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Research paper

Effects of interface mutations on the dimerization of alanine glyoxylate aminotransferase and implications in the mistargeting of the pathogenic variants F152I and I244T

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ABSTRACT

In this work the dimerization process of the minor allelic form of human alanine glyoxylate aminotransferase, a pyridoxal 5′-phosphate enzyme, was investigated. Bioinformatic analyses followed by site-directed mutagenesis, size exclusion chromatography and catalytic activity experiments allowed us to identify Arg118, Phe238 and Phe240 as interfacial residues not essential for transaminase activity but important for dimer-monomer dissociation. The apo and the holo forms of the triple mutant R118A-Mi/F238S-Mi/F240S-Mi display a dimer-monomer equilibrium dissociation constant value at least ~260- and 31-fold larger, respectively, than the corresponding ones of AGT-Mi. In the presence of PLP, the apo-monomer of the triple mutant undergoes a biphasic process: the fast phase represents the formation of an inactive PLP-bound monomer, while the slow phase depicts the monomer-monomer association that parallels the regain of transaminase activity. The latter events occur with a rate constant of ~0.02 $\mu M^{-1} min^{-1}$. In the absence of PLP, the apomonomer is also able to dimerize but with a rate constant value ~2700-fold lower. Thereafter, the possible interference with the dimerization process of AGT-Mi exerted by the mutated residues in the I244T-Mi and F152I-Mi variants associated with Primary Hyperoxaluria type 1 was investigated by molecular dynamics simulations. On the basis of the present and previous studies, a model for the dimerization process of AGT-Mi, I244T-Mi and F152I-Mi, which outlines the structural defects responsible for the complete or partial mistargeting of the pathogenic variants, was proposed and discussed.

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1. Introduction

Human liver peroxisomal alanine glyoxylate aminotransferase (AGT) (EC 2.6.1.44) catalyses the transamination of L-alanine and glyoxylate to pyruvate and glycine, respectively, by a classical ping-pong mechanism. In the first half-reaction the enzyme in the pyridoxal 5′-phosphate (PLP) form (AGT–PLP) reacts with L-alanine generating pyruvate and the enzyme in the pyridoxamine 5′-phosphate form (AGT–PMP). Then, in the second half-reaction, AGT–PMP binds glyoxylate and converts it to glycine regenerating AGT–PLP [1]. Human AGT is a dimer and belongs to the Fold Type I class of the PLP-enzyme family [2]. The crystal structure reveals that each subunit is composed of an N-terminal extension (residues 1–21) that wraps over the surface of the other subunit, a large domain (residues 22–282) containing most of the active site residues and of the dimerization interface, and a small domain (residues 283–392) containing a peculiar KKL type 1 peroxisomal targeting sequence (PTS1) at the C-terminus [2]. A deficit of AGT causes Primary Hyperoxaluria Type I (PH1) (OMIM 259900), a rare metabolic recessive disease due to inborn errors affecting the metabolism of glyoxylate in liver peroxisomes [3].

Abbreviations: AGT-Mi, minor allele of human alanine glyoxylate aminotransferase; PH1, Primary Hyperoxaluria type I; PTS1, type 1 peroxisomal targeting sequence; $K_{d(dim-mon)}$, dimer-monomer equilibrium dissociation constant; MD, molecular dynamics; ASA, solvent accessible area; $K_{d(PLP)}$, equilibrium dissociation constant for PLP; SEC, size exclusion cromatography; DLS, dynamic light scattering; ANS, 8-anilino-1-naphthalenesulfonic acid; RMSD, root mean square deviation; RMSF, root mean square fluctuation; ED, essential dynamics.

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The *AGXT* gene encoding AGT is present in humans in two polymorphic forms: the most common major allele encoding AGT-Ma, and the less common minor allele encoding AGT-Mi. The latter differs from the major allele for the presence of a 74-bp duplication in intron 1 and of two mutations leading to the Pro11-to-Leu and Ile340-to-Met amino acid substitutions [4]. The P11L replacement generates a structural motif of binding to the mitochondrial import receptor TOM 20, and this could partly explain why AGT-Ma is 100% peroxisomal, while AGT-Mi is 95% peroxisomal and 5% mitochondrial. Moreover, as compared with AGT-Ma, AGT-Mi has a reduced (~70%) catalytic activity and, importantly, a reduced dimeric stability under chemical stress [5, 6]. Nevertheless, since the true value of the dimer-monomer equilibrium dissociation constant ($K_{d(dim-mon)}$) for both AGT-Ma and AGT-Mi is only assumed to be lower than 0.3 μM [5], it is not possible to establish if the mutations P11L and/or I340M alter the dimer-monomer dissociation under native conditions. The AGT-Mi polymorphism is not pathogenic *per se*, but many mutations are pathogenic only when they functionally synergize with the minor allele. Some of them, like G170R, F152I, I224T, G47R and G41R cause a complete or partial aberrant targeting of AGT to mitochondria, thus resulting in a hampering of the metabolic function. Fargue et al. [7] advanced the possibility that all PH1-causing mutations segregating with the minor allele synergize with the P11L mutation unmasking the cryptic mitochondrial targeting sequence, and, as a consequence, leading to peroxisome-to-mitochondrion mistargeting. However, it should be noted that, when transiently expressed in CHO cells, the G161 variants co-segregating with the minor allele were found as cytosolic aggregates [8]. Again, it was reported that (i) the G41R-Mi shows a complex picture, some mithocondrial labelling and some peroxisomal in the shape of intraperoxisomal aggregates [9], and (ii) G47R-Mi shows a peroxisomal and mitochondrial localization [10]. Moreover, the structural basis of the suggested synergic interaction of P11L with each mutation leading to mistargeting is far from being elucidated. Since AGT in the monomeric form and containing the P11L mutation is a prerequisite for the aberrant targeting of the enzyme, we decided to study the associative/dissociative process of AGT-Mi in the apo and holo forms. Up to date, the self-association of the subunits of AGT including the structural elements which contribute to dimer formation is unknown. It was only demonstrated that the deletion of the N-terminal extension does not prevent AGT dimer formation [11], and it was suggested the involvement of the P11L mutation in the destabilization of the dimeric structure of AGT [5, 11].

In this work, guided by bioinformatic information, a series of single, double, and triple mutants was engineered and analyzed for their catalytic activity, spectroscopic features, and quaternary structure. Taken together, our studies allowed us to identify Arg118 as a hotspot dimer interfacial residue playing a relevant role in the dimerization process of AGT-Mi. Moreover, we noticed that the combination of R118A mutation with F238S and F240S mutations significantly alter the extent of monomerization of both apo and holoAGT-Mi. Starting from the folded apomonomer of the triple mutant the process of dimerization was investigated. We established that in the presence of PLP the formation of a PLP-bound monomer precedes the generation of the holodimer, which parallels the regain of transaminase activity. These events occur with a rate constant of 0.020 ± 0.002 $\mu M^{-1} min^{-1}$. The apomonomer, in the absence of exogenous PLP, is also able to dimerize even if with a rate constant 2700-fold lower that of the PLP-bound monomer. In addition, predictions by means of molecular dynamics (MD) simulations of the structural effects caused by the mutations F152I and I244T on the minor allele provide a possible interpretation and explanation for their interference with the dimerization process.

2. Materials and methods

2.1. Materials

PLP, L-alanine, NADH, rabbit muscle L-lactic dehydrogenase, and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Sigma. 8-anilino-1-naphthalenesulfonic acid (ANS) was purchased from Molecular Probes. All other chemicals were of the highest grade commercially available.

2.2. Bioinformatic analyses

Alanine scanning mutagenesis, analysis of the interfacial hydrophobic patches and inspection of the AGT structure and interface contacts were performed by the software Molecular Operating Environment (MOE 2015.1001) [12] of the Chemical Computing Group using the crystal structure of human AGT (pdb file: 1H0C). The dimeric structure of human AGT was obtained by means of the PISA web server [13] starting from the available coordinate file of the monomer (PDB id: 1H0C) [2]. In particular, each residue present on the dimerization interface involved in either intrachain or interchain contacts was subjected to alanine scanning mutagenesis using the Unary Quadratic Optimization (UQO) under the LowMode ensemble, which uses the LowModeMD [14] to search the conformational space of the mutants. The conformations that satisfied the required energetic and geometric criteria were saved to the output database. Atoms located at a distance greater than 4.5 Å from the mutation site were marked as inert, iterations were limited to 50, and conformations were limited to ten for each mutated complex. The dStability values (kcal/mol) were calculated with MOE 2015.1001 [12].

2.3. Site-directed mutagenesis

The mutant forms of human AGT-Mi were constructed by the QuikChangeTM II kit from Agilent Technologies using the pTrcHis2A vector encoding AGT-Mi (pAGT-His) [1] as template and the oligonucleotides listed in the Supplementary Table S1.

2.4. Expression and purification of AGT-Mi and mutants

E. coli JM109 cells transformed with vectors encoding AGT-Mi or mutated AGT-Mi were grown in 4.5 l of Luria broth at 37 °C to an absorbance at 600 nm of 0.4–0.6. Expression was induced after 6 h with 0.1 mM IPTG for 15 h at 30 °C. Cells were harvested and resuspended in 20 mM sodium phosphate buffer pH 7.4, containing 0.5 M NaCl, 20 mM imidazole and 100 μM PLP. Lysozyme was added to a final concentration of 0.2 μg/ml and the culture was incubated for 15 min at room temperature. After a freeze-thaw, leupetin (0.5 μg/ml) and pepstatin (0.5 μg/mL) were added and the suspension was centrifuged at 30,000 *g* for 30 min at 4 °C. The lysate was loaded on a HisPrep FF 16/10 equilibrated with 20 mM sodium phosphate buffer pH 7.4 containing 0.5 M NaCl and 20 mM imidazole. A linear gradient was then applied (0–100% in 240 ml) with the same buffer containing 500 mM imidazole. AGT-Mi and mutants elute between 200 and 300 mM imidazole. After addition of 100 μM PLP, the protein solution was concentrated; imidazole and unbound coenzyme were removed by extensive washing with 100 mM potassium phosphate buffer, pH 7.4, using Amicon Ultra 10 concentrators (Amicon). The protein concentration in the AGT-Mi and mutants samples in the dimeric form was determined using an extinction coefficient of $9.54 \times 10^4 M^{-1} cm^{-1}$ at 280 nm [15]. The

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