Biochemical, structural and functional characterization of a novel P450 oxidoreductase mutation causing virilization in a 46,XX patient

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Introduction

A broad spectrum of human diseases are caused by mutations in the NADPH cytochrome P450 oxidoreductase (POR)[1]. Mutations in POR cause mild to severe forms of CAH with and without bone malformation symptoms resembling Antley-Bixler syndrome. Here we are reporting a novel R550W mutation in POR identified in a 46,XX patient with signs of aromatase deficiency. Child was born of first pregnancy and mother presented with signs of virilization (deepening of voice and hirsutism) from the 6th month. Mother had elevated T (545 ng/dl) 5th day post-partum that later returned to normal (26 ng/dl) at 4th month post-partum. The daughter was born with body length of 49 cm and weighed 2.74 Kg at birth. At 7th day fused labioscrotal folds (genital tubercle 1.5 cm with urethral opening, Prader stage 3) were observed. Ultrasound examination revealed presence of uterus and ovaries. Slightly elevated 17OH-progesterone (4,700 ng/dl) and T (84 ng/dl) normalized, ruling out CYP21A2 deficiency and suggesting aromatase deficiency.

Sequencing of *CYP19A1* gene did not reveal any defects and later on candidate gene screening for DSD (NGS panel) revealed compound heterozygous mutations c.70_71delTC / p.Leu25PhefsTer93 and c.1648C>T / p.Arg550Trp in *POR*.

At 8 years, adrenal function is normal except for slightly elevated 17OH-progesterone.

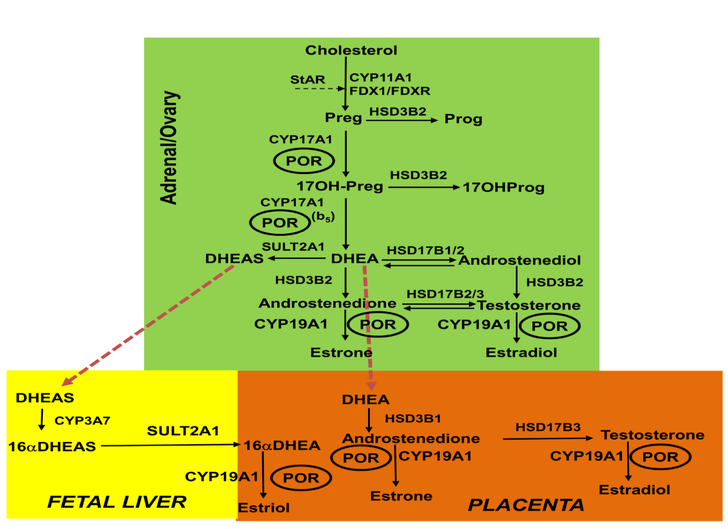


Fig 1: Steroid hormone biosynthesis. The qualitative regulator of steroidogenesis CYP17A1, converts pregnenolone to 17OH-pregnenolone and dehydroepiandrosterone (DHEA). DHEA, the precursor to androgens, is then metabolized in a series of steps involving HSD3B2 and CYP19A1 to estrogens either directly in the placenta or through intermediates formed in the fetal liver and then sent to the placenta.

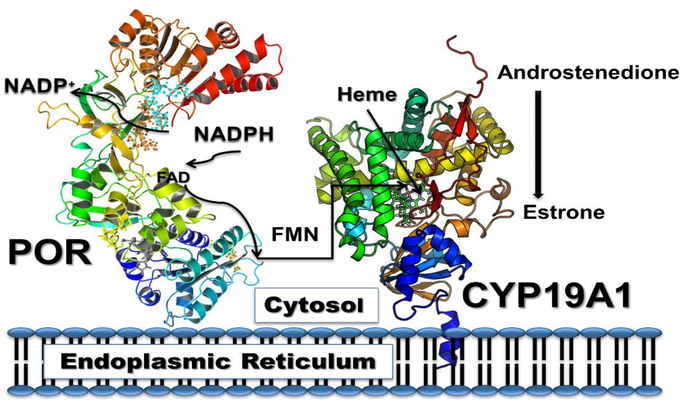


Fig 2: The CYP19A1 activity and role of POR. CYP19A1 interacts with POR in the endoplasmic reticulum to receive electrons used in metabolism of androgens to estrogens.

Objective

We performed detailed enzymatic and biochemical characterizations of the R550W variant of *POR* to study its metabolic profile and role in causing POR deficiency (PORD).

Methods

DNA was analysed with a custom-designed targeted Disorders of Sexual Development NGS panel (DSDSeq.V1, 111 genes) using SeqCap EZ technology (Roche Nimblegen) and sequenced on a NextSeq (Illumina) platform.

The wild type and mutant human POR proteins were expressed in bacteria. The ability of wild type POR and R550W variant to reduce ferricyanide, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and cytochrome c was monitored by measuring the change in absorbance at 420 nm, 610 nm and 550 nm. The ability of WT and R550W variant to support CYP19A1 aromatase activity was determined by tritiated water release assay.

Results

R550 residue is highly conserved and located in the NADPH binding domain of POR (**Figure 3**).

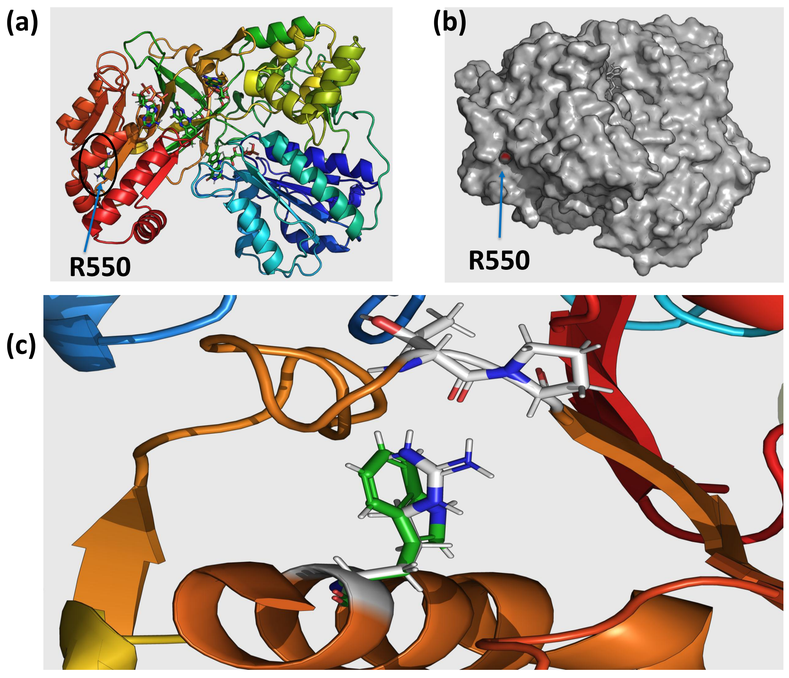


Fig 3: (a) Location of R550W residue in POR. **(b)** R550 is not directly at surface of POR. **(c)** R550 forms hydrogen bonds with T529 to stabilize the NADPH binding domain. Its mutation to W results in destabilization.

We found severe effects of R550W mutation on POR activities with different substrates. As compared to WT, R550W variant showed 41% cytochrome c and 27 % ferricyanide reduction activity, but had only 7.7 % MTT reduction activity (**Figure 4 and Table 1**).

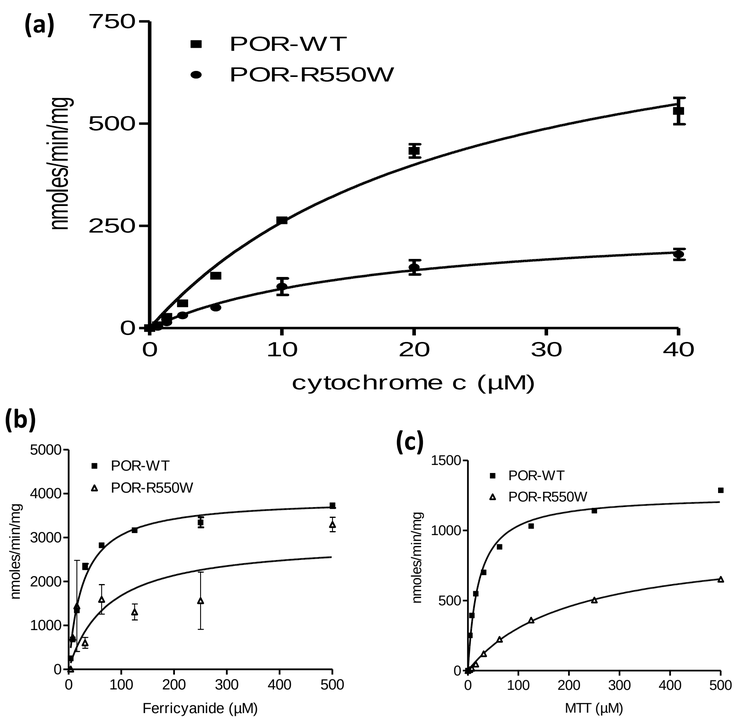


Fig 4: Kinetics of **(a)** cytochrome c, **(b)** ferricyanide and **(c)** MTT reduction by WT and R550W POR.

Results

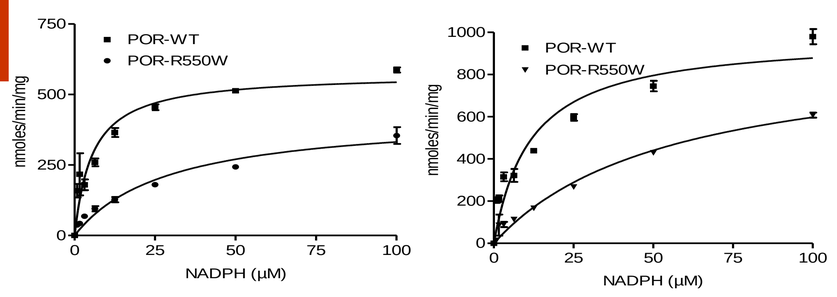
Table 1: Kinetic parameters for cytochrome c ferricyanide and MTT reduction by POR WT and R550W variant.

Cyt-c Reduction	POR-WT	POR-R550W
VMAX	873.2±68.1	267.3±29.8
KM	23.7±3.7	17.78±4.3
Vmax/Km	36.8	15.0
%WT	100.0	40.8

FeCN Reduction	POR-WT	POR-R550W
VMAX	3890±88.1	2939±712.4
KM	27.2±2.3	74.76±54.3
Vmax/Km	143.0	39.3
%WT	100.0	27.5

MTT Reduction	POR-WT	POR-R550W
VMAX	1254±22.7	916±24
KM	21.3±1.5	201±11.7
Vmax/Km	58.9	4.6
%WT	100.0	7.7

Severe effect on NADPH binding by R550W mutation was observed using both cyt-c and MTT as substrates with variable NADPH concentration (**Figure 5**).



Cyt-c Reduction	POR-WT	POR-R550W
VMAX	573.5±31.2	429±32.6
KM	5.5±1.1	29.4±5.5
Vmax/Km	103.8	14.6
%WT	100.0	14.1

MTT Reduction	POR-WT	POR-R550W
VMAX	980±61.2	929±83.4
KM	12±2.3	55±10
Vmax/Km	84	17
%WT	100.0	20

Fig 5. Measurement of NADPH binding and affinity in WT and R550W POR.

CYP19A1 activity was severely reduced in R550W mutant (**Figure 6**).

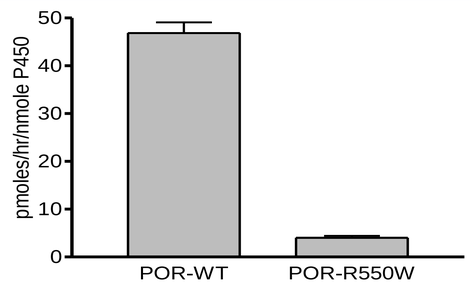


Fig 6: CYP19A1 activity supported by the R550W variant of POR compared to WT. Conversion of [3H] labeled androstenedione to estrone.

Conclusions

The mutation Arg550Trp is located in the NADPH binding region of POR. Computational analysis predicted instability in the NADPH binding region of POR by R550W mutation due to disruption of hydrogen binding, which may affect aromatase (CYP19A1) activity to a higher degree than other partner enzymes because CYP19A1 requires 6 molecules of NADPH per reaction cycle compared to 2 molecules of NADPH for other cytochrome P450 partners of POR. Computationally predicted adverse effect on aromatase activity as well as binding of NADPH were confirmed by experiments using recombinant proteins. These results suggest a pathological effect of *POR* R550W and a diagnosis of PORD in the patient with p.Arg550Trp/p.Leu25PhefsTer93 in *POR*.

1. A.V. Pandey and C.E. Flück. NADPH P450 oxidoreductase: Structure, function and pathology of diseases. Pharmacology & Therapeutics. 2013, 138:229-254
2. Burkhard FZ, Parween S, Udhane SS, Flück CE, Pandey AV. P450 Oxidoreductase deficiency: Analysis of mutations and polymorphisms. J Steroid Biochem Mol Biol. (2017) 165:38-50.

Acknowledgements

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