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Modeling the effect of 3 missense *AGXT* mutations on dimerization of the AGT enzyme in primary hyperoxaluria type I

Short Title: modeling AGXT mutations in PH1

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Conflict of interest: none

Abstract

Introduction

Mutations of the *AGXT* gene encoding the alanine:glyoxylate aminotransferase liver enzyme (AGT) cause primary hyperoxaluria type 1 (PH1). Here we report a molecular modeling study of selected missense *AGXT* mutations: the common Gly170Arg, the recently described Gly47Arg and Ser81Leu variants, predicted to be pathogenic using standard criteria.

Methods

Taking advantage of the refined 3D structure of AGT, we computed the dimerization energy of the wild type and mutated proteins.

Results

Molecular modeling predicted that Gly47Arg affects dimerization with a similar effect to that shown previously for Gly170Arg through classical biochemical approaches. In contrast, no effect on dimerization was predicted for Ser81Leu. Therefore, this probably demonstrates pathogenic properties via a different mechanism, similar to that described for the adjacent Gly82Glu mutation that affects pyridoxine binding.

Conclusion

This study shows that the molecular modeling approach can contribute to evaluating the pathogenicity of some missense variants that affect dimerization. However, *in silico* studies - aimed to assess the relationship between structural change and biological effects - requires the integrated use of more than one tool.

Key words:

AGXT gene, AGT enzyme, Primary Hyperoxaluria Type 1, molecular modeling

INTRODUCTION

Primary hyperoxaluria type I (PH1) is an autosomal recessive disorder caused by defects of the liver-specific peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT, EC 2.6.1.44) encoded by the *AGXT* gene (1). The AGT molecule has a homodimeric structure of 2x43 kD/ 392 aminoacid subunits. AGT rapidly dimerizes after synthesis in the cytosol, and is imported into the peroxisomes, where it catalyzes the transamination of glyoxylate to glycine, coupled with the conversion of alanine to pyruvate, with pyridoxal-5-phosphate as a cofactor.

Deficiency of AGT in PH1 patients impairs the hepatic detoxification of glyoxylate and results in increased oxalate and glycolate in plasma and urine. Excess oxalate saturates body fluids and accumulates as insoluble calcium salts in several organs (2, 3). The natural history of untreated PH1 is characterized by progressive decline in renal function, from nephrolithiasis nephrocalcinosis to generalized oxalosis and death (4).

A comprehensive algorithm for the clinical and biochemical diagnosis of hyperoxaluria has been recently proposed (5). *AGXT* gene testing has become an important diagnostic tool (6-8) and provided that the pathogenicity of mutations is verified, it renders the invasive liver biopsy (for direct assaying of AGT activity) unnecessary in most cases (9). Conversely, when only private variants of unknown pathogenicity are found, molecular diagnosis remains doubtful.

The mutational spectrum of the *AGXT* gene encompasses almost 100 different mutations so far, including nonsense, splice site, frameshift and missense point mutations, and rarer entire exon deletions (6,8,10). Missense mutations may have various pathogenic effects. The most frequent, Gly170Arg, causes a remarkable trafficking defect, whereby the newly synthesized AGT subunit is diverted away from its normal peroxisomal location, in part degraded, while the remaining in part is diverted to the mitochondria where eventually dimerizes (11). This effect is synergistically

influenced by the common Pro11Leu polymorphism whose Leu allele introduces a mitochondrial signal sequence (12), that targets a fraction of the AGT protein to the mitochondrion (13), and decreases the enzyme activity by 30% (14). Docking of the correctly folded monomers into stable dimers represents a critical step of AGT biosynthesis. Patients carrying Gly170Arg, or other mistargeting mutations synergic with the Leu11, like Phe152Ile (14), are most likely to respond to vitamin B6 (Pyridoxine) (16-18). Other missense mutations are associated with accelerated degradation or intra-peroxisomal aggregation, that probably result from misfolding at later stages of AGT biosynthesis (14). Finally, specific mutations of critical residues in the catalytic- or the cofactor-binding sites can affect the enzymatic activity without altering AGT dimerization or targeting (19).

Determination of the crystal structure of the AGT enzyme has enabled us to improve the understanding of the pathogenic effects of some recurrent missense mutations, in terms of folding, dimerization and stability (20). Such studies may open the way to new therapeutic strategies, based on the structure-based design of small molecules capable of rescuing the defective enzyme, and thus extending the chance of conservative treatment to other more severe, life-threatening and non-Pyridoxine responsive mutations (21).

In this paper we approach the question by using a new *in silico* molecular modeling method aimed to rationalize the effects of specific missense AGT mutations on dimerization thermodynamics and provide proof of principle evidence of its performance.

SUBJECTS AND METHODS

Patients in whom the modeled mutations were identified belong to the Italian multicenter study of primary Hyperoxaluria, and had given their informed consent to use their genetic and clinical data for research purposes, as already described (17,22). Age at onset, relevant clinical data and

residual liver AGT activity, when available, are shown in Table 1. Screening of *AGXT* gene mutations was performed on genomic DNA through PCR amplification and direct sequencing, whereas healthy controls were analysed by DHPLC. The refseq NP_000021.1 protein sequence of the human AGT gene was used as reference. Multiple sequence alignment of 22 orthologous species was generated using ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>, Supplementary material, Fig.2). The evolutionary conservation at the mutated positions was evaluated using the SIFT (23), SNPs3D (24) and PMUT (25) algorithms available on line. The “major” or “minor” haplotype were characterized by inspection of the relevant sequence features at codons 11 and 340 (26).

Molecular Modeling. Starting from the crystal structure of the *wt* AGT homodimer *WT:WT* (20) we prepared the refined three-dimensional (3D) structure of the wild type (*wt*) dimer bound to the pyridoxal-5'-phosphate (PLP) cofactor and the aminooxyacetic acid (AOA) inhibitor (Figure 1) available from *Protein Data Bank* (27) - and from there we prepared the structure of the *wt* AGT monomer.

In the 3D models of the mutant AGT forms (*mt*) we replaced the side chains at the mutated sites by the best rotamer of the substituting residue followed by careful conformational search and geometry optimization using the molecular modeling software *Insight-II* (Accelrys Software Inc., San Diego, CA, release 2000). We computed the conformational and interaction energies of each AGT monomer and dimer by molecular mechanics (MM) using class II consistent force field CFF91 and charge parameters (28).

The dimerization of two *mt* AGT monomers in aqueous solution to form a homodimer (*MT:MT*) can be represented by the energy of dimerization: $\Delta E_{\text{tot}} = E_{\text{tot}}[\text{MT:MT}] - 2E_{\text{tot}}[\text{MT}]$, composed of contributions from molecular mechanics (MM) potential energy (ΔE_{MM}) and solvent effects

(ΔE_{solv}) as: $\Delta E_{\text{tot}} = \Delta E_{\text{MM}} + \Delta E_{\text{solv}}$. When computing dimerization energy, we took into account the interaction between monomers in the dimer, the stability and molecular structure of the free monomers and the effect of solvent upon monomers association. We compared the different *mt* AGT forms via relative changes in the dimerization energy, $\Delta \Delta E_{\text{tot}} = \Delta E_{\text{tot}}[\text{MT:MT}] - \Delta E_{\text{tot}}[\text{WT:WT}]$ with respect to the reference *wt* homodimer *WT:WT*. Relative changes were defined in a similar way for the contributions of both the MM interaction energy and the solvent effect.

When computing the solvation energy, we also incorporated the effects of ionic strength through the solution of nonlinear Poisson-Boltzmann equation (29), using the software package *DelPhi* (Accelrys Software Inc., San Diego, CA, release 2000).

We calculated the molecular solvent-accessible surface area of the *wt* and *mt* residues by using the Connolly algorithm (30) implemented in the *Insight-II* software. Relative changes in the accessible surface area of the studied residues upon dimer formation ($\Delta \Delta S_{\text{bur}}$) were calculated as a difference in the solvent accessible surfaces of residues in the dimer and in the free monomers with respect to the corresponding residues in the reference *wt* form of AGT: $\Delta \Delta S_{\text{bur}} = (2S_{\text{Con}}[\text{mt}]_{\text{MT}} - S_{\text{Con}}[\text{mt,mt}]_{\text{MT:MT}}) - (2S_{\text{Con}}[\text{wt}]_{\text{WT}} - S_{\text{Con}}[\text{wt,wt}]_{\text{WT:WT}})$.

RESULTS

AGXT Mutations. The 3 selected mutations were all absent in 80 healthy ethnically-matched controls (160 chromosomes). Multiple sequence alignment showed evolutionary conservation at residue Gly47 and Gly170 in 22/22 species and at Ser81 in 17/22 (Supplementary material, Fig.2). The SIFT and SNPs3D software tools concordantly predicted Gly47Arg and Ser81Leu to be not tolerated, with similar or stronger scores than the common Gly170Arg mutation (Table 1),

and were considered as pathogenic. In contrast, the PMUT software predicted only Gly47Arg to be pathogenic, and Ser81Leu to be tolerated.

We then implemented a molecular modeling approach to study the effect of the three missense changes on AGT dimerization, using the known Gly170Arg mutation as a positive control. For this, we modeled the homodimeric AGT forms of each of the three changes, as well as the heterodimer expected in the Gly170Arg/Gly47Arg compound heterozygous patient 1. No heterodimer was expected in patient 2 who carried a null mutation on the second allele.

Molecular Modeling. Residue Gly47 is located on the dimerization interface of AGT, close to the C-terminal boundary of the long, irregular N-terminal coil (residues 1-21) that upon dimerization wraps around the cognate monomer (Figure 2). Residue Ser81 is similarly positioned on the dimerization interface, near the cofactor binding site. In contrast, residue Gly170 is located on the exposed surface without direct contact with the cognate monomer. Due to its position at the dimer interface, the substitution of the small Gly47 residue by a bulky cationic Arginine contributes more to the relative surface area buried upon AGT dimerization ($\Delta\Delta S_{bur}$) than the other mutations that either insert a smaller residue or are located outside of the dimerization interface (Table 2). The Gly47Arg and Gly170Arg substitutions stabilize the AGT homodimers via increased monomer-monomer interaction, $\Delta\Delta E_{MM}$, more than the Ser81Leu substitution (Table 2).

On the other hand, the attractive MM interactions caused by the non conservative Gly to Arg substitutions are largely compensated for upon AGT dimerization by the effect of the solvent. The *wt* AGT monomer bears a total molecular charge of $+1e^-$, while Arg47 and Arg170 increase its charge to $+2e^-$. In both cases, and especially for Arg47, the free *mt* monomers with the charged Arg residues exposed to the solvent are better stabilized by the interactions with water

($\Delta\Delta E_{\text{solv}}$) than the dimers, where the corresponding residues are partially buried (Table 2). Thus the stability of the mutated dimers is significantly diminished by the effect of protein hydration. In contrast, the Ser81Leu substitution does not change the charge. The overall dimer stability, which takes into account both the interactions between the residues of the associated monomers and the solvent effect ($\Delta\Delta E_{\text{tot}}$), is weaker for all the considered *mt* dimers than the native *WT:WT*. Our computational approach predicts a stronger decrease of dimer stability for the *mt* *G47R:G47R* > *G170R:G170R* dimers compared to the *WT:WT* reference, while only a minor change in dimer stability is predicted for the *mt* *S81L:S81L* homodimer (Table 2).

DISCUSSION

The pathogenic effect of recurrent AGT mutations has been extensively investigated with classical biochemical and biological approaches. A series of studies, both *ex vivo* and *in vitro*, have highlighted the peculiar effect of the recurrent Gly170Arg mutation on AGT biosynthesis (15). However, no comparable knowledge is available for the remaining missense AGT mutations, about 30-34% in Caucasians, in which a growing series of rarer or private changes are identified (10). In these cases, the current criteria of cosegregation of disease in families and lack of disease in controls does not always allow to discriminate true mutations from rare polymorphisms. The liver specific expression of AGT further hampers to evaluate the effect of promoter and splice-control mutations on mRNA biosynthesis. For missense mutations, determination of the crystal structure of AGT has opened the way to rationalize the pathogenic mechanisms in terms of folding, dimerization and stability of the enzyme (20). Here we describe the application of molecular modeling techniques as a means to explore the pathogenic potential of AGT missense mutations. Similar approaches was previously employed by Burnett et al (31)

for hemoglobin and Kobayashi et al (32) for lipoprotein lipases, but to our knowledge their use for AGT mutations is novel.

We assumed the working hypothesis that some mutations, in particular the Gly47Arg situated on the dimerization interface, affect the formation of AGT dimers and may exert effects similar to those demonstrated for Gly170Arg. Indeed, when we rationalized the stability of the mutated Gly170Arg and Gly47Arg dimers in terms of net charge and size of the replacing Arg residue and its location on the molecular surface with respect to the dimerization region, solvation resulted to exert a dominant effect and stabilize the free monomers more than the corresponding dimers. In our molecular modeling both mutations change the $\Delta\Delta E_{\text{tot}}$ by an order of tenth kcal·mol⁻¹, i.e. shift the equilibrium constant of the *mt* molecules from dimer to free monomeric *mt*. In other words, dimers with either mutation are predicted to be less thermodynamically stable at physiological conditions than the wild type AGT dimer.

Decreased stability of the mutated AGT dimers has been previously considered as a possible cause of the peroxisomes-to-mitochondria mistargeting (15) and accelerated AGT degradation or aggregation that have been associated with the Gly170Arg mutation (14). According to our modeling study, Gly47Arg destabilizes the AGT dimer even more than Gly170Arg. Interestingly, a similar destabilizing effect is predicted for both the Gly170Arg:Gly170Arg and Gly47Arg:Gly47Arg mutated homodimers and for the Gly47Arg:Gly170Arg heterodimer, whose corresponding *in vivo* situation is represented by the compound heterozygous patient 1. On overall, these data support the pathogenicity of Gly47Arg, although its clinical severity cannot be established in the presence of the mild Gly170Arg mutation, as in general true for new described mutations when found in compound heterozygosity with mild ones.

The effect of the Ser81Leu mutation predicted from molecular modeling is much less pronounced, with a minimal change of the $\Delta\Delta E_{\text{tot}}$, suggesting that secondary structures and dimers formation are not affected. This is in agreement with the PMUT results, based also on the profile of secondary structure and solvent accessibility. On the other hand, Ser81Leu should be considered pathogenic on the basis of absence in ethnically matched controls and high degree of evolutionary conservation. Its proximity to the cofactor and substrate binding sites suggests that it may affect the catalytic function of the enzyme. Indeed, the adjacent Gly82Glu mutation was previously described to affect pyridoxine binding (14).

The wide mutational spectrum of primary hyperoxaluria and the consequent variety of the AGT enzyme defects makes the use of multiple approaches to characterize novel mutations highly recommended. Since dimerization represents a critical step in AGT biosynthesis, the presented approach can significantly contribute to assess the pathogenicity of missense mutations, clarifying their contribution to the phenotype and providing some insight into the structural and functional changes of AGT responsible of the PH1 phenotype.

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Legends for figures

Figure 1

Dimerization site/active site view of the relaxed *wt* AGT monomer in a ribbon rendering. The co-factor pyridoxal-5'-phosphate (PLP) and the substrate aminooxyacetic acid (AOA) are shown in Corey-Pauling-Kultun (CPK) representation in cyan and brown. The locations of the Leu11 and Met340 residues of the minor polymorphic form are indicated by horizontal and vertical arrows respectively. The mutated residues are highlighted: Gly47 in yellow, Ser81 in green and Gly170 in white.

Figure 2

(A) Front view and (B) side view of the AGT dimer *WT:WT* in Corey-Pauling-Kultun (CPK) representation, with monomer A shown in red and monomer B in blue. Positions of the mutated residues GlyA47 and GlyB47 are shown in yellow, residues GlyA170 and GlyB170 in white. Residues SerA81 and SerB81 located at the dimerization interface are not visible. The long N-terminal extensions (coils of residues 1 – 21) wrap around the N-terminal segment of the opposing monomer.

TABLES

Table 1. Clinical, biochemical and molecular findings of PH1 patients.

Patient ID gender (origin)	Age at onset (yrs)	AGT enzyme activity	cDNA	Protein	AGXT haplotype*	Evolutionary conservation	Pathogenicity score [§]		
							SIFT	SNPs3D	PMut
1 male (Sicily)	35	2%	c.139G>A	G47R	minor	22/22	0.00 non toler.	-1.91 non toler	0.59 non toler
			c.508G>A	G170R	minor	22/22	0.00 non toler.	-0.55 non toler	0.56 non toler
2 female (Serbia)	1	Not done	c.242C>T	S81L	maior	17/22	0.01 non toler	-0.51 non toler	0.31 tolerated
			c.614C>A	S205X	maior				

* minor haplotype is defined by P11L + 74bp-duplication in intron 1 + I340M.

§ non tolerated: SIFT < 0.05 (23), SNPs3D < 0 (24) PMut > 0.5 (25).

Table 2. Buried surface and relative energies of dimerization of wild type (*wt*) and mutated (*mt*) human AGT monomers.

AGT dimer	Interacting monomers		Relative buried surface [Å ²]	Relative energy of dimerization [kcal·mol ⁻¹]		
	Monomer <i>MT_i</i>	Monomer <i>MT_j</i>	$\Delta\Delta S_{bur}$ ^a	$\Delta\Delta E_{MM}$ ^b	$\Delta\Delta E_{solv}$ ^c	$\Delta\Delta E_{tot}$ ^{d,e}
<i>wt:wt</i> WT:WT homodimer	<i>wt</i> AGT ($Q_{wt} = +1$) ^f	<i>wt</i> AGT ($Q_{wt} = +1$)	0	0	0	0
<i>G47R:G47R</i> <i>MT_i:MT_j</i> homodimer	<i>mt</i> AGT <i>Gly47Arg</i> ($Q_{mt} = +2$)	<i>mt</i> AGT <i>Gly47Arg</i> ($Q_{mt} = +2$)	158	-23	106	83
<i>G170R:G170R</i> <i>MT_i:MT_j</i> homodimer	<i>mt</i> AGT <i>Gly170Arg</i> ($Q_{mt} = +2$)	<i>mt</i> AGT <i>Gly170Arg</i> ($Q_{mt} = +2$)	44	-16	56	40
<i>S81L:S81L</i> <i>MT_i:MT_j</i> homodimer	<i>mt</i> AGT <i>Ser81Leu</i> ($Q_{mt} = +1$)	<i>mt</i> AGT <i>Ser81Leu</i> ($Q_{mt} = +1$)	14	1	2	3
<i>G47R:G170R</i> <i>MT_i:MT_j</i> heterodimer	<i>mt</i> AGT <i>Gly47Arg</i> ($Q_{mt} = +2$)	<i>mt</i> AGT <i>Gly170Arg</i> ($Q_{mt} = +2$)	110	-19	74	55

^a $\Delta\Delta S_{bur}$ – relative contribution of mutated residue to the buried surface upon dimer formation was calculated as the difference in Connolly surfaces (30) of the

- concerned residues in the dimer *MT:MT* and in free monomer *MT* with respect to the native residues in the homodimer *WT:WT* $\Delta\Delta S_{\text{bur}} = (2S_{\text{Con}}[mt]_{MT} - S_{\text{Con}}[mt,mt]_{MT:MT}) - (2S_{\text{Con}}[wt]_{WT} - S_{\text{Con}}[wt,wt]_{WT:WT})$, in [\AA^2];
- ^b $\Delta\Delta E_{\text{MM}} = (E_{\text{MM}}[MT:MT] - 2E_{\text{MM}}[MT]) - (E_{\text{MM}}[WT:WT] - 2E_{\text{MM}}[WT])$ is the relative molecular mechanics interaction energy contribution to the *MT:MT* AGT dimer formation with respect to the *wt* homodimer *WT:WT*;
- ^c $\Delta\Delta E_{\text{solv}} = (E_{\text{solv}}[MT:MT] - 2E_{\text{solv}}[MT]) - (E_{\text{solv}}[WT:WT] - 2E_{\text{solv}}[WT])$ is the relative solvation energy contribution to the *MT:MT* AGT dimer formation with respect to the *wt* homodimer *WT:WT*;
- ^d $\Delta\Delta E_{\text{tot}} = \Delta\Delta E_{\text{MM}} + \Delta\Delta E_{\text{solv}}$ is the relative total energy change connected with the *MT:MT* AGT dimer formation with respect to the *wt* homodimer *WT:WT*;
- ^e a change of $\Delta\Delta E_{\text{tot}}$ by $10 \text{ kcal}\cdot\text{mol}^{-1}$ corresponds to about 3.2% change in the energy of dimerization the *WT:WT* dimer. For comparison, an increase of Gibbs free of a dimerization reaction by $10 \text{ kcal}\cdot\text{mol}^{-1}$ will cause a shift of the equilibrium constant of this reaction by a factor of $5\cdot 10^8$ towards dissociated monomers;
- ^f Q_{wt} , Q_{mt} are the molecular charges of *wt* or *mt* AGT monomers including the co-factor PLP and substrate AOA.

Figure 1

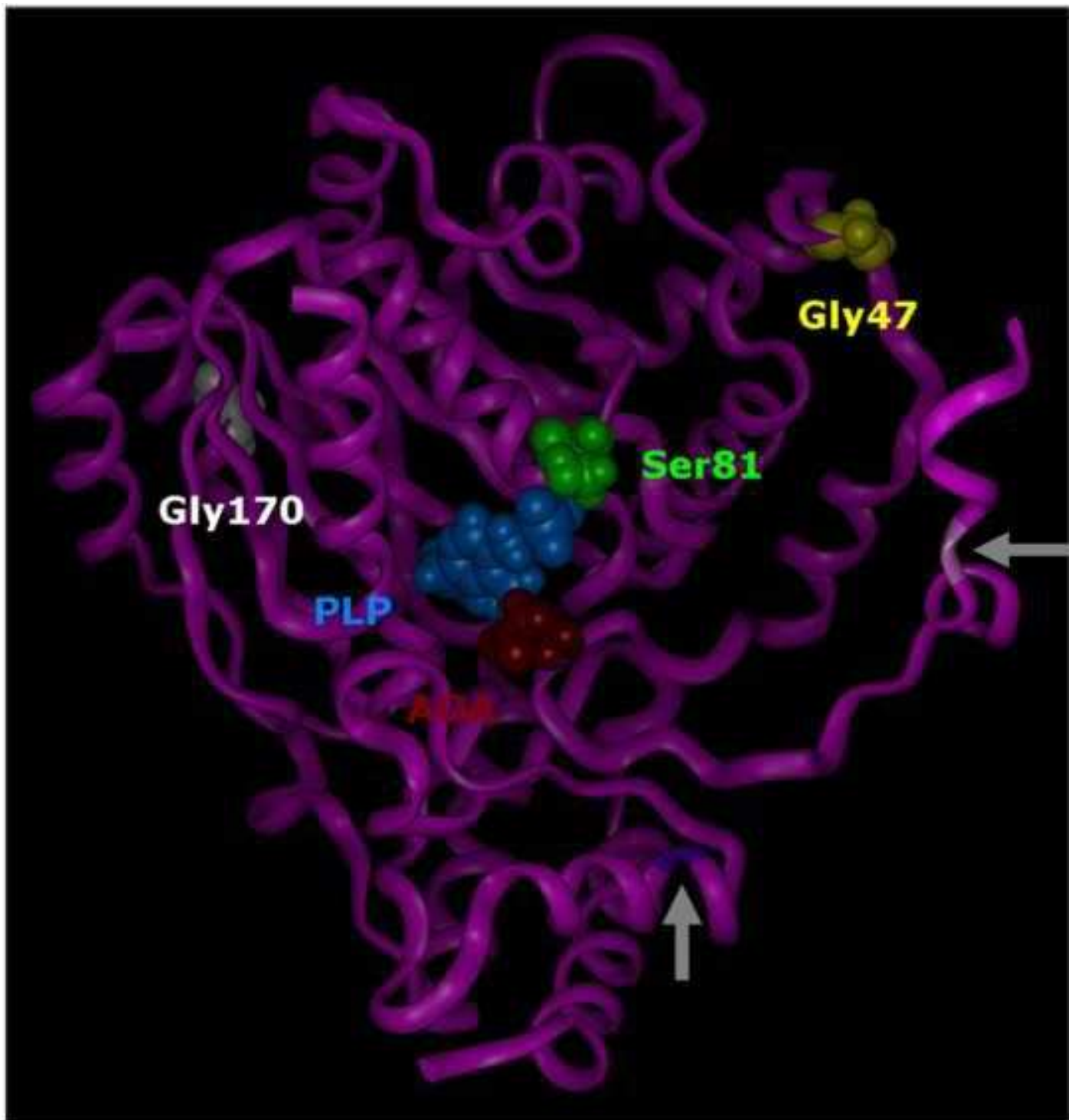
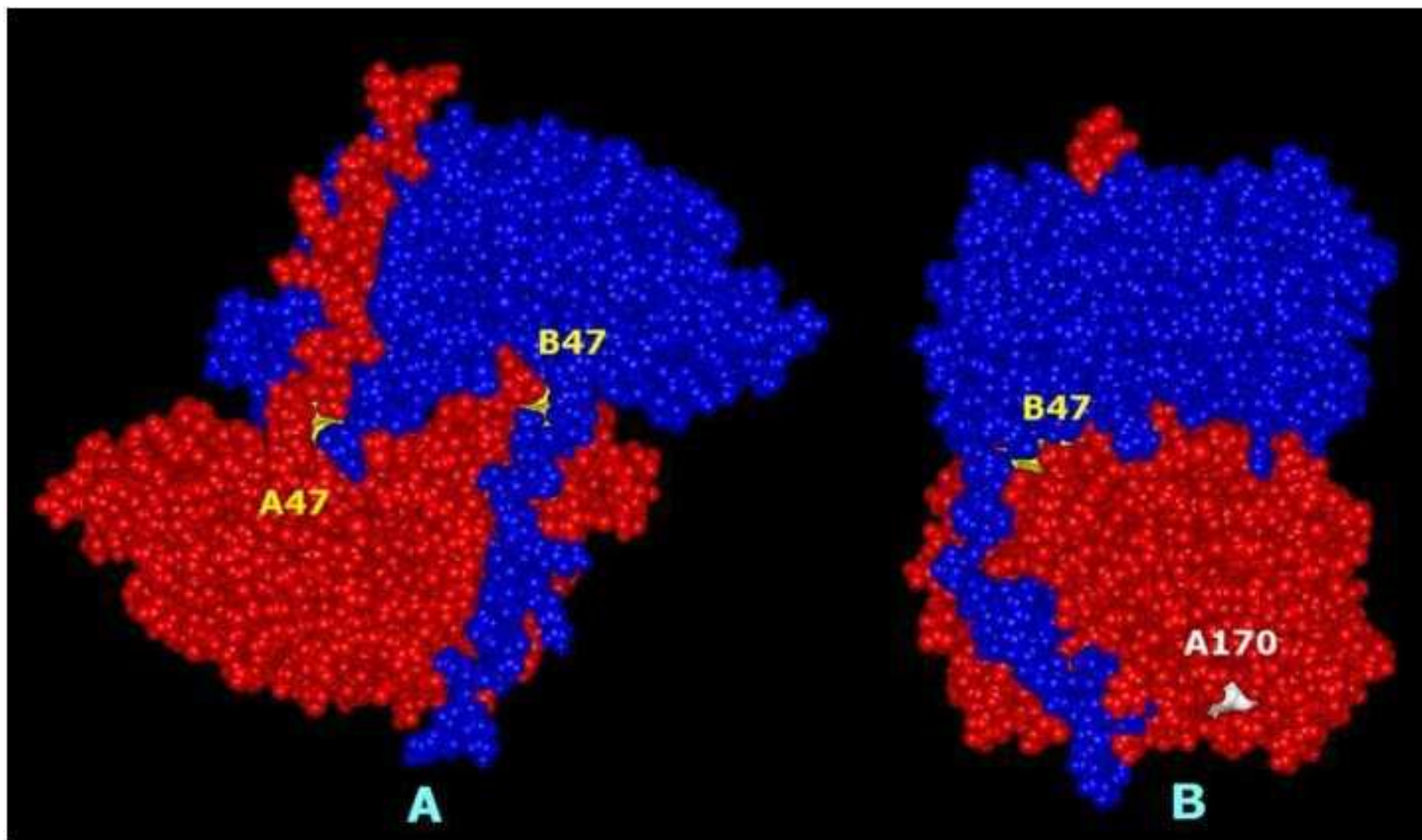


Figure2



Supplementary Material

Supplementary Material 1: Legend to Figure 1

Electropherograms of the three missense mutations studied

Supplementary Material 2.

Multiple sequence alignment of AGT orthologous sequences of 22 different species.

The four mutated residues are highlighted with boxes, and the substituting residue is indicated above the top line. The degree of evolutionary conservation of each residue is indicated below the bottom line, according to Clustal rules: * identity, : conservative substitution, . semi-conservative substitution

H.sapiens NP_000021.1, M.mulatta XP_001090301.1, P.pygmaeus Q5RDP0.1, C.jacchus P31029.1, C.familiaris XP_855949.1, F.catus NP_001036031.1, E.caballus XP_001497552.1, B.taurus NP_001095825.1, O.cuniculus NP_001075778.1, O.anatinus XP_001513675.1, R.norvegicus NP_085914.1, M.musculus NP_057911.2, X.laevis NP_001081948.1, D.rerio NP_001002331.1, D.melanogaster NP_511062.1, A.aegypti XP_001660875.1, N.vitripennis XP_001608017.1, A.mellifera XP_397119.2, C.elegans NP_495885.1, S.purpuratus XP_780986.2, Cyanothecce sp. CCY0110 ZP_01727410.1, Prochlorococcus marinus str. NATL2A YP_291337.1

Multiple sequence alignment of AGT orthologs using CLUSTAL 2.0.2.

```
H.sapiens -----
M.mulatta -----
Pongo_pygmaeus -----
C.jacchus -----MFQALAKASAALGP 14
Canis_familiaris -----MFRALARASVALGP 14
Felis_catus -----MFRALARASATLGP 14
Equus_caballus MPSQEAVADPGPPLLPCLPACKGFCFLTLQPLGLVWSRRGASREMLRALAMASVALGP 60
Bos_taurus -----MLWALTTARAVLGH 14
O.cuniculus -----
O.anatinus -----
Rattus_norvegicus -----
Mus_musculus -----MFRMLAKASVTLGS 14
Xenopus_laevis -----MQGSVRSISSLLCA 15
Danio_rerio -----
D.melanogaster -----
Aedes_aegypti -----
N.vitripennis -----
Apis_mellifera -----
C.elegans -----MISTRFLRP 9
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S.purpuratus
Cyanothece_bacter
Pr.coccus_marinus -----MQLSKKTPRLCLSPFRLGRY 20

L R
H.sapiens -----MASHKLLVTPPKALLKPLSIPNQLLLGPGPSNLPPRIMAAGGLQMI GMSKSD 52
M.mulatta -----MASHKLLVPPPKALLKPLSIPKRLLLGPGPSNLPPRIMSAGGMQI I GPM DKE 52
Pongo_pygmaeus -----MASHKLLVPPPKALLKPLSIPNRLLLGPGPSNLAPRIMAAGGLQI I GPM S K D 52
C.jacchus RAAGWVRTMASHQLLVAPPKALLKPLSIPTRLLLGPGPSNLPPRTMAAGGLQMLGHMHKE 74
Canis_familiaris QAAGWVRTMASQLLVAPPKALLKPLSIPNRLLLGPGPSNLAPRILAAGGLQMI G H M H K E 74
Felis_catus QVAGWARTMATCQLLVAPPEALLRPLSIPNRLLLGPGPSNLAPRVLVAGGKQMI G H M H K E 74
Equus_caballus QAAGWARTMASHQLLVAPPEALRKPLSIPNRLLLGPGPANLAPRVLAAGGLQV I G H M H K E 120
Bos_taurus RTAGWVRTMASHSLVAPPAAALSKPLFIPSRLLLGPGPSNLTPRVMAAGGLQVLGHMHQE 74
O.cuniculus -----MASRQLLVAPPEALRKPLCTPHRLLLGPGPSNLPPRVLAAGGLQMI G H M H E E 52
O.anatinus -----MSSQKLLVSPPSLLKPFV I PDKLLLGPGPSNLPPRVMAAGGLQMI G H M H K E 52
Rattus_norvegicus -----MGSHQLLVPPPEALSKPLSIPKRLLLGPGPSNLAPRVLAAGSLRM I G H M Q K E 52
Mus_musculus RAAGWVRTMGSYQLLVPPPEALSKPLSVPTRLLLGPGPSNLAPRVLAAGSLRM I G H M Q K E 74
Xenopus_laevis ARLSAPVRIMSSLAT I P P P S A L Q R P L N V P Q R L M L G P G P S N V P P R I Q A A G G L Q L I G H M H P E 75
Danio_erio -----MSSLSVL - P P E C L L K P F P V P Q R L M L G P G P S N V P A R I S A A G A Q P M L G H L H T E 50
D.melanogaster -----M E V P P P L V L K R P L Y V P S K T L M G P G P S N C S H R V L E A M S N P V L G H M H P E 47
Aedes_aegypti -----M E Y K V T P P A V L R E P L V T P N K L L M G P G P S N A P Q R V L D A M S R P I L G H L H P E 49
N.vitripennis -----M E V E P P K E L L K P L Q L P Q R I L T G P G P S N C S Q R V L K A L E Q Q V L G H T H P E 47
Apis_mellifera ---MNYKWNKLAVHKEPPQ I L R T K L Q L P I K T L M S P G P T N C S K R V L Q S L Q N Q I L G H L H P E 56
C.elegans SVSIFGFGIKSSMSSRAPKALLQDMVVP P R Q L F G P G P S N M A D S I A E T Q S R N L L G H L H P E 69
S.purpuratus -----M V G N K S M A V S S P K E L L L P M I V P Q K L L M G P G P S N V P P R I L A A G A L P I L G H M H P E 53
Cyanothece_bacter -----M T I P S Q P -----Q P L T I P P R L L M G P G P S N A N P R I L S A M S L P A I G H L D P F 44
Pr.coccus_marinus INRSVFLATQ I L P S V D N Q H R K D F G T V S P E R L L L G P G P S N A D P A V L K A L S Q P P I G H L D P F 80

* : : .***:* : : *

L
H.sapiens MYQIMDEIKEGIQYVFQTRNPLTLVISGSGHCALEAALVNVLEPGDSFLVGVNGI W G Q R A 112
M.mulatta MYQIMDEIKEGIQYVFQTRNPLTLVITGSGHCALEAALVNVLEPGDSFLVGVNGI W G Q R A 112
Pongo_pygmaeus MYQIMDEIKEGIRYVFQTRNPLTLVISGSAHCALEAALVNVLEPGDSFLVGVNGI W G Q R A 112
C.jacchus TYQIMDEIKEGIQYVFQTRNPLTLVISGSGHCALEAALINVLEPGDSFLVGVNGI W G Q R A 134
Canis_familiaris MFQIMDEIKEGIQYVFQTKNPLTLAVSGSGHCALEAALFNLEPGDSFLVGVNGI W G Q R A 134
Felis_catus MFQIMDDIKQGIQYVFQTKNPLTLAISGSGHCALEAALFNLEPGDPFLVGVNGI W G Q R A 134
Equus_caballus MYQIMDEIKQGIQYVFQTRNPLTLAISGSGHCALEAALFNLEPGDSFLVGVNGI W G Q R A 180
Bos_taurus VYQIMDEIKQGIQYVFQTRNPLSLAISGSGHCALEAALFNLEPGDSFLVGVNGI W G Q R A 134
O.cuniculus MYQVMDEIKQGIQYAFQTRNALTAVSGSGHCALETALFNLEPGDAFLVGVNGI W G Q R A 112
O.anatinus MFQIMDEIKEGIQYAFQTKNQITLAVSGSHSAMEAALFNLEPGDSVMVGVNGI W G Q R A 112
Rattus_norvegicus MFQIMDEIKQGIQYVFQTRNPLTLVSVSGSGHCAMETALFNLEPGDSFLVGTNGI W G I R A 112
Mus_musculus MLQIMEEIKQGIQYVFQTRNPLTLVSVSGSGHCAMETALFNLEPGDSFLVGTNGI W G M R A 134
Xenopus_laevis MFQIMDDIKQGIQYAFQTKNNLTFVAVSGSGHCAMETAIFNVVEKGDVVLVAVKGI W G E R A 135
Danio_erio TIEIMNQIKSGIQYAFQTKNRVTLAVSGPGHAAAMECAIFNSLEPGDKILIAVNGI W G E R A 110
D.melanogaster CLQIMDEIVKEGIKYIFQTLNDATMCISGAGHSAMEAALCNLEEDGDVVLVAVKGI W G H R A 107
Aedes_aegypti TLKIMDDIKQGIQYVFQTKNNLTFVAVSGSGHCAMETAIFNVVEKGDVVLVAVKGI W G E R A 109
N.vitripennis MFQIMDEIKAGLRYAFQTKNNLTLAISASGHGMEAAIGNVLERENMLIVKAGI W A E R A 107
Apis_mellifera FCMLMDEIKEGLQYIFQTKNNLTLALSASGHGMEACLTLNLEPGETVLI V K S G I W S E R A 116
C.elegans FVQIMADVRLGLQYVFKTDNKYTFVAVSGTGHSGMECAMVNLEPGDKFLVVE I G L W G Q R A 129
S.purpuratus TLKIMDDIKGLQYVFQTKNELTFVAVSGSGHCMEAMMNLIEPGDVLIVASNGI W G E R I 113
Cyanothece_bacter YLEMMDQIQDLLRYVWQTEENELTISISGTGSAGMEASLANVVEPGDVLVIGVMGYFGHRL 104
Pr.coccus_marinus YVDLMSEVQELRLRYAWQTSNRLTLPMSTGSAAMEATLANVVEPEDTVLVAIKGYFGHRL 140

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R
H.sapiens VDIGERIGARVHPMTKDPGGHYTLQEVEEGLAQHKPVLLFLTHGESSTGVLQP -LDGFGE 171
M.mulatta MDIGERMGARVHPMTKDPGGHYTLQEVEEGLAQHKPVLLFLTHGESSTGVLQP -LDGFGE 171
Pongo_pygmaeus VDIGERMGARVHPMTKDPGGHYTLQEVEEGLAQHKPVLLFLTHGESSTGVLQP -LDGFGE 171
C.jacchus ADIGERLIGARVHPMTKDPGGHYTLQEVEEGLAQHKPVLLFLTHGESSTGVLQP -LDGFGE 193
Canis_familiaris ADIGERIGARVHSMVKDPGDYTLQDVEEGLAQHKPALLFLTQGESSTGVLQP -LDGYGD 193
Felis_catus ADIGERIGARVHPMIKDPGNHYTLQELEEALAQHKPVLLFLTQGESSTGVLQP -LDGYGE 193
Equus_caballus ADIGERIGARVHMMVKDPGGHYTLREVEEGLAQHKPVLLFLTQGESSTGVLQP -LNGYGE 239
Bos_taurus QDIAERIGARVYPMVKAPGGHHTLQEVEEALARHKPALLFLAHGESSTGVLQP -LDGYGE 193
O.cuniculus AEVGERIGARVHPMIKDPGGHYTLQEVEEGLAQHKPVLLFLTHGESSTGVLQP -LDGFGE 171
O.anatinus ADIAERIGGKVHQLVKSPGGYTLQDIEKGLIQHKPVLLFLTQGESSTGVLQP -LDGYGD 171
Rattus_norvegicus AEIAERIGARVHQMIIKPPGHEHYTLQEVEEGLAQHKPVLLFLTHGESSTGVLQP -LDGFGE 171
Mus_musculus AEIADRIGARVHQMIIKPPGHEHYTLQEVEEGLAQHKPVLLFLVHGESSTGVVQP -LDGFGE 193
Xenopus_laevis GDIAERIGADVRYVSKPVEFAFTLKDVEKALAEHKPSLFFITHGESSTGVVQP -LDGLGD 194
Danio_erio SEIAERIGAKVNTVETMAGGYLTNEEIEKALNKYRPAVFFLTHGESSTGVVHP -IDGIGP 169
D.melanogaster GDMARRYGAEVHYVEASFGRALSHEEITFAFEAHRPKVFFIAQGDSSGTGIIQQNIRELGE 167
Aedes_aegypti ADMATRYGADVRYVSKVQSLSLDEIRDALLIHKPSVFLTQGDSSGTGVLQ -LEGVGA 168
N.vitripennis ADMAGRLGIRVDFMETEFVAVGLREFELAVSEYRPAKAVFVHSESSAGLKQP -LEGLGD 166
Apis_mellifera ACMANRLGANVKLLETDTYTGITLKELEIALEQHRPVVFMVHAESSTGVKQP -LEDFGV 175
C.elegans ADLANRMGIEVKKITAPQQAQVVEDIRKAIADYKPNLVFVCQGDSSGTGVAQP -LETIGD 188
S.purpuratus ADLGKRLGANVKVLQNP TGVAFLKQIEQSIHVYKPAIFAITHGESSTGVLQP -LQIGIN 172

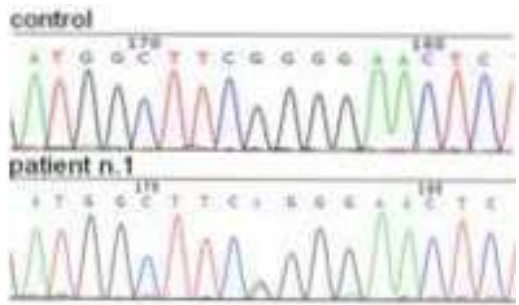
Cyanothece_bacter	VDMATRYGADVKTINKPWGENFSLAELKEAIEETHKPAILGLVNAETSTGVRQP-LEGVGE	163
Pr.coccus_marinus	ADMAGRYKANVETIHKEWGNFSLQEIEDALKKHTPAVLAIVHAETSTGVCQP-MDGIGD	199
	: . * * : : . : * . : : : * * : : * *	
H.sapiens	LCHRYKCLLLVDSVASLGGTPLYMDRQGGIDILYSGSQKALNAPPGTSLSISFSDKAKKKMY	231
M.mulatta	LCHRYKCLLLVDSVSVSLGGTPLYMDQGGIDILYSGSQKVLNAPPGTSFISFSDKAKKKMY	231
Pongo_pygmaeus	LCHRYKCLLLVDSVSVSGGTPVYMDQGGIDILYSGSQKALNAPPGTSLSISFSDKAKKKMY	231
C.jacchus	LCHRYKCLLLVDSVASLGGAPLYMDQGGIDILYSGSQKVLNAPPGTSLSISFSDTAKNKIY	253
Canis_familiaris	LCHRYKCLLLVDTVAALGGVPIYMDQGGIDVLYSGSQKVLNSPPGTSLSISFSDKAKSKIY	253
Felis_catus	LCHRYNCLLLVDSVASLGGTPIYMDQGGIDVLYSGSQKVLNSPPGTSLSISFSDKAKNKIY	253
Equus_caballus	LCHRYKCLLLVDSVASLGAVPYMDQGGIDVLYSGSQKVLNAPPGTSLSISFSDKAKNKIY	299
Bos_taurus	LCHRHQCLLLVDSVASLGGAPVYMDQGGIDVLYSGSQKVLNAPPGTSLSISFSDKAKNKIY	253
O.cuniculus	LCHRYKCLLLVDSVASLGGAPYMDQGGIDVLYSGSQKALNAPPGTSLSISFSDKAKSKIY	231
O.anatinus	LCHRYNCLLLVDCVASLGGTPIHMDKQAIDILYSGSQKVLNAPPGTSLSISFSEKAKKIF	231
Rattus_norvegicus	LCHRYQCLLLVDSVASLGGVPIYMDQGGIDILYSGSQKVLNAPPGTSLSISFNDKAKSKVY	231
Mus_musculus	LCHRYQCLLLVDSVASLGGVPIYMDQGGIDIMYSSSQKVLNAPPGTSLSISFNDKAKYKVVY	253
Xenopus_laevis	LCHRYNCLLLVDSVASLGGAPYMDKQGGIDILYSGSQKVLNAPPGTAPISFSEAAASKMF	254
Danio_gerio	LCRKYSCLFLVDSVAALGGAPICMDEQGGIDILYSGSQKVLNAPPGTAPISFSEKAKKIF	229
D.melanogaster	LCRKYDCFLIVDTVASLGGTEFLMDEKVDVAYTGSQKSLGGPAGLTPISFSKRALTRIR	227
Aedes_aegypti	LCHQHNCCLLIVDTVASLGGAPMFMDRWEIDAMYTGSQKVLGAPPGITPVVFSHRAVERYK	228
N.vitripennis	IVHKYGLLLIVDTVASLGGAPFFADAWGIDVYVYTGSKALGAPAGITPISFSPAEEKIL	226
Apis_mellifera	LIHKYNALLIVDVVASLGGEPFFMDSWDIDAAYAGSQKAIGAPPGLAPISFSPRAEKKLF	235
C.elegans	ACREHGALFLVDTVASLGGTPFAADDLKVDCVYSATQKVLNAPPGLAPISFSDRAMEKIR	248
S.purpuratus	VCCRNDCLFLVDSVASLGGVPMFVDEWGDIAIYSGSQKVLGAPPGTAPISFSQRAISKFK	232
Cyanothece_bacter	LCREHNCLLLVDAVTSLGGVPFYTDKVGWDLAYSQKGLGCPPLSPFTMSDRAIEKLQ	223
Pr.coccus_marinus	LCRKYNCLLLVDTVTSLGGVPLYLDEWKIDLAYSQKGLSCPPGLGPFMSMNERAENKMS	259
	: . : : * * * : : . . * : * * : * * : : * * : : : . * * : : : * :	
H.sapiens	SRKTKPFSFYLDIKWLANFWGCDD-QP-RMYHHTIPVISLISLRESLALIAEQGLENSWR	289
M.mulatta	SRKTKPFSFYLDIKWLANFWGCDG-QP-RMYHHTIPVISLISLRESLALIAEQGLENSWR	289
Pongo_pygmaeus	SRKTKPFSFYLDIKWLANFWGCDD-QP-RMYHHTIPVISLISLRESLALIAEQGLENSWR	289
C.jacchus	SRKTKPSSFYLDVVKLANLWGCDD-QP-RMYHHTIPVVISLISLREGLALLSEQGLENSWR	311
Canis_familiaris	ARKTKPVSFYLDKMKLANIWGCDD-QP-RVYHHTIPVISLISLRESLALIAEQGTARAR	311
Felis_catus	TRKTKPVSFYLDKMKLANIWGCDD-KP-RIYHHTIPVVISLISLRESLALIAEQGLENSWR	311
Equus_caballus	TRKTKPFSFYLDIKWLANFWGCDD-QP-RMYHHTIPITGLYSLRESLALIAEQGLENSWR	357
Bos_taurus	ARKTKPVSFYLDIQWLANFWGCDD-KP-RTYHHTIPVVISLISLRESLAHLAEQGLENSWR	311
O.cuniculus	ARKTKPFSFYMDVQLLANIWGCDD-KP-RMYHHTIPVIGIFALRESLALLVEQGLEKSWQ	289
O.anatinus	SRKTKPVSFALDMSWLANFWGCDD-KP-RIYHHTIPVISLISLREGLAILAEQGLEKSWK	289
Rattus_norvegicus	SRKTKPVSFYTDITYLSKLGWCEG-KT-RVIHHTIPVISLISLRESLALISEQGLENSWR	289
Mus_musculus	SRKTKPVSFYTDITYLAKLWGCCEG-ET-RVIHHTIPVVISLISLRESLALIAEQGLENSWR	311
Xenopus_laevis	GRKTKPVSFYLDIKWLANFWGCDD-KP-RIYHHTIPVVISLISLREGLAILAEQGLENSWA	312
Danio_gerio	NRKTKPISFYLDLNLWLANYWGCDD-KPVSRYHHTIPVVISLISLREGLAILAEQGLENSWK	288
D.melanogaster	KRKTKPKVYFYFDILLIGQYWGCGY--TPRIYHHTISSTLLYGLREALAHFCAVGLKAVVR	285
Aedes_aegypti	RRNTKVKVYWDMSLVGDYWGCFG--RPRIYHHTISSTLLYGLREALIAMEEGLPALIA	286
N.vitripennis	TRKSPVPVYFWDMTWLGRYWNCDFPRTPRPYHHTISATLVYGLREALAQLAEGLAASWA	286
Apis_mellifera	ERKTKPSSFYWDLTILGNWYKCFG-NEDRVYHHTMSATLLYGLREALAEIAEGLQALWN	294
C.elegans	NRKQVASFYFDAIELGNWYGCDD--ELKRYHHTAPISTVYALRAALSIAAKEGIDESI	306
S.purpuratus	SKKIRVPSFYLDLNLWLANYWGCDD-GP-RRYHHTCPVTNLYQLREGLAMVAEEGLEECWK	290
Cyanothece_bacter	KRTTSVSNWYLDLNMNLSQYWGEP-----KYHHTAPCNMNYGLREALALIVEEGLNCWQ	278
Pr.coccus_marinus	NRKDKVPNWYLDVSLNKNYWGSDR-----VYHHTAPVNMNFGIREALRLLAEEGLEVSWG	314
	: . * : * * * . : : * . :	
H.sapiens	QHREAAAYLHGRLQALGLQFLVKDPALRLPTVTTTAVPAGYDWRDIVSYVIDHFDIEIMG	349
M.mulatta	QHRETTAYLHGRLQALGLQFLVKDPALRLPMVTTTAVVVPAGYDWRDIVSYVDHFDIEIMG	349
Pongo_pygmaeus	KHREAAAYLHGRLQALGLQFLVKDPALRLPTVTTTAVPAGYDWRDIVSYVMDHFDIEIMG	349
C.jacchus	KHREAAAYLHGRLQALGLRFLVKDPALRLPTVTTTAVPAGYDWRDIVSYLIERFGIEITG	371
Canis_familiaris	GAGGRTPRAP---SSAGPEPSRVPQAARLPTVTTTAVPAGYDWRDIVSYVMDHFDIEITG	368
Felis_catus	QHREVTAYLHGRLQGLGLQFLVKDPALRLPTVTTTAVPAGYDWRDIVSYVMDHFDIEITG	371
Equus_caballus	QHRETMAYLHGRLQGLGLRFLVKDPALRLPTITTTAVPAGYNWRDIVSYVMDHFDIEITG	417
Bos_taurus	QHREASEYLHACLQGLGLQFLVKDPALRLPTITTSVVVPTGYDWRDIVKYIMDHHDEIAG	371
O.cuniculus	RHREVAQHLYRRLQELGLQFLVKDPALRLPTVTTTAVPAGYDWRDIVSYVMDHFDIEITG	349
O.anatinus	HHEEVTQYLYEELQKLGKLFVKEPAVRLPTVTTTAVPAGYNWKDIVDFLNMKYGIEITG	349
Rattus_norvegicus	RHREATAHLHKCLRELGLKFFVKDPEIRLPTITTTVTPAGYNWRDIVSYVDHFDIEISG	349
Mus_musculus	RHREATAHLHKHLQEMGLKFFVKDPEIRLPTITTTVTPAGYNWRDIVSYVDHFDIEISG	371
Xenopus_laevis	VHQENALKLHKGLEALGIKLFVKDPALRLPTVTTISVPNGYEWKDIITTFIMKNHAEITG	372
Danio_gerio	RHKEVAEYFHKGLEQMGKLFVQDKKARLPTVTTIVAPPAGYDWRITGYIMKTFNIEISG	348
D.melanogaster	RHQECSKRLQLGIEELGLEMFVSREEERLPTVNTIKVPFGVDWKKVAEYAMRKYSEISG	345
Aedes_aegypti	RHEDCAKRLYRGLQDAGFELYAD-PKDRLLSTVTTIKVPQGVVDWLLKAAQYAMKTYLVEISG	345
N.vitripennis	RHANVSQKFHEGLARRGYQLFVKQPQHRKLTVTAIIMLPDGVAEQPIIRYAMDRYNLEFSG	346
Apis_mellifera	RHAAAARLRLKGLERLGLQSYVKIPQYQLSTVIVSVQLPQGVVDKVVIVQAMEKYKVEISR	354
C.elegans	RHKDNAQVLYATLKKHGLEPFVVEDEKRLRPLCLTTVKVPEGVVDWLVAGKMMTN-GTEIAG	365
S.purpuratus	RHKAAARALYAGLEKLGKLFVEDEATRLPTVSAIILVPPGTDWKKVVVVVMQYRIEISG	350
Cyanothece_bacter	RHQQNAELLWEGLEKLGKLVCHVE-KQYRLPLTTVRIPEGVNGKAVSIYLLKEYNIEIGG	337

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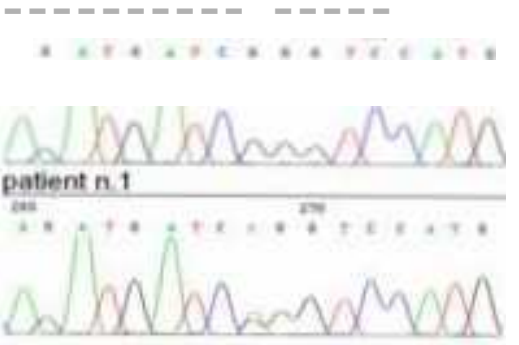
Pr.coccus_marinus      RHRTNAKSLWNSLENIGLELHVK-EELRLPTLT*TTVKIPEGLD*GKAFTK*HLLNNFNGVEIGG 373
                        *           :*      :  :  * .           :           *:

H.sapiens              GLGPSTGKVLRI*GLLGCNATRENVDRVTEALRAALQHCPKKKL----- 392
M.mulatta              GLGPSAGKVLRI*GLLGCNATRENVDRVTEALGAALQHCPKKNL----- 392
Pongo_pygmaeus        GLGPPTGKVLRI*GLLGCNATRENVDRVTEALRAALQHCPKKKL----- 392
C.jacchus              GLGPSTGKVLRI*GLMGCNATRENVDLVTEALREALQHCPKKKL----- 414
Canis_familiaris      GLGPSVGKVLRI*GLLGCNATRENVDRVTHALQEALRHCPRHKL----- 411
Felis_catus           GLGPSMGKVLRI*GLLGCNATRENVDRVIQALQEALQRC*SRNKL----- 414
Equus_caballus        GLGPSMGKVLRI*GLMGCNATRENVDRVIEALREALQRC*PRNKL----- 460
Bos_taurus            GLGPSAGKVLRI*GLLGCNATRENVDRVTRALREALQRC*PRSKL----- 414
O.cuniculus           GLGPSADKVLRI*GLLGCNATRENVDR*LATALREALQHCAQ*SQL----- 392
O.anatinus            GLGPSIGMVLRI*GLLGCNATRENVDR*V*QALREALKQCPK*NKI----- 392
Rattus_norvegicus     GLGPSEDKVLRI*GLLGCNATRENVDR*V*EALREALQHCPK*KNL----- 392
Mus_musculus          GLGPTEERV*LRIGL*GCNATRENVDR*V*EALREALQHCPK*KNL----- 414
Xenopus_laevis        GLGPSTGKVLRI*GLMGCNATRENVDRVLEALRDALQ*CPK*KKM----- 415
Danio_rerio           GLGPSAGMVLRI*GLMGCNATRENVDR*V*EALADAL*KH*CH*KS*RV----- 391
D.melanogaster        GLGPTVEHV*FRIGL*MG*ENATRENVDR*V*LSILNEAIQ*SS*KLGI*KT*DL*SKI 394
Aedes_aegypti         GLGPTAGQV*FRIGL*MQ*ATRENVDRV*LV*QV*FQEAVA*AV*KPDV*QV*KG-KM 393
N.vitripennis         GLGPTAGK*VIRIGL*MG*V*ATRENVDR*V*GLD*DAV*KY*AK*SC*QL*KS*NI-- 393
Apis_mellifera        GLGPTVGK*FLRI*GLL*GV*ATRENVDR*V*PEK*V*DL*V*LR*GL*DE*GL*Y*ASE*SKL----- 397
C.elegans             GLGATVGK*IWRIGL*TF*G*INS*TKI*EN*V*E*LL*SK*S*IG*EK*SK----- 405
S.purpuratus          GLGPSSGK*VWRIGL*MG*Y*ANQDNV*KR*V*LR*V*LE*DG*LN*H*SR*TT*SR*L----- 394
Cyanotheca_bacter    GLGELAGK*VWRIGL*MG*Y*NSR*PEN*V*LL*LL*EAL*RK*V*L----- 372
Pr.coccus_marinus     GLGDLAGK*VWRIGL*MG*Y*NSR*PEN*V*DKI*IN*IF*E*TE*LP*K*FR----- 412
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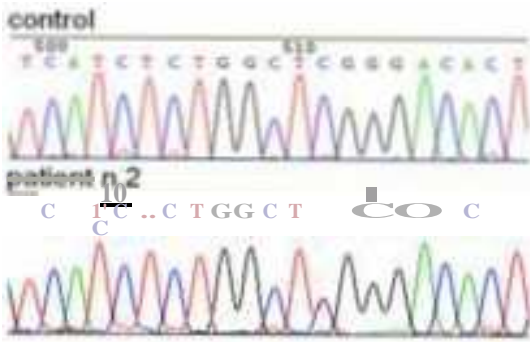
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c.508G>A leading to p.Gly170Arg in patient 1



c.139G>A leading to p.Gly47Arg in patient 1



c.242C>T leading to p.Ser81Leu in patient 2