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Protein expression, crystallization and *in-silico* studies on cytochrome P450 Oxidoreductase wildtype and mutant variants

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ABSTRACT: Cytochrome P450 oxidoreductase (POR) is a crucial membrane-bound enzyme that facilitates the transfer of electrons to all cytochrome P450 (CYP450) enzymes. Several mutations in the POR gene have been reported to cause cytochrome P450 oxidoreductase deficiency (PORD), an autosomal recessive genetic disorder. This study explored the consequences of seven POR missense mutations (Y181D, A287P, R457H, R498P, C569Y, Y607C, and H628P), which have been documented in PORD patients, on the structural integrity and stability of the POR enzyme *in vitro*. The comparison between these mutants and the wild-type POR focused on *in vitro* protein expression, purification, and crystallization characteristics. The mutation-induced alterations in the POR architecture significantly influenced the protein's expression and crystallization capabilities. The magnitude of these effects on the enzyme's behavior varied from moderate to severe, contingent on the mutation's nature and position. This research illuminates the influence of specific mutations on POR stability, underlining the necessity of understanding mutation-driven effects on enzyme stability to devise personalized therapeutic approaches for PORD patients. Future studies will involve the functional characterization of these mutant enzymes to further understand their impact on the POR enzyme's activity and stability

KEYWORDS: Cytochrome P450 oxidoreductase (POR); cytochrome P450 oxidoreductase deficiency (PORD); POR protein expression; POR crystallization; POR mutants

1. INTRODUCTION

Cytochrome P450 oxidoreductase (POR) is a membrane-bound flavoprotein of critical importance in human metabolism, serving as the electron donor for all cytochrome P450 (CYP450) enzymes [1-3]. POR is involved in the synthesis of steroid hormones, xenobiotic metabolism, and drug metabolism, underlining its central role in maintaining cellular homeostasis [3]. The human POR gene is located on chromosome 7q11.23 and contains altogether 16 exons (15 protein-coding and 1 non-coding exon) and codes for a 680 amino acids membrane-bound protein [3]. The human POR interacts with CYP450 enzymes with the help of its N-terminus hydrophobic domain which is anchored at the endoplasmic reticulum [4]. The reported three-dimensional structure of the human POR enzyme (PDB: 5FA6) consists of three cofactor binding domains, i.e., the flavin adenine mononucleotide (FMN) binding domain, the flavin adenine dinucleotide (FAD) binding domain, and NADPH binding domain (Figure 1) [5,6]. The two flavin cofactors (FAD and FMN) binding domains are linked via a flexible hinge region which helps bring the FAD nearer to the FMN domain (Figure 1) [4]. The first step of electron transfer starts with NADPH getting oxidized as it donates its hydride anion (H-) to FAD which then gets reduced. The generated H- ion is then accepted by the FMN which then finally facilitates the transfer of electrons to all Cytochrome P450 enzymes (Figure 1) [7].

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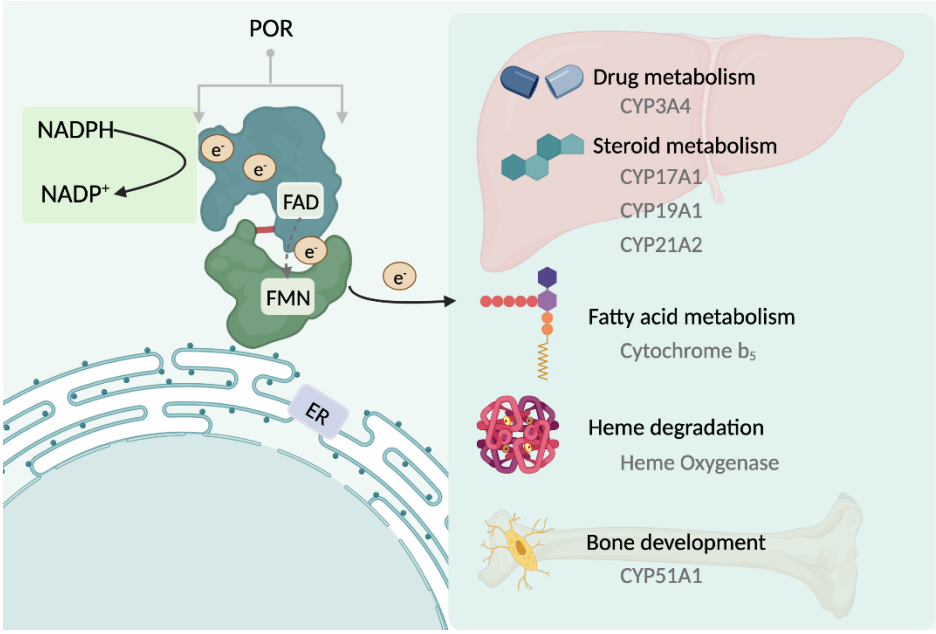

Figure 1. Role of human cytochrome P450 oxidoreductase (POR). POR provides electrons to microsomal P450 enzymes, which are involved in metabolizing drugs and xenobiotics compounds, steroid hormone synthesis, and bone development, as well as to other enzymes, e.g., cytochrome b_5 and heme oxygenase. The two binding domains for the flavin cofactors (FAD and FMN) are linked by a flexible hinge region, which facilitates the proximity of the FAD to the FMN domain. The initiation of electron transfer occurs through the oxidation of NADPH, leading to the reduction of FAD. Subsequently, the generated H- ion is accepted by FMN, ultimately enabling the transfer of electrons to all Cytochrome P450 enzymes. The figure was created using BioRender software.

1.1. Reported mutations in POR enzyme

Mutations in the POR gene can cause alterations in the 3D structure of the POR enzyme, leading to its malfunction. This condition is known as cytochrome P450 oxidoreductase deficiency (PORD), an autosomal recessive genetic disorder (OMIM: 613571) [2,8]. Given POR's involvement in steroid hormone synthesis and drug metabolism, POR mutations could compromise normal reproductive system development and drug metabolizing pathways in patients. A46, XY, six-month-old infant exhibiting a female phenotype and abnormal genital with unusual steroid profile was assessed by Peterson et al. (1985) [9], and it was assumed that the reported phonotype is due to the loss of function of CYP17A1 and CYP21A2. But this hypothesis was discarded with the discovery of embryonic lethality in POR knockout mice [10,11]. This had led Miller et al. (1986) to propose that there could be a defect in POR enzyme, responsible for providing electrons to CYP17A1 and CYP21A2. In 2004, Flück et al. [2] confirmed this hypothesis by discovering POR gene mutations in four individuals suffering from PORD. These mutations can detrimentally affect the enzyme's 3D structure, potentially causing the behavior of the mutant enzyme to deviate significantly from that of the wild-type enzyme [12,13]. Such deviations, which impact the overall stability of the enzyme structure, can be studied by analyzing their *in vitro* protein expression pattern, purification, crystallization and in-silico 3D structure analysis.

The most common POR mutations reported in PORD patients is missense mutations. Our previous study mapped the reported missense mutations on the 3D X-ray crystal structure of the human POR enzyme [13]. The current study aims to investigate the enzyme behavior of selected POR mutations (Y181D, A287P, R457H, R498P, C569Y, Y607C, and H628P), reported in PORD patients [13]. The analysis involves comparing their *in vitro* protein expression, purification, and crystallization properties against those of the wild-type POR enzyme, and an in-depth in-silico structure analysis. The goal is to establish a potential correlation between the severity of the clinical phenotype observed in PORD patients and mutation-driven structural changes in the POR enzyme. Understanding the impact of specific mutations on the stability of the POR enzyme is crucial for designing personalized treatments for patients with PORD.

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2. RESULTS AND DISCUSSION

2.1. Protein expression and crystallization of POR mutants and wildtype enzyme

To study the effects of POR mutants (Y181D, A287P, R457H, R498P, C569Y, Y607C, and H628P) (Table 1) on the 3D structure integrity of POR enzyme, the protein expression-purification studies were conducted initially on a small scale (50 ml) in E. coli. The selected POR mutants were expressed and purified together with the wild-type POR enzyme (Figure 2A). The expression results revealed a moderate to high protein expression level of wild-type POR as well as the mutants Y181D, R457H, R498P, C569Y and Y607C (Figure 2A). Out of the remaining three mutant variants, the mutants A287P and H628P showed a very low level of protein expression (Figure 2A), whereas the C569Y mutant protein exhibited a diffuse expression pattern (Figure 2A). The level of POR enzyme architecture impairment due to the presence of different mutations drives the clinical phenotype, such as skeletal malformations, irregular steroid hormone profile and Disorders of sex development (DSD), as reported in PORD patients [2,4,8]. The mutant A287P, which exhibits features resembling Antley-Bixler Syndrome (ABS) [2,14] in reported PORD patients, is not in direct contact with any POR domain i.e., FMN, FAD and NADPH, instead, it is located near the interface of FAD and NADPH domains, interconnected by the help of the connecting loop (Figure 2B). The impact of a single amino acid substitution in protein is crucial for its stability and folding [15-18], as reflected with low or diffuse protein expression pattern of mutants (A287P, C569Y and H628P) (Figure 2A). Furthermore, the three well-expressed mutants (Y181D, R498P, and Y607C) and one low-expressing mutant (A287P), together with the wild-type enzyme were subjected to large-scale (10-20 liters) protein expression-purification (Figure 2C) for the purpose of protein crystallization studies. The wild-type POR and mutants (Y181D, R498P, and Y607C) display a high level of protein expression (Figure 2C), as compared to a low protein expression display of mutant A287P (Figure 2C) on a large scale.

Table 1. List of analyzed POR mutant variants.

No.	Variant	Mutation Site	Mutation Residue	DNA Change	Exon	Domain Affected	References
1	Y181D	Tyr181	Asp	541T>G	5	FMN	[11,19][12,24]
2	A287P	Ala287	Pro	859G>C	8	Below FAD	[2,8] [10,11,14,19-24]
3	R457H	Arg457	His	1370G>A	11	FAD	[2,11,23,25-38]
4	R498P	Arg498	Pro	1493G>C	12	FAD	[11,20]
5	C569Y	Cys569	Tyr	1706G>A	13	NADPH	[2,11,22,23]
6	Y607C	Tyr607	Cys	1820A>G	14	NADPH	[11,31]
7	H628P	His628	Pro	g.32234A>C	14	NADPH	[11,20]

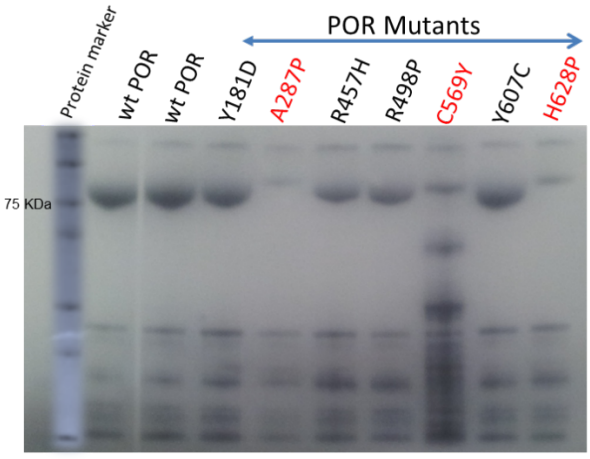

Figure 2A. Small-scale (50 ml) protein expression analysis of wild-type POR and mutant enzymes (Y181D, A287P, R457H, R498P, C569Y, Y607C, and H628P). The three POR mu-tants (A287P, C569Y and H628P) mentioned with red fonts display little or diffuse expression patterns.

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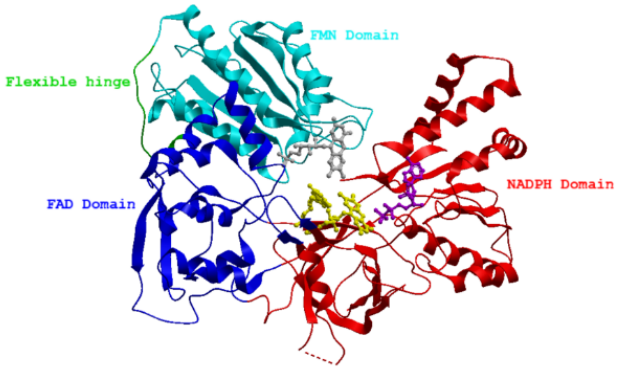

Figure 2B. A 3D model of human wild-type POR structure (PDB: 5FA6), showing the flexible hinge region (green), the three cofactor binding domains i.e., the NADPH (red ribbon), FMN (cyan ribbon), and FAD (blue ribbon). The NADPH, FMN, and FAD ligands, are indicated as balls and sticks with colors pink, grey and yellow, respectively). The initiation of electron transfer starts via the oxidation of NADPH first, leading to the reduction of FAD cofactor. Subsequently, the generated H-ion is accepted by FMN cofactor, ultimately enabling the transfer of electrons to all Cytochrome P450 enzymes.

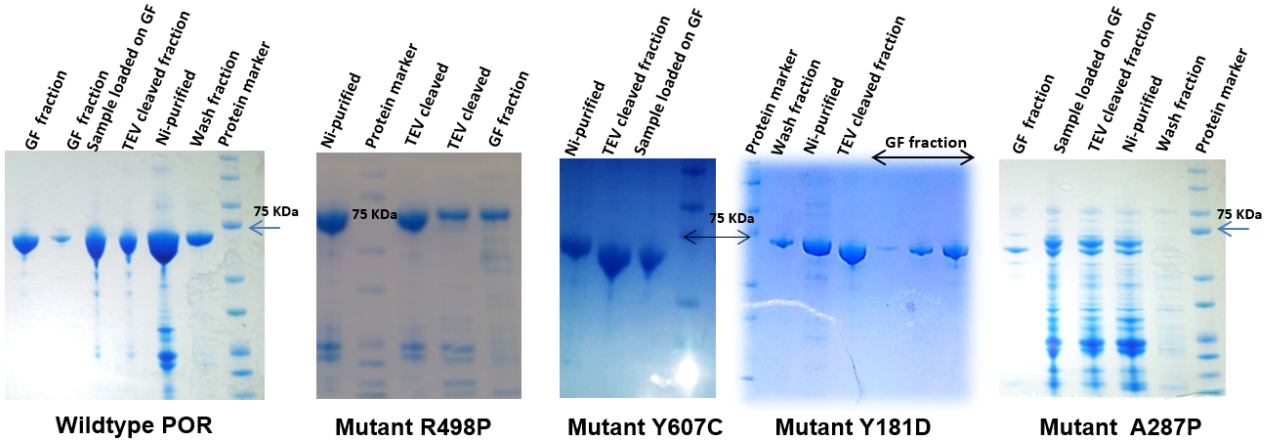

Figure 2C. Large scale (> l0 L) protein expression analysis of wild-type POR and mutant enzymes (R498P, Y607C, Y181D and A287P).

Furthermore, to analyze the impact of POR mutations on the structural stability of POR enzyme, the selected POR mutants (A287P, Y181D, and Y607C) and wild-type POR protein were subjected to protein crystallization studies (Figure 3A), using a variety of protein crystallization screens (Figure 3A), for the purpose of their 3D structural determination. The initial protein crystallization results revealed a thin crystalline formation of wild-type POR protein (Figure 3A), whereas the mutants, i.e., A287P showed ambiguous crystalline growth; Y181D showed very heavy crystalline precipitate; and mutant Y607C displayed few very thin needle shape crystals (Figure 3A). The crystallization of wild-type POR protein and mutant variants were further optimized under a different set of conditions i.e., wtPOR (ethylene glycol (0.12 M), MES/Imidazole buffer (pH: 6.5), MPD_PIK_P3350 (37.5%)); A2879 (monosaccharides 0.12M, buffer system (1) - 0.1M pH:6.5, P550MME_P20K (30%)); Y181D (0.12 M Bis-Tris (pH 6.5), 25% (w/v) polyethylene glycol (PEG) 3350, 0.1 M ammonium sulfate); Y607C (caesium chloride (0.05 M), 0.1 M MES (pH: 6.5), v/v Jeffamine m-600 (30%)). The optimization of initial protein crystals revealed a rectangular shape crystal for wtPOR protein; no defined crystal growth for mutant A287P; the appearance of rod-like crystals for Y181D; and thin sheet crystalline formation for mutant Y607C (Figure 3B). The presence of mutation(s) in proteins exerts dramatic changes in terms of folding and stability [16,17], and therefore, imposes a challenge for successful protein crystallization [12], as seen in the case of mutant A287P.

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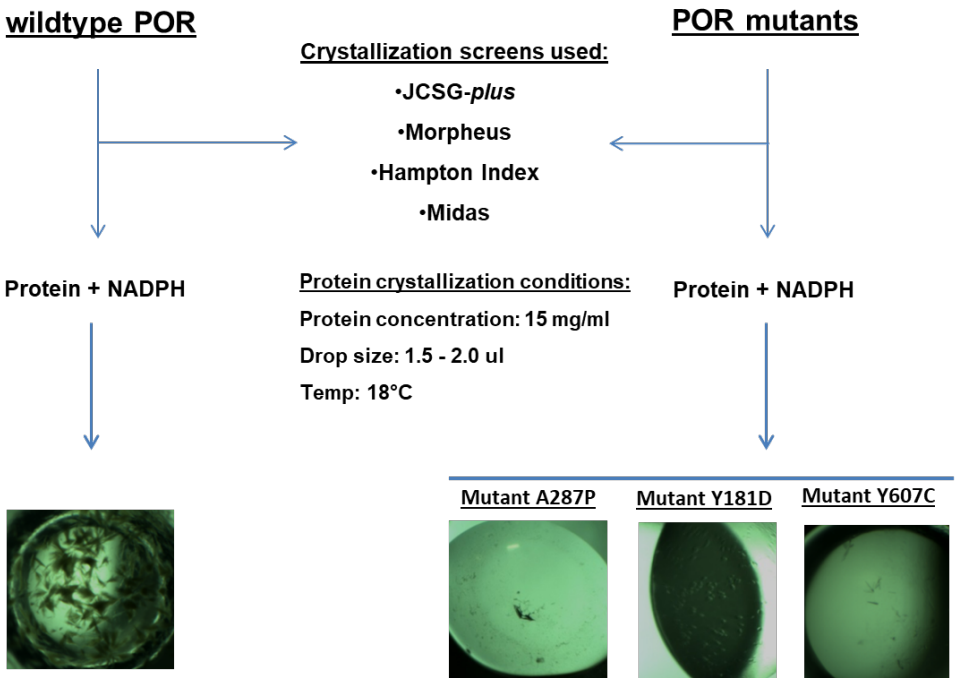

Figure 3A. Initial protein crystallization of POR wild-type and mutant proteins (A287P, Y181D, Y607C) was performed using the JCGG-plus, Morpheus, Hampton Index and Midas crystallization screens. Crystallization experiments were performed at 18°C using a sitting drop consisting of 100 nl of POR wildtype or mutant proteins (15 mg/ml) and 200 nl of well solution containing different crystallization reagents. The crystals appear after 14 days of experiment setup.

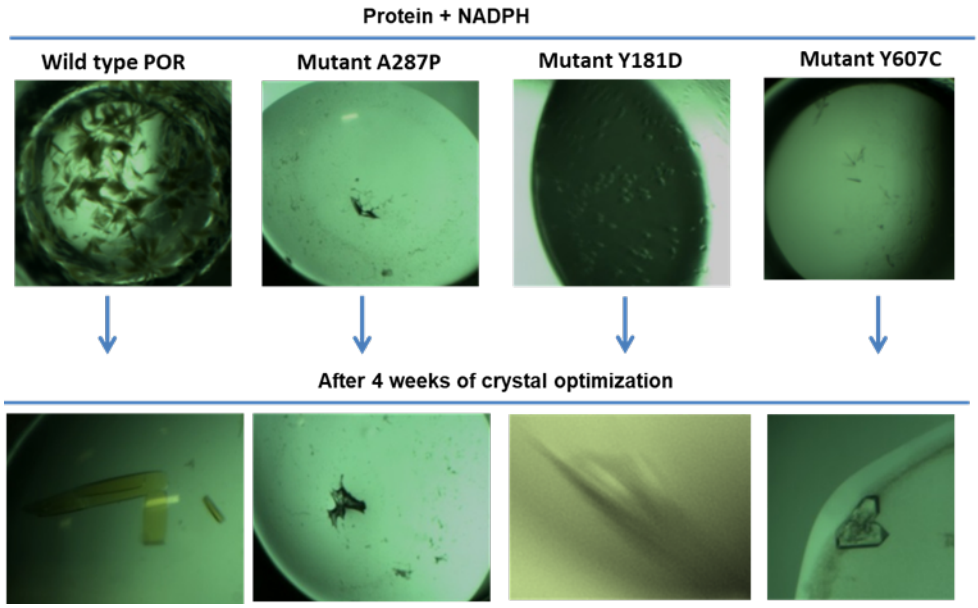

Figure 3B. Crystal optimization of POR wild-type and mutant (A287P, Y181D, Y607C) proteins was performed under a different set of conditions, i.e., wtPOR (ethylene glycol (0.12 M), MES/Imidazole buffer (pH: 6.5), MPD_PIK_P3350 (37.5%)); A287P (monosaccharides 0.12M, buffer system (1) - 0.1M pH:6.5, P550MME_P20K (30%)); Y181D (0.12 M Bis-Tris (pH 6.5), 25% (w/v) polyethylene glycol (PEG) 3350, 0.1 M ammonium sulfate); Y607C (caesium chloride (0.05 M), 0.1 M MES (pH: 6.5), v/v Jeffamine m-600 (30%)). Crystallization experiments were performed at 18°C using 10 -15 mg/ml protein concentration.

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2.2. In-silico analysis of POR mutant variants

The in-silico mapping of POR mutants was performed on the published 3D X-ray crystal structure of wild-type POR protein (PDB: 5FA6) using the computational modelling software Molsoft ICM browser (Molsoft LLC, California, USA). The in-silico analysis reveals the location of POR mutants (Y181D, A287P, R457H, R498P, C569Y, Y607C, and H628P) within or at the surrounding regions of the different cofactor binding domains (Figure 4). The POR mutants (Y181D, Y607C, R498P, and A287P) used for protein crystallization were further subjected to an in-depth in-silico analysis. The Y181D which is located at the FMN domain interacts using a pi-pi bond interaction with the isoalloxazine ring of FMN [13] and with the neighboring amino acid residues glutamic acid (E) 182 and threonine (T) 180 (Figure 5A). The replacement of T181 with aspartic acid (D) would result in the destabilization of FMN cofactor. Although the substitution of Y181 with the aspartic acid (D) is associated with the significant decrease of activity of CYP17A and cytochrome C enzyme [3,39], the Y181D mutant showed a moderate protein expression (Figures 2A & 2C) and crystallization pattern (Figure 3B) reflecting no major impact on the structure stability in terms of affecting protein expression level and crystal packing.

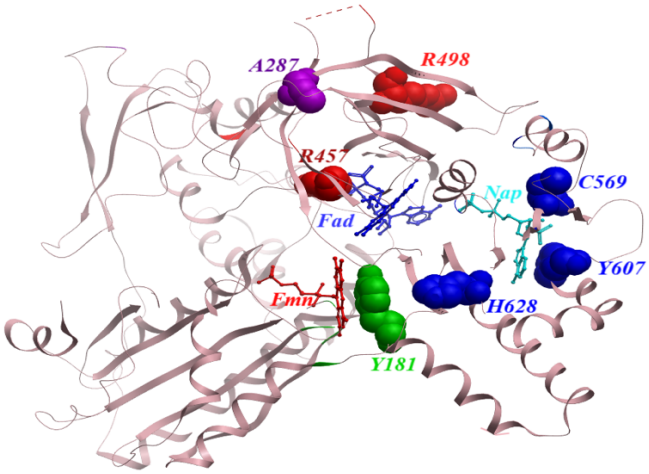

Figure 4. 3D enzyme structure of human POR (PDB: 5FA6) represented in a ribbon and balloon model showing the location of mutated amino acid residue on their respective binding domains. Residue Y181 is located near the FMN domain; residues C569, Y607 and H628 are located near the NADPH domain; residues R457 and R498 are located near the FAD domain; residue A287 is located near the loop connecting the FAD and NADPH domain.

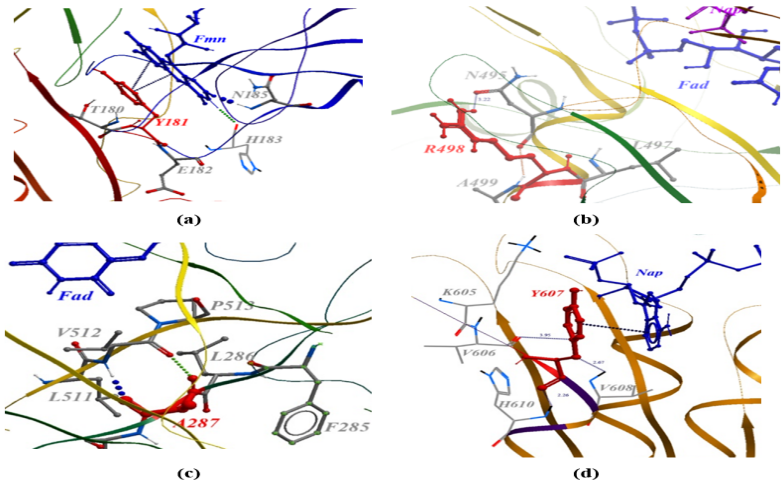

Figure 5. An in-silico analysis displaying interaction of the POR mutants with the neighboring amino acid residues. (A) Y181 interactions with the neighboring residues and FMN cofactor. (B) R498 interactions with the surrounding amino acid resides. (C) A287 interactions with the neighboring residues below the FAD cofactor. (D) Y607 interactions with the NADPH cofactor and neighboring residues within the NADPH binding domain.

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The other two mutants i.e., R457H and R498P are located at the FAD binding domain (Figure 4). Between the two, R457H is the most abundant mutation among the Asian population with a mild to severe patient phenotype. [2,11,25,26,31-34,38]. The amino acid arginine (R) 498 which is not in direct contact with the FAD cofactor closely interacts with the neighboring asparagine (N) 495, alanine (A) 499 and leucine (L) 497 (Figure 5B). The substitution of arginine (R) with the cyclic proline (P) would introduce a bend in the beta-sheet and break the bonding with the neighboring residues. Although both R457H and R498P mutants showed a decent protein expression pattern (Figure 2A), none of them showed any protein crystal growth on several crystallization attempts reflecting their potentially deleterious impact on the stability of the FAD binding domain (Figures 4 & 5B). Several reported *in vitro* studies have associated Arg to His mutation with the 3% residual activity of the CYP17A1 enzyme [23]. On the other hand, the A287P mutant which is not located within the three domains of POR but situated below the FAD-binding domain displays very weak protein expression levels (Figure 2C) and no protein crystal growth (Figure 3B)**.** The A287P mutant accounts for 40% residual activity of the POR enzyme and is mostly found in the Caucasian population [2]. The amino acid alanine (A) 287 is involved in hydrogen bonding with the surrounding valine (V) 512 (Figure 5C) and also closely interacts with the amino acid residues leucine (L) 511 and 286 (Figure 5C). The alanine (A) 287 is involved in stabilizing the beta-sheet structure and its replacement with proline (P) would result in the distortion of the beta-sheet secondary structure, as reflected within the severe clinical symptoms of bone defects and sexual development disorder in PORD patients [2,8,32,40,41]**.**

The remaining three investigated mutants i.e., the C569Y, H628P and Y607C are located at the NADPH binding domain as revealed by in-silico mapping (Figure 4). The C569Y mutant showed a very diffuse protein expression pattern (Figure 2A), whereas the mutant H628P displayed a very low protein expression (Figure 2A) and hence no protein crystallization attempts were made on the C569Yand H628P mutants. On the contrary, the mutant Y607C interacts with the NADPH cofactor using a hydrogen bond and pi-pi interactions [12], and with the neighbouring amino acid residues valine (V) 606 and 608 and histidine (H) 610 (Figure 5D), display very good protein expression (Figure 2A & 2C) and crystallization pattern (Figure 3B). The substitution of an aromatic tyrosine (Y) residue with the cysteine (C) will hinder the interactions with the NADPH cofactor and hence destabilize the domain. Tyrosine (Y) 607 substitution to cysteine (C) residue is reported to exhibit a significant decrease in aromatase and 17,20 lyase enzyme activities [39,42].

3. CONCLUSION

The POR enzyme plays a significant role in providing electrons to all the cytochrome P450 enzymes, aromatase, heme oxygenase and cytochrome b5 which is vital for the proper functioning of these enzymes. Mutations in the POR enzyme interfere with the transfer of electrons and are hence responsible for the total or significant decrease in the activity of partner enzymes resulting in several clinical phenotypes as observed in patients with PORD. The level of functional impairment caused by the mutants varied from moderate to severe, depending on the nature and location of impaired residues within or around the POR cofactor binding domains, i.e., the FAD, FMN and NADPH domains. The impact of a specific mutation on the stability of the POR enzyme, in terms of its expression and crystallization, depends on its size, charge and interactions with the neighboring amino acid residues, as revealed by an in-silico analysis. Therefore, the current study highlights the importance of understanding the consequence of mutated residues on the stability of enzyme structure through an in-depth in-silico analysis before designing any personalized treatments against the observed clinical phenotypes reported in PORD patients.

4. MATERIALS AND METHODS

4.1. Expression and purification

The POR wildtype and mutant clones, as described [25], were gifted by Professor Wiebke Arlt (University of Birmingham, UK). E. coli Bl-21 DE3 cells (Invitrogen) were transformed with pNIC-28-BSAI expression vectors containing cDNA of POR WT or its mutants for protein expression. Bacteria were cultured in Terrific Broth at 37°C using INFORS shaker at 180 rpm (81 x g) until OD_{600} ≥1. Cells was induced with 0.6 mM IPTG (isopropyl β-D-thiogalactopyranoside) (Sigma) and cultured overnight at 18°C. Cells were harvested using Beckman Coulter centrifuge for 12 min at 6500 rpm (10,533 x g) (4°C) and resuspended in binding buffer (50mM HEPES (n-(2-hydroxyethyl)piperazine-n'-(2-ethanesulfonic acid) pH 7.5, 500mM NaCl, 5% glycerol, 5mM imidazole and 1mM PMSF). In addition, 0.5mM TCEP (tris(2-carboxyethyl)phosphine) and protease inhibitor cocktail tablet, EDTA (Ethylenediaminetetraacetic acid) free were further added to the

buffer. Cells were lysed using Bugbuster® Protein Extraction Reagent (Novagen). The cell pellet was removed by centrifugation using Beckman Coulter centrifuge (50 min, 17000 rpm (11.770 x g), 4°C) and the supernatant was subjected to immobilized metal ion affinity chromatography. His-tagged proteins were eluted from Ni^{2}-sepharose (Sigma-Aldrich) with elution buffer containing 240mM imidazole. His-tag was removed using TEV (Tobacco Etch Virus) protease (Invitrogen). POR enzymes were later purified by gel filtration using HiLoad 16/60 Superdex 200 prep grade (GE Healthcare). The eluted proteins from the Superdex 200 column were treated with TEV protease overnight at 4◦C and passed over Ni-Sepharose resin pre-equilibrated with GF buffer. The total yield of purified protein from large scale (12 liters) cell culture was POR wildtype: 19mg; Y181D: 13mg; A287P: 7.5mg; R498P: 15.5mg; Y607C 14 mg. The final purified proteins were concentrated to 15-20 mg/ml (wild-type and mutant POR) and stored at −80◦C.

4.2. Selection of POR mutants

To study the effects of POR mutations on the 3D structure of POR enzyme, seven POR mutants were selected based on their interactions with the three different cofactor bonding domains, i.e., Y181D interact with FMN binding domains; R457H and R498P interacts with the FAD binding domain; C569Y, Y607C, and H628P interacts with the NADPH binding domain of the POR enzyme. The A287P mutant, which is located below the FAD binding domain, was selected due to its severe clinical phenotype, as reported in the PORD patients [2].

4.3. Protein crystallization

Prior to crystallization, 5 mM NADPH (Nicotinamide adenine dinucleotide phosphate) (Sigma) was added to the wildtype POR and mutant proteins. Crystals were grown by vapour diffusion in sitting drops at 18◦C. A sitting drop consisting of 100 nl of POR wildtype or mutant proteins (10-15 mg/ml) and 200 nl of well solution was equilibrated against well solution containing 0.08M ethylene glycol, 0.1M MES/Imidazole buffer (pH: 6.0), 37.5% MPD_PIK_P3350 (wtPOR); 0.15M monosaccharides, buffer system (1) - 0.12M pH:6.5, 35% P550MME_P20K (A287P); 0.15 M Bis-Tris (pH 6.5), 30% (w/v) polyethylene glycol (PEG) 3350, 0.12 M ammonium sulfate (Y181D); 0.01M caesium chloride, 0.1 M MES (pH: 6.5), 30% v/v Jeffamine m-600 (Y607C) (Figure 3A). Crystals were mounted directly from the crystallization solution and flash-cooled in liquid nitrogen.

4.4. In silico analysis of 3D X-ray crystal structure of POR protein

A 3D X-ray crystal structure of wild-type POR protein (PDB: 5FA6) was retrieved from the Protein Data Bank (PDB) for in-silico structure analysis. The 3D structure of POR enzyme was analysed using the Molsoft ICM browser (Molsoft LLC, California, USA). In addition, an in-silico mapping of mutations in the POR enzyme was performed using the reported human wild-type 3D X-ray crystal structure (PDB: 5FA6).

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Author contributions: Conceptualization, N.S.; methodology, N.S.; software, N.S. and L.C.M.; validation, N.S.; formal analysis, N.S. and S.N.A; investigation, N.S.; resources, N.S., S.N.A. and L.C.M; data curation, N.S.; writing—original draft preparation, N.S.; writing—review and editing, N.S., S.N.A. and L.C.M.; visualization, N.S.; supervision, N.S.; project administration, N.S. and L.C.M; funding acquisition, N.S. All authors have read and agreed to the published version of the manuscript.

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