants can be made. It is unlikely that the behavior of Gly161 could depend on their inability to interact with the peroxisomal carrier Pex5p, as the mutation site is far from the interaction site [5] and both G161S-Mi and G161C-Mi show a low peroxisomal localization. Nevertheless, considering that the aggregation kinetics of purified apoG161S-Mi and apoG161C-Mi under physiological conditions is faster than that previously reported for the G41R-Mi variant [14], it seems likely that the self-assembly of Gly161 variants occurs so rapidly that large aggregates are formed before the import can take place. This model can also explain the absence of a mitochondrial localization of G161S-Mi and G161C-Mi. Fargue S. et al. [24] reported that some destabilizing mutations cosegregate with the minor allele polymorphism lead to a mitochondrial mistargeting of the protein. The mislocalization is the result of a synergism between the polymorphic P11L mutation, which unmask a putative mitochondrial targeting sequence [8,41], and the pathogenic mutations, which promote the accumulation of unfolded or partly folded monomeric species compatible with the mitochondrial import machinery. In the case of the G161S-Mi and G161C-Mi variants, the pathogenic mutation does not allow the accumulation of monomeric species, which would favor the mitochondrial import. However, it cannot be excluded that the mitochondrial localization is prevented by the short-term expression of the protein, which could foster a peroxisomal localization [24].

4.3. PLP is able to partly reduce the effects of Gly161 mutation by decreasing the aggregation extent of the variants

Treatment with vitamin B6 is a common strategy for the therapy of many metabolic diseases caused by PLP-dependent enzymes. However, the degree of responsiveness is extremely variable depending on the disease, the genotype and the enzymatic phenotype of the patients [42–47]. Pyridoxine administration is effective in about 25–35% of PH1 patients and clinical data suggest that the responsive mutations are those associated with AGT mistargeting [48–50]. Although it is known that pyridoxine administration increases the intracellular concentration of PLP, the molecular bases of the effect of the coenzyme are still under investigation with both biochemical and cell biology approaches [10,15,38,21]. The evidences reported in this study suggest that (i) in the CHO cellular model, pyridoxine treatment increases the enzymatic activity of Gly161 variants by preventing protein aggregation and (ii) the addition of PLP reduces the aggregation extent of purified G161S-Mi and G161C-Mi variants by converting the apo- to the more stable holo-form. Thus, the coenzyme reduces the aggregation of the variants both in a non-cellular and in a cellular environment by shifting the equilibrium from the apodimer to the holodimer (Fig. 11A). Therefore, the coenzyme seems to play essentially a prosthetic role. This is also confirmed by the finding that the total amount of AGT was not increased in the presence of added pyridoxine, thus suggesting that the coenzyme does not prevent protein degradation, in agreement with previous evidences indicating that the addition of PLP does not increase the half-life of the G161R-Ma variant [17]. However, it should be considered that the medium used for pulse-chase experiments has a standard pyridoxine content of 19.6 μM, which could promote holoenzyme formation and prevent the detection of the effect of added coenzyme.

4.4. Conclusions

A particular feature of PH1 is the extreme variability of enzymatic phenotypes by which missense mutations can lead to AGT deficiency.
Previous biochemical and cell biology studies have highlighted that while some mutations only cause a functional defect, the large majority of them interfere with AGT folding causing different downstream effects (Fig. 11B). In our work, we analyzed the effects of the Gly161 mutation by combining studies on a mammalian cellular system, which give insights into the effect of the mutation on the expression level, subcellular localization and intracellular stability of the protein, with analyses on the purified variants, which give insights into the effect of the mutation on the kinetic properties, the tertiary and the quaternary structure of the protein. The combination of the results obtained allowed us to identify a new enzymatic phenotype leading to AGT deficiency, i.e. the formation of cytosolic apopaggregates (Fig. 11B). Although PH1 patients bearing mutations at Gly161 identified until now are compound heterozygous [28,51], it should be predicted that at least 25% of the AGT expressed by these patients is homodimers of Gly161 variants whose enzymatic phenotype could be partially interpreted on the basis of our results in the cellular model system. Moreover, it cannot be excluded that the pathogenic mechanism identified in this work could apply to other pathogenic mutations leading to effects similar to those caused by Gly161 substitution, thus affecting a larger number of patients. Finally, on the basis of our results, pyridoxine administration can be proposed as a first line option for PH1 patients bearing mutations at Gly161. However, considering the high susceptibility to intracellular degradation of Gly161 variants and the limited effect of pyridoxine on their specific activities, vitamin B6 administration should be associated with other molecules able to either specifically improve the folding efficiency of the variants, such as pharmacological chaperones, or to enhance the general folding ability of the cell, such as proteostasis regulators [52].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.09.002.

Acknowledgements

This work was supported by Telethon Foundation (GPP10092). E.O. is supported by a Fellowship from the Hyperoxaluria and Oxalosis Foundation. We thank Prof. Carla Borri Voltattorni for critically reading the manuscript. We would also like to acknowledge Elena Polishchuk and Simona Iacobacci (TIGEM, Naples) as well as the Telethon Electron Microscopy Core Facility (IPB, CNR, Naples) for IEM support (Telethon grant #GTF08001).

References


