P450 Oxidoreductase Deficiency: Loss of Activity Caused by Protein Instability From a Novel L374H Mutation

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Context: P450 oxidoreductase (POR) is required for the activities of steroid-metabolizing cytochrome P450 enzymes in the endoplasmic reticulum. POR deficiency (PORD) is a form of congenital adrenal hyperplasia.

Objective and Aim: Enzymatic and structural analysis of a novel L374H POR mutation from a patient with 46,XX disorder of sexual development.

Design, Setting, Patient, and Intervention: The patient was a 46,XX girl with nonconsanguineous Turkish parents. She had virilized external genitalia at birth, a uterus and ovaries, and no sign of Antley-Bixler syndrome. The initial diagnosis was CYP21A2 deficiency with no mutations in CYP21A2, but POR mutations were found. Functional testing was done after producing recombinant POR proteins for analyzing enzymatic and structural properties.

Main Outcome: Novel mutations were causing severe loss of POR activities for metabolism of steroids and small molecules.

Results: The L374H mutation reduced activities by 80% in cytochrome c, 97% in thiazolyl blue tetrazolium bromide, and 86% in ferricyanide reduction assays. The catalytic efficiency of the 17 α-hydroxylation of progesterone and the 17,20-lyase reaction of 17-OH pregnenolone was decreased by 87 and 90%, respectively; 21-hydroxylation of progesterone was decreased by 96%, and androstenedione aromatization was decreased by 90%. Analysis of the mutant structure by molecular dynamics simulations revealed structural instability. Flavin release and fast proteolysis assays showed that the L374H mutant is less stable than wild-type POR, confirming the bioinformatics prediction.

Conclusions: This is the first report of a mutation causing PORD by affecting protein stability that causes severe reduction in POR activities. Detailed characterization of individual mutations in POR is required for understanding novel molecular mechanisms causing PORD. **(*J Clin Endocrinol Metab* 101: 4789–4798, 2016)**

Cytochrome P450 oxidoreductase deficiency (PORD) (OMIM 613571 and OMIM 201750) is a rare form of congenital adrenal hyperplasia (1–6). Cytochrome P450 oxidoreductase (POR) (OMIM 124015) is required for the metabolism of drugs, xenobiotics, and steroid hormones (1, 5). Cytochrome P450 enzymes in the endoplas-

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Abbreviations: 3D, three-dimensional; DHEA, dehydroepiandrosterone; DOC, deoxycorticosterone; FAD, flavin adenine dinucleotide; FASTpp, fast proteolysis assay; FMN, flavin mononucleotide; MTT, thiazolyl tetrazolium blue bromide; NADPH, nicotinamide adenine dinucleotide phosphate; POR, P450 oxidoreductase; PORD, POR deficiency; TL, thermolysin; WT, wild-type.

mic reticulum receive reductive equivalents for their enzymatic activities from the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) through POR (1, 7). Defects in many steroid pathway proteins have been reported, including CYP17A1, CYP21A2, and CYP19A1, but in several cases, a correct genetic diagnosis could not be made. Some patients showed steroid hormone profiles that suggested combined defects in 17- and 21-hydroxylase genes and resembled the bone malformation disorder Antley-Bixler syndrome (8). An index case was a 46,XY infant with a female phenotype who had an unusual steroid profile with low plasma levels of all measured C19 steroids, which remained unaffected upon stimulation by chorionic gonadotropin or ACTH (8). Genetic analysis of CYP17A1 and CYP21A2 did not reveal any mutations, and a possible defect in redox partners like POR (which is required for all cytochrome P450 proteins in the endoplasmic reticulum including CYP17A1 and CYP21A2) and cytochrome b_5 (which is required for 17,20-lyase activity of CYP17A1) was suggested by Walter Miller at the University of California, San Francisco, but no experiments could be performed at that time to test this hypothesis (9). Kelley et al (10) analyzed the CYP51A1 gene in a patient with ambiguous genitalia who had no mutations in the fibroblast growth factor receptor 2 and showed symptoms of CYP51A1 deficiency, but no variations in CYP51A1 were detected. Several years later, sequencing of the POR gene in a 46,XX girl who had a steroid profile of combined 17- and 21-hydroxylase deficiencies revealed mutations in POR (2), and multiple reports of PORD followed soon afterward (2, 11–13). These studies established PORD as a rare form of congenital adrenal hyperplasia that is caused by mutations in POR that affect the enzymatic activities of CYP17A1, CYP19A1, CYP21A2, and CYP51A1 (2, 3). PORD is now considered a separate metabolic disorder, independent of Antley-Bixler syndrome (2, 6, 11–14). Analysis of steroid hormone data from many PORD patients from several laboratories suggests that PORD affects the metabolism of steroids in the adrenal glands and the gonads and causes genital ambiguity at birth in both males and females (2, 11–16). Many POR mutations found in patients with PORD are in cofactor binding regions of POR. However, mutations in proteins may also cause structural and conformational instability, which may in turn impact enzymatic activities, protein-protein interactions with redox partners, and the half-life of the protein.

The aim of this study was to perform enzymatic and structural analysis of a novel L374H POR mutation from a patient with 46,XX disorder of sexual development. This is the first report of a novel conformational mutation in human POR, L374H, which affects protein stability and function. We analyzed the mutation by bioinformatics methods, which predicted protein instability. We then produced wild-type (WT) and L374H mutant of POR in recombinant forms and performed enzyme kinetic experiments to check the impact of the L374H mutation on CYP17A1-, CYP21A2-, and CYP19A1-catalyzed reactions. We found reduced activity of the L374H mutant of POR in the metabolism of small molecules and steroid-metabolizing enzymes CYP17A1, CYP19A1, and CYP21A2. Structural analysis by fast proteolysis and urea-mediated release of flavins revealed that the L374H variant of POR is less stable than WT POR. These results provide further insight into a novel mechanism for the impairment of steroidogenesis by conformational mutations in POR. All of the issues associated with PORD described in our first report also apply to the patient described in this study (2).

Patient and Methods

Patient

The patient was a 46,XX girl of Turkish origin born to nonconsanguineous parents. She was their first child, born after four previous pregnancies. Earlier there were two spontaneous abortions and two voluntary abortions. The pregnancy was normal, and no virilization of the mother was detected. The patient had virilization of external genitalia at birth, with posterior labial fusion, rugged major labia, no palpable gonad and clitoromegaly. The presence of a uterus and ovaries was detected; however, there were no clinical signs of Antley-Bixler syndrome. Salt-wasting symptoms were not observed. Treatment by glucocorticoid and mineralocorticoid was started after hormonal analysis (day 3), which suggested a diagnosis of 21-hydroxylase deficiency. After no mutations in the CYP21A2 gene were detected, the treatment was stopped at 5 months. An ACTH test was performed at 7 months of life to confirm the diagnosis (Table 1). After the ACTH test, progesterone was increased, 17-OH-progesterone was in the range of patients heterozygous for 21-hydroxylase deficiency, 21-deoxycortisol was in the lower range of the nonclassical form of 21-hydroxylase deficiency, and dehydroepiandrosterone (DHEA) and Δ4-androstenedione were low and did not respond to ACTH stimulation. In cases of aromatase deficiency, the mothers have a degree of virilization during pregnancy, and at birth all steroids return to normal values. In the current case, the mother had no virilization, and her steroid pattern was not normal. Therefore, aromatase deficiency was discounted. Another genetic disorder with disorder of sexual development symptoms, the AKR1C2/AKR1C4 deficiency, was also discounted based on the steroid analysis pattern and the karyotype of the patient (17, 18). The steroid pattern of the patient indicated a diagnosis of PORD based on predicted deficiencies of both CYP17A1 and CYP21A2, and the *POR* gene was sequenced to confirm this diagnosis. Because compound heterozygote mutations in the *POR* gene were found, no further genetic analysis was performed, as per the current laboratory practice.

Table 1. Patient's Steroid Measurement Data

	Day 18		7 Months 14 Days		
Steroid	RIA	UPLC-MS/MS	UPLC-MS/MS − 30 Min	0	60 Min
17-OH pregnenolone	44.96			1.93	4.90
DOC		0.37			
Corticosterone		81.98	29.95		
Progesterone	2.01			1.40	21.01
17-OH progesterone	6.56	2.36	2.44	1.90	20.81
11-Desoxycorticol		1.25	0.71	0.75	2.15
Cortisol				449	765
21-Desoxycorticol		<0.125	1.00	0.45	7.95
DHEA	17.37			0.485	0.40
Δ4-Androstenedione	0.765	0.64	0.15	0.11	0.14
T	0.16	<0.1	<0.1	0.18	
DHT		<0.1	<0.1		

Abbreviations: DHT, dihydrotestosterone; UPLC-MS/MS, ultra performance liquid chromatography–tandem mass spectrometry. Data are expressed as nmol/L throughout table.

Genetic analysis

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the institutional review board at Lyon University Hospital. After obtaining informed consent from the parents of the patient immediately after birth, genomic DNA was extracted from blood leukocytes. The *CYP21A2* gene analysis was performed twice, first at the 18th day of life, and then at day 45, but no mutations in the *CYP21A2* gene were found. Analysis of the *POR* gene was done by PCR amplification of the gene exon by exon using specific primers as described earlier (19). Sequencing of PCR-amplified products was done after purification in an automated sequencer (Applied Biosystems) using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's specifications. Some sequencing primers were different from the primers used for PCR amplification. The National Centre for Biotechnology entry of human POR (NM_000941) was used for comparing the sequencing results. The patient was compound heterozygous with novel POR mutations p.L374H/c.-5+4A>G. Analysis by SpliceSiteFinder-like (SSF), MaxEntScan (MaxEnt), NNSplice, GeneSplicer, and Human Splicing Finder (HSF) concluded that the c.-5+4A>G mutation diminishes or suppresses the physiological donor sites (SSF, −12%; MaxEnt, −37.1%; NNSplice, loss of donor site; GeneSplicer, −38.6%; and HSF, −9%, with creation of a new donor site at the mutation position and a score of 68.55). This mutation is not reported in the dbSNP database and has not been found in 100 healthy controls.

Steroid assays

Steroid assays were performed in the Centre de Biologie et de Pathologie Est, Lyon, France, using immunological and ultra performance liquid chromatography–tandem mass spectrometry methods as described in the Supplemental Data.

Three-dimensional (3D) protein models

To study the potential impact of mutations on structure, 3D models of WT and L374H were prepared using previously published model-building protocols (20).

Molecular dynamics simulations for model refinement

The molecular dynamics simulations were performed using AMBER03 force field (15, 21). The simulation cell was filled with water, pH was fixed at 7.4, and the AMBER03 (22) electrostatic potentials were evaluated for water molecules in the simulation cell and adjusted by the addition of sodium and chloride ions. The final molecular dynamic simulations were then run with AMBER03 force field at 298K, 0.9% NaCl, and pH 7.4 for 1000 pico seconds to refine the model. The best models were selected for analysis and evaluation of the effect of mutations.

Expression of WT and mutant POR in bacteria

WT and L374H POR were expressed in bacteria, and membranes were prepared as described previously (14). Details are provided in the Supplemental Data.

Expression of human CYP17A1, CYP19A1, CYP21A2, and POR in yeast

Yeast strain W(B) lacking the endogenous yeast CPR1 gene (23) was used for transient expression by cotransfecting pYcDE2 (expressing full-length WT or mutant POR cDNAs) and pYeSF1-pgk (expressing human WT CYP17A1, CYP19A1, or CYP21A2) as described earlier (19).

POR assays

The ability of bacterially expressed POR to reduce cytochrome c or to oxidize NADPH was assayed by measuring the absorbance change at A_{550} nm (21.1 mM^{-1} cm^{-1}) as described before with slight modification (24). Details of the assays with cytochrome c, thiazolyl tetrazolium blue bromide (MTT), and ferricyanide are shown in the Supplemental Data. The activities of steroidogenic enzymes (CYP17A1, CYP19A1, and CYP21A2) supported by WT or L374H POR as electron donors were assayed with 40 μg of yeast microsomes (2, 15, 19). The 17α-hydroxylase and 17,20-lyase activities of CYP17A1 were assayed using ^{14}C progesterone and ^{3}H 17-OH pregnenolone as substrates for the 17α-hydroxylase reaction and the 17,20-lyase reaction, respectively (2). Fifty picomoles of cytochrome b_5 were

also added in the 17,20-lyase reaction, and the reactions were started by the addition of 1 mM NADPH. The reactions were stopped by ethyl acetate:iso-octane (3:1); steroids were extracted, separated by thin layer chromatography (silica gel 60 F-254 thin layer chromatography plates; Merck) using ethyl acetate:chloroform (3:1) as the solvent system, and quantified by phosphorimaging (25). The activity of CYP19A1 was assessed by tritiated water release assay during aromatization of 1_{β}-^{3}H substrates as described earlier (15). For all assays, the data were fitted with nonlinear regression using the Michaelis-Menten equation, and kinetic constants (V_{max} and Km) were calculated using PRISM (GraphPad Software Inc.). A Lineweaver-Burk plot was also generated for the visual inspection (not used for calculations) of the differences between the activities and kinetic constants of the WT and L374H mutant (Supplemental Figure 1).

Flavin analysis of WT and L374H POR

Flavin content of POR was determined as described previously (26). Flavins were released by treating the protein samples with 2 M urea or by boiling it for 5 minutes and removing precipitated proteins by centrifugation at 13 000 rpm for 10 minutes. The fluorescence of released flavin molecules was measured at pH 7.7 and pH 2.6 (excitation at 450 nm, emission at 535 nm) to determine the flavin mononucleotide (FMN)/flavin adenine dinucleotide (FAD) ratio.

Fast proteolysis assay (FASTpp)

The FASTpp was carried out using a previously described protocol (27). The proteolysis assay mixture contained 0.4 mg/mL of bacterial membranes expressing WT or L374H POR, 0.05 mg/mL of thermolysin (TL; Sigma-Aldrich) (28) in 10 mM $CaCl_2$, and 20 mM potassium phosphate buffer (pH 7.6), and digestion was performed in a TGradient Thermoblock (Biometra), generating a gradient from 39 to 70°C. Proteins were analyzed by SDS-PAGE and coomassie staining.

Results

Structural changes caused by L374H mutation

We analyzed the location and structural features of the leucine 374 and the changes caused by the L374H mutation in POR by computational bioinformatics methods. A multiple sequence alignment of human POR with homologs from other species revealed that the leucine 374 residue is conserved across all species (Figure 1A) and buried inside the core of the POR structure (Figure 1B). We made an in silico mutation L374H in POR and analyzed the structural changes after molecular dynamics simulations. We found structural instability and loss of secondary structural features in the L374H variant of POR (Figure 1C). The leucine 374 residue is involved in the structural stability of the POR protein and makes several contacts with amino acids on other helices (D330, D379). The change to histidine from leucine causes rearrangement of amino acid side chains and affects the intramolecular contacts (Figure 1D). The overall structure of POR was significantly altered after the L374H mutation (Figure 1E), and these structural changes could affect the interaction of POR with its redox partners as well as domain movements in POR that are responsible for opening and closing of the POR molecule to cycle NADPH molecules for supplying redox equivalents to partner proteins and small molecules.

Effect of POR L374H on cytochrome c, MTT, and ferricyanide reduction activities

To assess the effect of the L374H mutation on POR activity, we expressed WT and L374H POR in *Escherichia coli* and measured their ability to receive electrons from NADPH and donate them to cytochrome c, MTT, or ferricyanide. The POR L374H mutation markedly reduced the capacity to both reduce cytochrome c and oxidize NADPH (Table 2 and Figure 2, A and B). Compared to WT POR, the L374H variant lost approximately 80% of its activity to reduce cytochrome c and approximately 90% of its activity to oxidize NADPH. Similarly, the MTT (Figure 2C) and ferricyanide reduction activities (Figure 2D) were also severely affected. The L374H POR had 2.9% of MTT reduction and 14.3% of ferricyanide reduction activity compared to WT POR (Table 2 and Figure 2). The loss of activities with MTT and ferricyanide indicates a disruption of electron transport from NADPH to FAD, which could be due to poorer binding of NADPH in the L374H variant of POR or conformational instability affecting domain movements that affect the transfer of electrons from NADPH to FAD.

Effect of POR L374H on CYP17A1 – 17α-hydroxylase and 17,20-lyase activities

L374H mutation disrupted both the 17α-hydroxylase and 17,20-lyase activities of CYP17A1. Compared to WT POR, the 17α-hydroxylase activity of CYP17A1 supported by the L374H mutant was reduced by 87%, and 17,20-lyase activity was reduced by 90% (Table 3). The reaction velocity of 17α-hydroxylase and 17,20-lyase was reduced by 79 and 82.5%, respectively. Higher Km for the substrate was observed for both 17α-hydroxylase and 17,20-lyase reaction, suggesting that the L374H mutation affects either the interaction of CYP17A1 with substrate or the CYP17A1-POR interaction.

Effect of POR L374H on CYP19A1 – aromatase activity

The aromatase converts androgens to estrogens requiring three pairs of electrons, compared to the one pair required for many other cytochrome P450 reactions, and therefore is more sensitive to changes in NADPH binding than other electron transfer partners of POR (15, 29). For

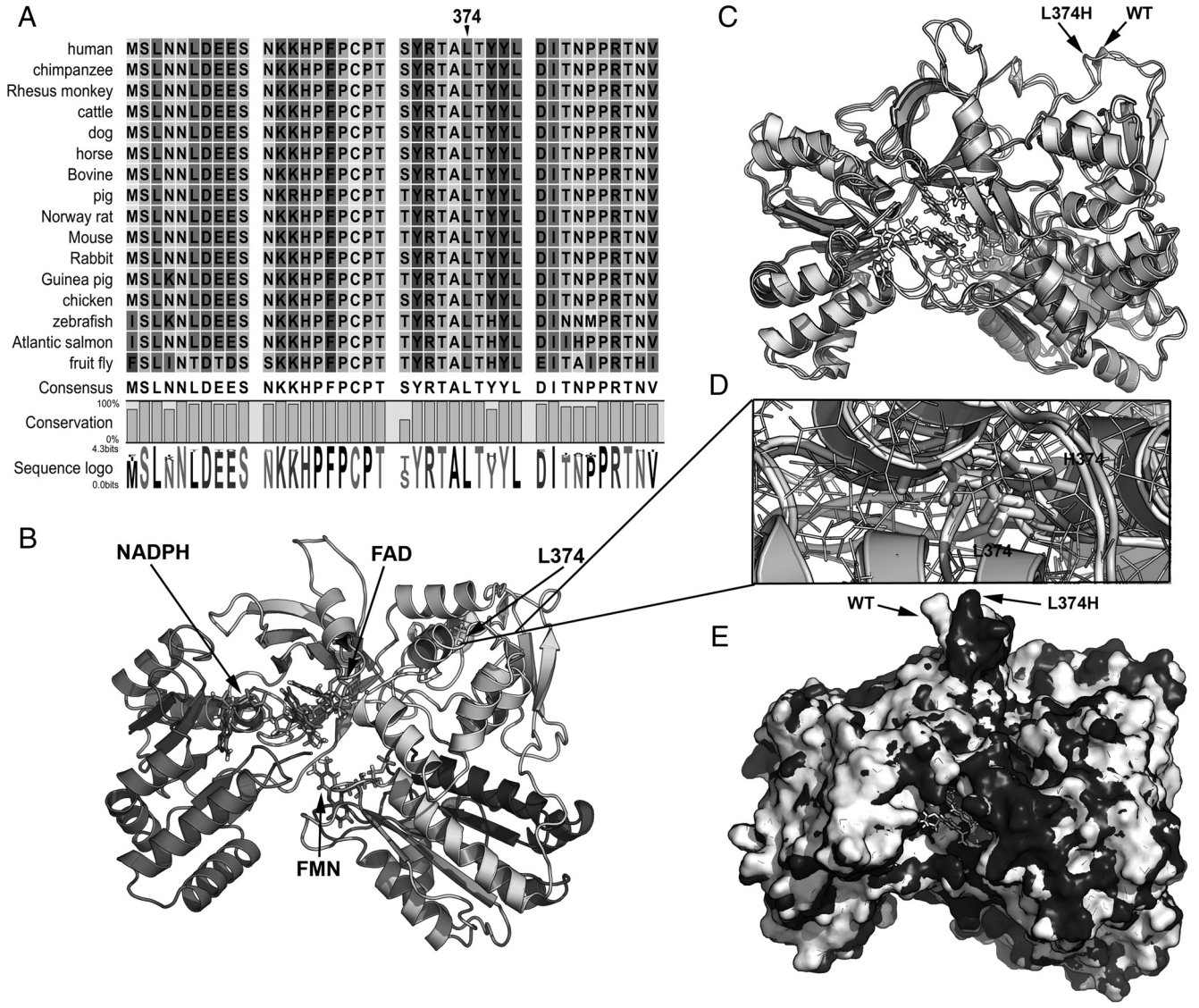

Figure 1. Location of the L374H mutation in the POR structure. A, Multiple sequence alignment of POR amino acid sequences from different species. The leucine 374 residue is conserved across all species, which indicates a crucial structural role of this residue. B, A 3D model of POR protein in closed conformation showing the position of the L374 residue. The leucine 374 residue is buried inside the core POR structure. C, The L374H mutation in POR caused structural instability and changed secondary structural features in the POR structure. D, The leucine 374 residue makes several intramolecular contacts with the neighboring residues in POR for structural stability, and a change to histidine alters the side-chain arrangement and atomic contacts in POR. E, Overall structural changes caused by the L374H mutation in POR. The WT POR is in a light shade, whereas the L374H mutant is in dark shade. Structure models of POR are based on a known 3D structure of the POR protein.

the L374H mutation, the aromatase activity was reduced by 90% compared to WT POR (Table 3). The apparent Km in the reaction with L374H was 0.28 μM and was comparable to Km for the WT POR reaction (0.29 μM). However, the apparent V_{max} for the L374H reaction decreased to 0.26 pmol/min/mg protein from 2.75 pmol/min/mg protein for the WT reaction.

Effect of POR L374H on CYP21A2 – 21-hydroxylase activity

The 21-hydroxylase activity of CYP21A2 supported by L374H was reduced by 96%, with a 90% reduction in V_{max} and a 113% higher Km compared to the WT POR reaction for the conversion of progesterone to 11-deoxycorticosterone (DOC) (Table 3). The loss of activities for all three steroidogenic cytochrome P450 enzymes by the POR variant L374H indicates problems with POR-P450 interaction as well as electron transport within the POR, as deduced from experiments with small molecules.

Flavin content of WT and mutant POR

Loss of POR activity could also be due to effects on binding of cofactors FMN and FAD. To assess the effect of mutation on cofactor binding, total flavin content of WT and L374H POR was measured. Total flavin content of the L374H mutant was comparable to that of WT POR

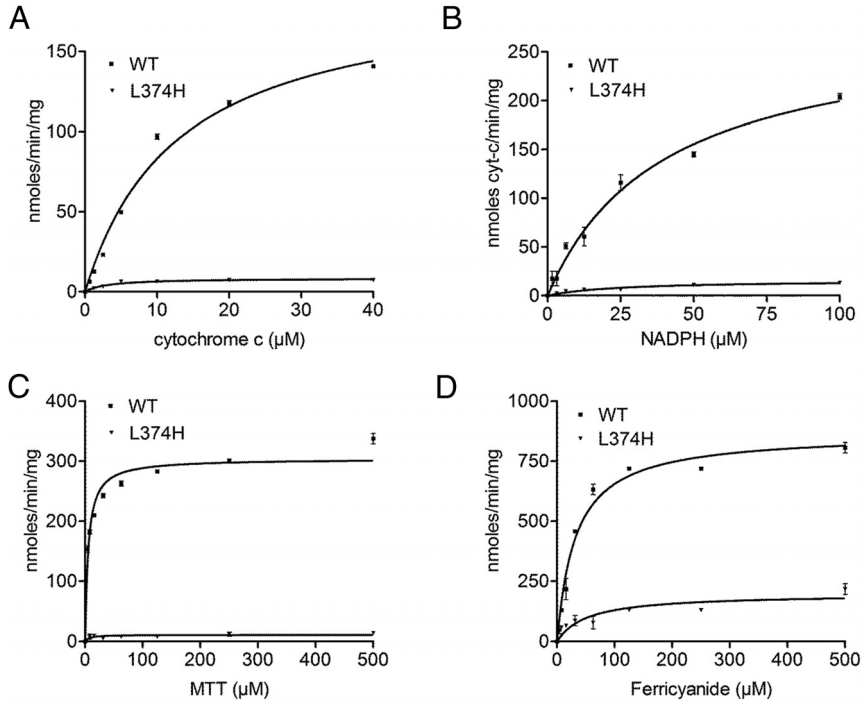

Figure 2. Steady-state kinetics of a small molecule reduction by the WT and L374H POR. The curves represent the best nonlinear fits to the Michaelis-Menten equation. A and B, Cytochrome c reduction assays by the WT and L374H POR expressed in bacteria. Fitted plots of cytochrome c reduction using variable cytochrome c (A) and variable NADPH (B) concentration. C and D, MTT (C) and ferricyanide (D) reduction assay with WT and L374H variant of POR.

using both thermal and urea-mediated denaturation of the POR proteins (Figure 3, A and B), suggesting that the mutation did not affect the binding of FMN or FAD, and the disruption of activities is likely to be due to conformation changes that affect interaction with partner proteins and affect electron transport from NADPH to FAD and FMN.

L374H mutation affects the stability of POR protein

After several assays indicated an effect on protein stability due to the L374H mutation in POR, we tested the stability of the WT and L374H mutant by performing fast proteolysis assay. WT and the mutant POR were treated with TL in parallel to a range of different temperatures. TL is a thermostable protease that readily cuts the unfolded but not the folded part of a protein and preferentially cleaves near the hydrophobic residues Phe, Leu, Ile, and Val. The temperature-dependent changes in the degradation pattern represent the readout for the stability of the proteins. SDS-PAGE analysis of the parallel FASTpp of WT and L374H (Figure 3C) showed that upon the addition of TL, WT is slightly degraded at 4°C, whereas L374H is partially degraded on ice (4°C). Degradation of L374H was complete at 55°C, whereas there was some residual WT POR even at 66°C. This clearly indicated that the L374H mutant is less stable toward TL-mediated proteolysis.

To evaluate the relative conformational stability of WT and mutant POR, the effect of urea on flavin release was measured. Treatment of POR with different concentrations of urea produced a concentration-dependent increase in fluorescence for flavin molecules. The rate of total fluorescence change for L374H was faster than that for the WT POR, indicating the dissociation of flavins at lower concentrations of urea (Figure 3D). This suggests increased structural instability of L374H compared to WT POR protein.

Discussion

The PORD has a very broad clinical profile (1, 5, 6, 30, 31). Many patients with PORD (~85%) have skeletal malformations that were not found in the current patient (Supplemental Table 1) (6). The largest number of skeletal anomalies has been observed when POR mutation R457H (primarily seen in the Japanese population) is present together with a loss of function mutation (14, 30, 31). Recent urine steroid analyses suggest that in PORD, the biosynthesis of androgens may shift from the classic pathway to the alternative pathway, causing virilization in some 46,XX patients as observed here, and some mothers carrying male fetuses may also be affected during pregnancy (31–33). Severe loss of aromatase activity observed for mutation L374H may further influence genital development in 46,XX patients, as seen in the case of the current patient, but this may not cause maternal virilization in mothers of 46,XX fetuses.

Biochemical effects of amino acid changes caused by mutations in POR are influenced by the nature of POR redox partners and their substrates (1, 7, 29, 34). In the first reports describing PORD, mutations in POR caused variable effects on different target proteins (2–4, 14). In general, the assays that were based on activities of cytochrome P450 enzymes correlated well with the clinical observations (2–4, 14). The POR mutations that cause a loss of FMN (Y181D) or FAD binding (R457H, Y459H, V492E) are severe in physiological impact and result in the loss of enzymatic activities in all assays (1, 2, 7, 14, 35). The POR mutations in the NADPH binding domain

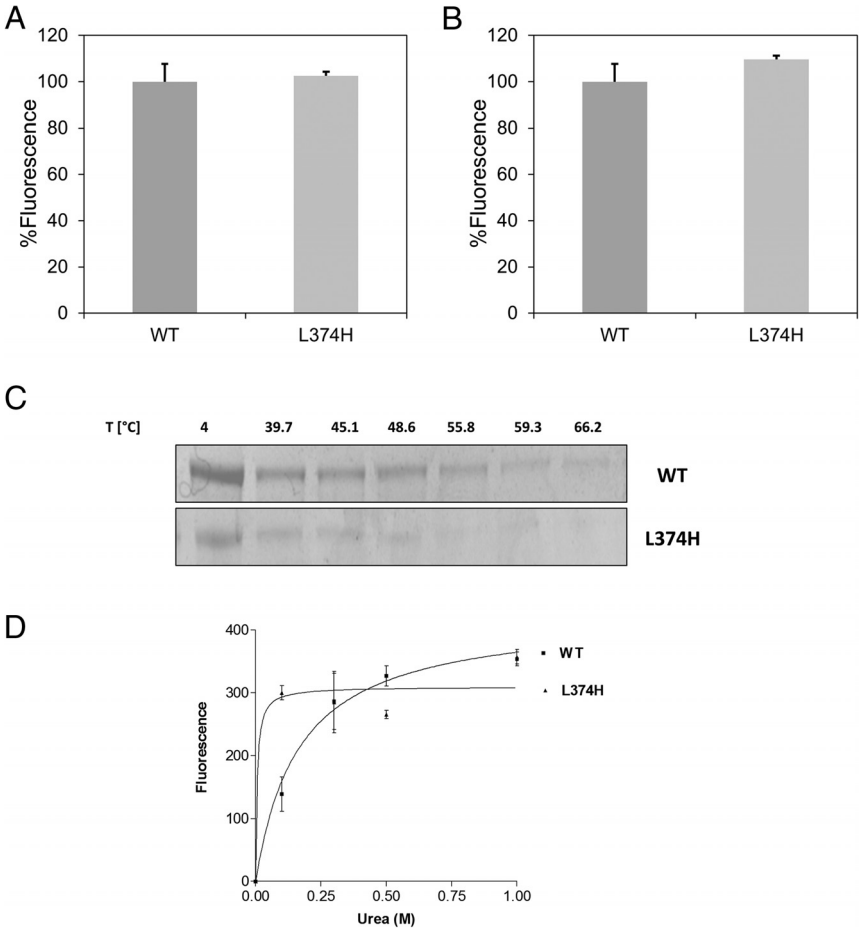

Figure 3. Comparative flavin content and stability of WT and L374H POR. Total flavin fluorescence with excitation at 450 nm and emission at 535 nm was measured. A and B, Flavins were released by heat denaturation of proteins (A) and treatment of POR proteins with 2 M urea (B). C, Analysis of WT and L374H stability by FASTpp assay. FASTpp of WT and L374H analyzed by SDS-PAGE and coomassie staining as described in *Patient and Methods*. The L374H mutant of POR was less stable than the WT POR protein and was degraded at much lower temperatures upon TL treatment. D, Analysis of WT and L374H stability by urea-mediated flavin release assay. For the flavin release assay, urea concentration was gradually increased, and fluorescence of flavin molecules was monitored. The L374H mutant of POR was less stable than the WT protein and released flavin molecules at much lower concentrations of urea.

(C569Y, V608F) disrupt NADPH binding and also have severe effects in many P450-based assays, but these mutations affect the aromatase activity to a greater extent compared to other P450 enzymes (2, 15, 29).

The POR mutations that do not affect cofactor binding are located in all regions of POR structure and cause variable effects, but there are major differences in activities with different redox partners. POR interacts with most of the 50 human cytochrome P450 enzymes located in the endoplasmic reticulum. The P450-redox partner interactions are based on the shape of the molecules as well as atomic charge pairs, which changes with different partners and may therefore depend on the molecular geometry of the redox-partner binding sites in each partner molecule (36–38). In addition, conformational mutations may cause POR to interact with certain redox partners in a different way and may, therefore, impact activities of some partners while showing activity similar to WT with other partner proteins. Protein-protein interactions between the POR, cytochrome P450s, and their substrates in the membranes of the endoplasmic reticulum may cause conformational changes in the flavin- and NADPH-binding domains of POR and may also induce conformational changes in the binding site of the interacting P450s and other redox partners. The precise mechanisms for the interaction of POR with different cytochrome P450s and other electron acceptors may be different in each case. Further complex variations for these interactions may be caused by the binding of substrates to cytochrome P450s, which would then undergo conformational changes. Therefore, no individual assay could reliably provide an indication of activities of variations in POR for all redox partners, and the specific metabolic effects of many POR variants may depend on individual redox partner proteins as well as their substrates.

Many single amino acid variations in the POR gene have been found from the study of the normal population (6, 7). The most common variant of POR is A503V, which is present in about 26% of all alleles and varies from 16% to more than 40% in different subpopulations (6, 7). Some of these variants may cause harmful effects on POR activities, as we have recently found for POR variant Y607C (SNP: rs72557954), which is predominantly found in south Asian populations (6, 29). There are still many instances of disordered steroid metabolism without any defect in known genes, and a search for novel targets and pathways in steroid biosynthesis is required to identify the genetic causes behind these cases (18, 32, 39). We have recently described mutations in patients with disordered steroidogenesis in the genes for aldo-keto reductases AKR1C2 (OMIM: 614279) and AKR1C4 (OMIM: 600451) (18). This has established the existence of two pathways in humans for the production of androgens in the fetal testis and the importance of the alternate pathway for development

Table 2. Steady-State Kinetics Parameters For Reactions Catalyzed by Recombinant WT or L374H POR

	V_{max}, nmol/min*mg	Km, μM	V_{max}/Km (% of WT)
Cytochrome c reduction activity			
WT	190.6 ± 8	12.9 ± 1.3	14.7 (100)
L374H	8.4 ± 0.6	3.1 ± 0.8	2.7 (18.3)
NADPH consumption activity			
WT	278.2 ± 16.9	39.5 ± 5.7	7 (100)
L374H	16 ± 1.6	24.8 ± 7	1 (9)
MTT reduction activity			
WT	303.8 ± 6.3	5.3 ± 0.6	57.1 (100)
L374H	10.9 ± 1.4	6.7 ± 5.7	1.6 (2.9)
Ferricyanide reduction activity			
WT	866.5 ± 26.4	32.3 ± 3.7	26.8 (100)
L374H	198.0 ± 22	51.7 ± 18.9	3.8 (14.3)

Comparison of the activities of WT or mutant POR expressed in bacteria in various assays. All data are expressed as V_{max}/Km, shown as a percentage of the WT control, set at 100%.

of external genital organs in males (17, 18, 39). The POR is also required for reactions in the alternate pathway (1, 17, 39).

Conclusions

In this report, we have shown that a novel conformational mutation in POR identified from a patient caused severe loss of activities of CYP17A1, CYP19A1, and CYP21A2 and also affected direct metabolism of small molecules. Many mutations in POR that reduce activities in P450-based assays still retain activities for metabolizing small molecules like ferricyanide and MTT. In these situations, small molecules can get reducing equivalents directly from FAD or FMN bound to POR without the requirement for a protein-protein interaction. The mutation L374H is the first one to be identified and analyzed in PORD that alters protein stability. Studies on further protein-destabilizing mutations in POR located in different domains may be able to identify crucial residues in POR that provide conformation stability as well as flexibility to interact with various partner proteins. The study of conformational instability in disease-causing mutations provides novel insights about the role of individual amino acids, and in conservative changes involving structurally and chemically similar residues, a surprising activity or stability change would point to subtle structural features that balance evolutionary changes and preserve protein function. Information from detailed structural and enzymatic analysis of mutations in POR is crucial for our understanding of the pathophysiology of PORD, which is a complex disorder affecting many different target proteins and pathways. In the case of the current patient, detailed enzymatic and structural studies obtained from our analysis together with the patient's hormonal and clinical data allowed a definitive assignment of PORD. The transla-

Table 3. Calculated Kinetic Parameters For Activities of CYP17A1, CYP19A1, and CYP21A2 Supported by WT and L374H POR

	V_{max}, pmol/min*mg	Km, μM	V_{max}/Km (% of WT)
CYP17A1: 17-hydroxylase activity (Prog to 17-OHP)			
WT	0.91 ± 0.32	6.07	0.15 (100)
L374H	0.19 ± 0.11	9.07	0.02 (13)
CYP17A1: lyase activity (17-OHPreg to DHEA)			
WT	40.29 ± 10.56	2.34	17.2 (100)
L374H	7.15 ± 5.03	4.02	1.7 (10)
CYP19A1: aromatase (D4A to E_1)			
WT	2.75 ± 0.39	0.29	9.45 (100)
L374H	0.26 ± 0.04	0.28	0.92 (10)
CYP21A2: 21-hydroxylase activity (Prog to DOC)			
POR WT	30.35 ± 12.96	1.99	15.25 (100)
L374H	2.68 ± 1.14	4.24	0.63 (4)

Abbreviations: D4A, androstenedione; DHEA, dehydroepiandrosterone; DOC, 11 deoxycorticosterone; E1, Estrone; Prog, progesterone; 17-OHP, 17 hydroxy progesterone; 17-OHPreg, 17 hydroxy pregnenolone.

Comparison of the steroidogenic enzyme activities supported by WT or mutant POR expressed in yeast. All data are V_{max}/Km, shown as a percentage of the WT control, set at 100%.

tional nature of such analysis was further useful in understanding the exact blockages in the steroid pathways of the patient and helped in the choice of treatment. The exact diagnosis of PORD allowed the clinicians to stop the treatment for CYP21A2 deficiency at an early stage, which has close resemblance to PORD. Currently, the patient is fine without additional treatments.

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