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DATA REPORT

Functional Characterization of Five *Protoporphyrinogen oxidase* Missense Mutations Found in Argentinean Variegate Porphyria Patients

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Abstract A partial deficiency in protoporphyrinogen oxidase (PPOX) produces the acute/cutaneous (or mixed) variegate porphyria (VP), the third most frequent porphyria in Argentina. This autosomal dominant disorder is clinically characterized by skin lesions and/or acute neurovisceral attacks. The precise diagnosis of patients with a symptomatic VP is essential to provide accurate treatment. It is also critical to identify asymptomatic relatives to avoid precipitating factors and prevent acute attacks.

Functional consequences of five *PPOX* missense mutations were evaluated in a prokaryotic expression system. Three mutations were found in families previously reported c.101A>T (p.E34V), c.670T>G (W224G), c.995G>C (G332A) and two were novel findings c.227C>T (p.S76F), c.1265A>G (p.Y422C). All mutations were identified in heterozygotes with reduced PPOX activity and variable clinical expression of the disease, including asymptomatic cases. Prokaryotic expression showed that all five missense mutations decreased the PPOX activity, demonstrating their detrimental effect on enzyme function, and thus, providing evidence for their causative role in VP. These results reinforce the importance of molecular genetic analysis for VP diagnosis and especially the usefulness of prokaryotic expression of missense mutations to assess their deleterious effect on PPOX activity.

MM and BXG contributed equally to the publication. RES and MVR share senior authorship.

Expression of *PPOX* Missense Mutations

Introduction

The porphyrias are a group of inherited metabolic disorders that result from the partial deficiency of specific heme biosynthetic pathway after the first enzyme. The different porphyrias exhibit acute or cutaneous symptoms or both together, depending on the enzyme affected and therefore the porphyrin precursors and/or porphyrins accumulate (Anderson et al. 2001).

Variegate porphyria (VP; OMIM 176200) is one of the acute/cutaneous or mixed porphyrias and it is the third in prevalence in Argentina (1:500,000 to date). VP is an autosomal dominant disorder that results from the deficiency of protoporphyrinogen oxidase (PPOX; EC 1.3.3.4) (Anderson et al. 2001). This mitochondrial enzyme catalyzes the penultimate step in the heme pathway, the oxidation of protoporphyrinogen IX (PROTOgen IX) to protoporphyrin IX (PROTO IX), using flavin adenine dinucleotide (FAD) as a cofactor (Dailey and Dailey 1996; Dailey 2002).

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Patients with VP can present acute and/or cutaneous symptoms. The first are characterized by intermittent attacks of neurological dysfunction, abdominal pain, constipation, vomiting, hypertension, tachycardia, and various peripheral and central nervous system manifestations due to the accumulation of neurotoxic precursors, δ-aminolevulinic acid (ALA), and porphobilinogen (PBG). Its expression is highly variable determined in part by environmental, metabolic, and hormonal factors, and can be fatal if the correct diagnosis is delayed and/or treatment is inadequate (Anderson et al. 2001; Kauppinen 2005). Acute attacks frequently result from exposure to diverse porphyrinogenic drugs, alcohol ingestion, reduced calorie intake due to fasting or dieting, infections, and hormones, which stimulate porphyrin precursors induction of the first and rate-limiting enzyme, aminolevulinate synthase (Batlle 1997; Anderson et al. 2001; Bickers and Frank 2003; Kauppinen 2005). The cutaneous symptoms include photosensitivity leading to blistering in sun-exposed areas, skin fragility, hyperpigmentation, and hypertrichosis due to the accumulation of porphyrins, especially coproporphyrin III (COPRO III) and PROTO IX (Anderson et al. 2001).

The human *PPOX* gene has been mapped to chromosomal region 1q22-23 (Roberts et al. 1995; Taketani et al. 1995), spans a genomic interval of 5.5 kb and contains one noncoding and 12 coding exons (Taketani et al. 1995). Its mRNA is 8 kb and encodes a 477 amino acid polypeptide with a molecular weight of 50.8 kDa (Nishimura et al. 1995).

To date about 130 different mutations have been identified in the *PPOX* gene causing VP (Human Gene Mutation Database (HGMD), http://www.hgmd.cf.ac.uk/ac/index.php). These mutations included 70 missense, 22 splicing defects, 29 small deletions, 15 small insertions, 1 small indels, 1 gross insertion, and 1 duplication. In several of the missense mutations, their functional effect on protein activity has been studied using in vitro expression systems (von und zu Fraunberg et al. 2001; Qin et al. 2011). Most patients are heterozygous, exhibiting approximately 50% reduced PPOX activity (Brenner and Bloomer 1980; Deybach et al. 1981). However, since the first description of a homozygous VP case in 1984 (Korda et al. 1984), several homozygous and compound heterozygous cases have been reported (Hift et al. 1993; Kauppinen et al. 2001; Poblete-Gutiérrez et al. 2006).

In the present study, we have expressed in a prokaryotic system five *PPOX* missense mutations to evaluate their functional consequences. Two of these mutations are novel and three were previously identified in our laboratory (Rossetti et al. 2008).

Methods

Patients and Biochemical Determinations

Biochemical and molecular studies were performed in two unrelated Argentinean patients. Moreover, the molecular defect was characterized in these and four families previously studied in our laboratory (Rossetti et al. 2008). All patients gave informed consent prior to their inclusion in the study. The study was conducted in accordance with the Declaration of Helsinki and the study protocol was approved by the Ethical Committee of the Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP, Hospital de Clínicas, CONICET-UBA).

Urinary ALA, PBG, and porphyrins as well as fecal porphyrins were determined as previously described (Batlle 1997). Plasma porphyrin index (PPI) was determined as described by Rossetti et al, 2008. Lymphocyte PPOX activity was measured as described by Brenner and Bloomer, 1980 and Deybach et al, 1981. The diagnosis of VP was made on the basis of a clinical history of at least one acute attack and/or typical cutaneous lesions associated with a plasma porphyrin peak at 625 nm as well as increased excretion of porphyrins in urine and feces.

Patient P1 is a 40-year-old female who had at least one true acute attack and typical cutaneous lesions. Unfortunately, no family members were available for this study.

Patient P2 is a 34-year-old female whose mother had been diagnosed as VP clinically and biochemically in the CIPYP, but she died as a consequence of an acute porphyric attack before the molecular study could be done. It is important to note that although she was clinically asymptomatic, she has biochemically VP features (Table 1). She has four daughters; two of them also carry the mutation with a reduction of PPOX activity of about 50%, and only one has biochemically VP features (Table 1). The already reported mutations characterized in this study were three missense mutations c.101A>T (p.E34V), c.670T>G (p.W224G), and c.995G>C (p.G332A) indentified in Argentinean families (Rossetti et al. 2008): mutation c.101A>T (p.E34V) was identified in two members of a family who presented cutaneous symptoms alone, and in an unrelated patient who developed only acute symptoms. Mutation c.670T>G (p.W224G) was identified in two members of a family who experienced both cutaneous and acute symptoms, and in an asymptomatic relative. Mutation c.995G>C (p.G332A) was identified in two members of a family who had only acute symptoms and in two asymptomatic relatives.

Acute symptoms included abdominal pain, paresthesia, muscle weakness, paralysis, and/or data of at least one

Table 1 Novel Argentinean VP families: clinical, biochemical and molecular data

	Patient P1	Patient P2	Daughter 1 of P2	Daughter 2 of P2
Sex/age (years)	F/40	F/34	F/30	F/34
Symptoms (C/A)	+/+	−/−	−/−	−/−
Urinary ALA (mg/24 h)	1.0	5.5	3.5	1.7
Urinary PBG (mg/24 h)	1.3	3.5	3.8	1.8
Urinary porphyrins (μg/24 h)	318	108	108	128
Fecal porphyrins (μg/g dry weight)	1562	1020	1030	30
PPI (at λ: 625 nm)	8.40	2.33	2.33	1.20
PPOX activity (%)	33.7	47.0	43.7	42.8
Mutation (effect)	c.227C>T (p.S76F)	c.1265A>G (p.Y422C)	c.1265A>G (p.Y422C)	c.1265A>G (p.Y422C)

C cutaneous symptoms; *A* acute symptoms

PPOX activity is expressed as the percentage of normal value. Normal values were: urinary ALA: 2–4 mg/24 h, urinary PBG: 1–2 mg/24 h, urinary porphyrins: up to 250 μg/24 h, fecal porphyrins: up to 130 μg/g dry weight, PPI: up to 1.30 (at λ: 618 nm). PPOX activity: 33.25 ± 6.32 nmol protoporphyrin/h/mg protein

acute attack. Cutaneous symptoms included blisters, erosions, scarring in sun-exposed areas, and hypertrichosis. The clinical and biochemical profiles of these patients are present at the age of diagnosis and the genotype of the novel patients studied is shown in Table 1. Data from the families carrying the reported mutations were previously published (Rossetti et al. 2008).

Mutation Analysis

Genomic DNA was extracted from peripheral blood leukocytes using the GFX Genomic Blood DNA Purification Kit (Amersham, Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. The *PPOX* gene of each proband and 50 Argentinean control individuals was PCR amplified using primer pairs and conditions described by Rossetti et al, 2008. Mutation detection was performed by automated sequencing as previously described (Rossetti et al. 2008). All mutations were confirmed by sequencing both strands of at least two different PCR products. Nucleotides were numbered according to the cDNA sequence derived from the *PPOX* genomic sequence (GenBank accession number X99450.1), in which the A of the ATG translation-initiation codon is numbered as +1.

Prokaryotic Expression and Characterization of Missense Mutations

The normal and mutant *PPOX* alleles were expressed in *Escherichia coli* strain JM109 (Promega Corporation, Madison, WI, USA) using the expression vector pTrcHis B (Invitrogen, Carlsbad, CA, USA). The normal PPOX cDNA construct was kindly provided by Professor Dr P. Meissner (University of Cape Town, South Africa). In this vector, the wild-type human PPOX cDNA had been cloned into the pTrcHis B plasmid (Dailey and Dailey 1996). The normal construct was maintained in *E. coli* JM109 and was designated pTrc-PPOX-wt. Plasmid DNA was further purified by the QIAGEN Plasmid Midi Kit (QIAGEN GmbH, Hilden, Germany) and used to make the mutant constructs.

To generate each mutant constructs, a fragment of the *PPOX* cDNA, containing the desired mutation and restriction sites for cloning, was generated by PCR-based site-directed mutagenesis in one or two amplification steps (Cormack 1991; Méndez et al. 1998). PCR reactions were performed using primers indicated in Table 2 and the pTrc-PPOX-wt as template. Then, for each mutation the final PCR product was digested with the appropriated restriction enzymes and exchanged with the corresponding fragment in the pTrc-PPOX-wt. The resulting plasmids were transformed in *E. coli* JM109 generating the mutant constructs pTrc-PPOX-E34V, pTrc-PPOX-S76F, pTrc-PPOX-W224G, pTrc-PPOX-G332A, and pTrc-PPOX-Y422C.

For construction of pTrc-PPOX-E34V, two overlapping PCR products were obtained using primers PPOX(S1)/PPOX-34(AS) (PCR1A) and PPOX-34(S)/PPOX(AS1) (PCR1B). Then, a final PCR product containing the mutation and *CpoI/SacI* restriction sites, was obtained using the two PCR products together as templates and primers PPOX(S1)/PPOX(AS1) (PCR2). This product was then digested with the *CpoI* and *SacI* restriction endonucleases (New England Biolabs, Beverly, MA, USA) and purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK). The purified *CpoI/SacI* fragment was ligated as a cassette into the corresponding sites in pTrc-PPOX-wt construct and the resulting plasmid was transformed into *E. coli* JM109.

Table 2 Primers used for expression studies

Primer	Sequence (5′→3′)
PPOX(S1)	ATGGGCCGGACCGTGGT
PPOX-34(AS)	TCCCAGACGCTCACTGCTC**a**CCACTAGGA
PPOX-34(S)	TCCTAGTGG**t**GAGCAGTGAGCGTCTGGGA
PPOX(AS1)	ATGCTGAGCTCACGGCTGTTGCCT
PPOX-76(AS)	CCAAGCTCA**a**AAACCAGGAGCA
PPOX-76(S)	TGCTCCTGGTTT**t**TGAGCTTGG
PPOX(S2)	TGGAGTGTTTGCAGGCAACAGCCGT
PPOX-224(AS)	AACATCTCTAGACCTCCACGAAGTGACCACTGGCTCC**c**GCGCT
PPOX(S3)	AGTGGTCACTTCGTGGAGGT
PPOX-332(AS)	GGCACCAAATGT**g**CAAATCCCTGGA
PPOX-332(S)	CCAGGGATTTG**c**ACATTTGGTGCCATCTTC
PPOX(AS2)	ATCCGCCAAAACAGCCAAGCTTTCAGC
PPOX-422(AS)	CCAGTGACCTAGTGTA**c**ACTGGGGAAT
PPOX-422(S)	ATTCCCCAGT**g**TACACTAGGTCACTGG

S sense, *AS* antisense

The *underlined nucleotides* indicate the restriction sites for endonucleases *CpoI* (in PPOX(S1)) *SacI* (in PPOX(AS1)), *XbaI* (in PPOX-224(AS)), and *HindIII* (in PPOX(AS2). In the primers used for mutagenesis, the mutated base is indicated by *bold lower case letters*

A similar procedure was employed to construct the expression vectors for the mutations c.227C>T (p.S76F) (pTrc-PPOX-S76F), c.995G>C (p.G332A) (pTrc-PPOX-G332A), and c.1265A>G(p.Y422C) (pTrc-PPOX-Y422C), using the restriction enzymes *CpoI/SacI* for c.227C>T (p.S76F) and *XbaI/HindIII* for c.995G>C (p.G332A) and c.1265A>G (p.Y422C). For construction of pTrc-PPOX-S76F, the primer pairs for the three PCR were PPOX(S1)/PPOX-76(AS), PPOX-76(S)/PPOX(AS1), and PPOX(S1)/PPOX(AS1), respectively. For mutation c.995G>C (p.G332A), the two sets of primers for the first PCR step were PPOX(S3)/PPOX-332(AS) and PPOX-332(S)/PPOX (AS2), whereas for mutation c.1265A>G (p.Y422C), the primer pairs were PPOX(S3)/ PPOX-422(AS) and PPOX-422(S)/PPOX(AS2). Then, for both mutations, the two PCR fragments were used together as templates to obtain each final PCR product employing the primer pair PPOX(S3)/PPOX(AS2).

In the case of c.670T>G (p.W224G) mutation, the mutagenesis was performed in only one PCR step using the primers PPOX(S2) and PPOX-224(AS). The product obtained was digested with *SacI* and *XbaI* restriction enzymes (New England Biolabs), purified and ligated as was indicated for the two PCR step protocol.

The integrity of each expression construct was checked by automated sequencing in an ABI 3130 xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems). Bacterial clones containing either the pTrcHis B vector or any of the pTrc-PPOX expression constructs were grown to log phase and induced with 5 mM isopropylthiogalactoside (IPTG) for 3 h. Cells were harvested by centrifugation and washed twice with phosphate-buffer saline. The cell pellets were resuspended in 0.02 M Tris-HCl buffer, pH 8.7, containing Tween 20% (v/v), and disrupted by sonication three times for 30 s on ice. The bacterial lysates were centrifuged and the supernatant was used as source of enzyme. PPOX activity was determined in strict darkness and anaerobiosis conditions following the described methodology (Rossetti et al. 2008). The specific activity was calculated as nmol of protoporphyrin/h/mg protein.

Results

The two novel mutations were unrelated patients who exhibited the typical biochemical profile of this disease, with PPOX activity values around 50% of the control value (Table 1). Moreover, patient P1 also presents clinical signs of the disease.

Both P1 and P2 patients carried novel mutations. These mutations were transitions leading to an amino acid change. One was a C to T substitution at nucleotide position 227 (c.227C>T) (Patient P1, Fig. 1a) that results in a missense mutation that changes the serine 76 to a phenylalanine residue in exon 4 (p.S76F). The other mutation was an A to G substitution at nucleotide position 1265 (c.1265 A>G, p.Y422C) (Patient P2, Fig. 1b) that changes a tyrosine amino acid residue to a cysteine residue in exon 12. This proband has two daughters who inherited the mutation and

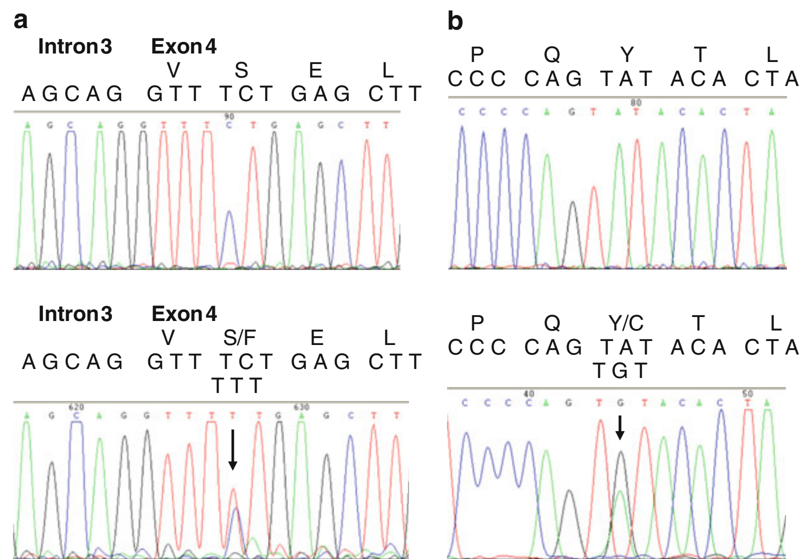

Fig. 1 Novel mutations identified in the *PPOX* gene. (**a**, **b**) electropherograms showing the relevant part of the sequence in a control individual (*top*) and in the affected patients (*bottom*). (**a**) Mutation S76F identified in exon 4 from patient P1. (**b**) Mutation Y422C identified in exon 12 from patient P2. The positions of the mutated nucleotides are indicated by *arrows* and the amino acid sequences are shown

the three females have a reduced PPOX activity to about 50%. The proband and one of her daughters have the typical biochemical VP profile, although they do not present clinical symptoms (Table 1). These two novel mutations were absent in 100 normal alleles.

The other three missense mutations c.101A>T (p.E34V), c.670T>G (p.W224G), and c.995G>C (p.G332A) were previously described (Rossetti et al. 2008). The five missense mutations were expressed in *E. coli* and, as shown in Table 3, all of these mutant alleles expressed proteins with little or no enzyme activity.

Discussion

In the present study, two novel missense mutations were identified in the *PPOX* gene. Furthermore, these and three missense mutations previously identified in Argentina were expressed in a prokaryotic system to study the functional consequences of these alterations on enzyme activity. Three of these mutations c.101A>T (p.E34V), c.995G>C (p.G332A), and c.1265A>G (p.Y422C), affect amino acids highly conserved throughout evolution, whereas the mutations c.227C>T (p.S76F) and c.670T>G (p.W224G) change less conserved residues, but preserved in different mammals species such as *Macaca fascicularis* (monkey), *Bos taurus* (bovine), *Sus scrofa* (pig), *Mus musculus* (mouse), and *Rattus novergicus* (rat) (Protein Knowledgebase (UniProtKB), http://www.uniprot.org). In the crystal structure of human PPOX, amino acids E34, S76, W224, and Y422 are located in the FAD-binding domain, and G332 resides in the substrate-binding domain (Qin et al. 2011). Besides, residues E34 and G332 are involved in the

Table 3 Prokaryotic expression of *PPOX* missense mutations

Construct	PPOX activity (nmol protoporphyrin/h/mg) mean ± SD (range)	Residual activity (%)
pTrcHis B	0.21 ± 0.03 (0.17–0.23)	0
pTrc-PPOX-wt	13.27 ± 1.20 (12.30–14.34)	100
pTrc-PPOX-E34V	0.51 ± 0.02 (0.47–0.55)	2.3
pTrc-PPOX-S76F	0.66 ± 0.03 (0.63–0.70)	3.4
pTrc-PPOX-W224G	0.76 ± 0.03 (0.70–0.81)	4.2
pTrc-PPOX-G332A	0.20 ± 0.02 (0.16–0.24)	<1.0
pTrc-PPOX-Y422C	0.62 ± 0.02 (0.60–0.66)	3.1

PPOX specific activity (SA) was determined in four independent experiments. Residual activity was calculated by dividing 100($SA-SA_{pTrcHisB}$) by ($SA_{pTrc\text{-}PPOX\text{-}wt}-SA_{pTrcHisB}$)

binding to cofactor and substrate, respectively (Qin et al. 2011). Moreover, tryptophan at position 224 is located in the hydrophobic core and so this mutation confers a hydrophilic side chain probably destabilizing the core and affecting the stability of the domain conformation (Qin et al. 2011). It would likely also be that this residue could be in the internal mitochondrial targeting signal (Morgan et al. 2004; Davids et al. 2006). Interestingly, another missense mutation at the same residue c.670T>A (p.W224R) has been described to cause VP (Lecha et al. 2006). Additionally, the residue S76 would fall within the alpha helix (helix 2) where it would most likely affect the secondary structure of the protein and stability. With respect to Y422 residue, it is very close to a beta bridge (http://www.rcsb.org/pdb/explore/remediatedSequence.do?structureId=3NKS) The prokaryotic expression studies showed that these five mutant alleles encode polypeptides with very low or no residual activity (Table 3), demonstrating the deleterious effect of these mutations on the structure and/or function of the enzyme. In consequence, the mutations studied are most likely the cause of the reduced PPOX activities observed in patients carrying these defects. Moreover, Qin et al. (2011) also found minimal or no residual activity for c.670T>G (p.W224G) and c.995G>C (p.G332A) mutations. No correlation was observed between these functional consequences and the expression or features of the disease in the patients carrying the respective mutations. This is expected, since in VP other factors contribute to disease expression (Anderson et al. 2001).

In symptomatic patients with VP, the clinical presentation is variable and can be similar to that from other diseases (Anderson et al. 2001). This may delay the precise diagnosis and therefore the accurate treatment of the disease. In patients with only cutaneous symptoms, the lesions are similar to those observed in porphyria cutanea tarda (OMIM 176100), the most common cutaneous porphyria. Moreover, the acute attacks in VP patients are identical to those of other acute porphyrias such as acute intermittent porphyria (OMIM 176000), and the signs are nonspecific resembling certain disorders other than porphyrias. Also, it is very important to identify latent carriers to provide adequate counseling to avoid precipitating factors that trigger acute attacks. Plasma fluorescence scanning is the most reliable and easy method to detect patients with VP (Enriquez de Salamanca et al. 1993; Hift et al. 2004). However, it may be negative in asymptomatic carriers and children (Hift et al. 2004; Schneider-Yin and Minder 2006). Therefore, when a disease-causing mutation has been identified in a proband, the screening of this mutation in asymptomatic relatives is recommended to detect latent carriers within a family (Hift et al. 2004). For mutations with no obvious negative consequences, it is important to prove their deleterious effect with expression assays and exclude neutral polymorphisms. In the case of missense mutations, prokaryotic expression studies provide a valuable tool to assess the likelihood of their pathogenicity (Von und zu Fraunberg et al. 2001; Qin et al. 2011).

These results show the deleterious effect that mutations described have on PPOX activity. Genotyping of these mutations would now allow us the accurate diagnosis of asymptomatic carriers in each family. These results also emphasize the importance of using molecular techniques in VP porphyria diagnosis.

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