CLINICAL STUDY

Clinical and biochemical description of a novel *CYP21A2* gene mutation 962_963insA using a new 3D model for the P450c21 protein

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Abstract

Objective: A severely virilized 46, XX newborn girl was referred to our center for evaluation and treatment of congenital adrenal hyperplasia (CAH) because of highly elevated 17 α -hydroxyprogesterone levels at newborn screening; biochemical tests confirmed the diagnosis of salt-wasting CAH. Genetic analysis revealed that the girl was compound heterozygote for a previously reported Q318X mutation in exon 8 and a novel insertion of an adenine between nucleotides 962 and 963 in exon 4 of the *CYP21A2* gene. This 962_963insA mutation created a frameshift leading to a stop codon at amino acid 161 of the P450c21 protein.

Aim and methods: To better understand structure–function relationships of mutant P450c21 proteins, we performed multiple sequence alignments of P450c21 with three mammalian P450s (P450 2C8, 2C9 and 2B4) with known structures as well as with human P450c17. Comparative molecular modeling of human P450c21 was then performed by MODELLER using the X-ray crystal structure of rabbit P450 2B4 as a template.

Results: The new three dimensional model of human P450c21 and the sequence alignment were found to be helpful in predicting the role of various amino acids in P450c21, especially those involved in heme binding and interaction with P450 oxidoreductase, the obligate electron donor.

Conclusion: Our model will help in analyzing the genotype–phenotype relationship of P450c21 mutations which have not been tested for their functional activity in an *in vitro* assay.

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Introduction

Congenital adrenal hyperplasia (CAH) consists of a group of autosomal recessive disorders of adrenal steroid hormone biosynthesis due to enzyme defects. CAH may lead to a lack of glucocorticoids, a decrease or increase of mineralocorticoids and adrenal C19 steroids (also known as adrenal androgens). In Western Europe, 90–95% of CAH is caused by mutations of the *CYP21A2* gene. *CYP21A2* encodes the steroidogenic enzyme 21 α -hydroxylase (21OHase), which catalyzes the conversion of progesterone to 11-deoxycorticosterone in the zona glomerulosa of the human adrenal cortex, and the conversion of 17 α -hydroxyprogesterone (17OHP) to 11-deoxycortisol in the zona fasciculata (1). Genotype–phenotype correlation studies of *CYP21A2* mutations have shown that there are essentially three groups of 21OHase deficiency: (a) mutations causing a complete loss of 21OHase activity resulting in the classical form of salt-wasting (SW) CAH, which is clinically manifest as a severe disturbance in water and salt homeostasis due to the mineralocorticoid deficiency, and for which the affected girls often show virilization of their external

genitalia at birth ranging from Prader stages 2–5 (2); (b) mutations encoding enzymes which have 1–2% of normal activity permitting normal mineralocorticoid synthesis, thus resulting in the simple virilizing (SV) form of CAH; and (c) mutations encoding enzymes with 20–60% activity resulting in the non-classical (NC), late-onset form of CAH manifesting in later life.

We studied a completely virilized 46, XX baby girl who presented with the SW form of CAH. She was found to be compound heterozygote for the previously described Q318X mutation in exon 8 of the *CYP21A2* gene (3), and a new mutation in exon 4. Furthermore, we built a new three dimensional (3D) model of the human P450c21 protein, which allows us to predict the structure–function relationship of different mutations in the P450c21 protein.

Methods

Case report

The patient was born at term by spontaneous, vaginal delivery after a normal pregnancy. Birth weight was 3.385 g (+0.6 s.d. score (SDS)) and birth length was

50 cm (+0.5 SDS). Initially, the baby was assigned to the male sex. The parents were not consanguineous and the family history was unremarkable. A 3-year-old sister was healthy. Newborn screening of our patient at day 3 of life revealed a highly elevated serum 17OHP (468 nmol/l; normal <30), thus the child was referred to our center for evaluation of CAH. On admittance, the weight was 3.060 g and the skin was icteric and dry. Detailed examination of the external genitalia revealed a hypoplastic scrotum, no palpable gonads and a penis without ventral swelling body consistent with the possibility of severely virilized female external genitalia. Accordingly, we found a normal 46, XX karyotype, and pelvic ultrasound revealed a normal uterus and enlarged adrenals. Biochemical tests revealed serum sodium of 137 mmol/l (132–142) (normal values in parentheses), potassium of 6.0 mmol/l (3.6–6.1), and chloride of 105 mmol/l (95–116). Serum glucose was 5.4 mmol/l (3.1–4.9) and total bilirubin was 230 µmol/l (2–14). Serum 17OHP was 778 nmol/l (<30), adrenocorticotropin 171 ng/l (9–50), cortisol 134 nmol/l (140–470), androstenedione 279 nmol/l (0.62–1.82), dehydroepiandrosterone-sulfate 12 µmol/l (0.23–1.5), aldosterone 2171 pmol/l (<5000), and plasma renin activity was 379.3 ng/l (20–120). Because all these clinical and biochemical findings were consistent with the diagnosis of SW CAH, the child was started on replacement therapy with hydrocortisone and fludrocortisone. In addition, the child was reassigned to a female sex form of rearing. She is now 2 4/12 years of age and developing normally.

CYP21A2 mutation analysis

Genomic DNA was extracted from peripheral blood leucocytes after obtaining informed consent from both parents. Three fragments of the *CYP21A2* gene were amplified by PCR using highly selective primers differentiating the *CYP21A2* gene from the *CYP21A1P* pseudogene by the 8 bp deletion located in exon 3 of *CYP21A1P* as well as specific primers for sequencing (4). In addition, a fragment of *CYP21A2* was PCR amplified independently of the 8 bp deletion in exon 3 with the primers CYP-5 (5'-AGCTATAAGTGG-CACCTCAGG-3') and CYP-14 (5'-GCATCTCCAC-GATGTGA-3'), with CYP-14 located in exon 6 being highly specific for *CYP21A2*. All PCR fragments, including the 10 exons, the exon/intron boundaries, intron 2, intron 7, and 380 bp of the promoter region of the *CYP21A2* gene were subjected to direct sequencing. Sequencing reactions were performed with the Thermo Sequenase Cycle sequencing kit from Amersham with IRD700 and IRD800 labeled oligonucleotides (MWG-Biotech, Ebersberg, Germany) on a Licor 4200L-2 automatic DNA sequencer (Licor, Lincoln, NE, USA). Large deletions of the *CYP21* gene locus on chromosome 6 and deletions leading to a loss of the 8-nucleotide sequence in exon 3 of the *CYP21A2* gene

were screened and quantified by polyacrylamide electrophoresis of IRD41 labeled unspecific PCR products of exon 3 as previously reported (5). For numbering of nucleotides and amino acids the reference sequence of White *et al.* was used (6).

Sequence alignment and molecular modeling of human P450c21

We performed a Phi-BLAST search of the protein structures in the protein data bank (PDB) database using the P450c21 amino acid sequence as a template. This method was chosen to provide preferential search parameters that allow structure-based alignment of proteins and consideration for both amino acid identity and similarity. Recently, solved structures of the rabbit P450 2B4 protein (7, 8) appeared near the top of our list and the inhibitor-bound structure of P450 2B4 was used as a template for building a new model of human P450c21.

An analysis of structurally conserved regions of P450 proteins was performed by multiple sequence alignment with two other human P450 structures from the PDB database, P450 2C8 and 2C9 (PDB id 1PQ2 and 1OG5) that show significant sequence and structural homology with P450c21. We also included the closely related human P450c17 enzyme, which has been studied for structure–function association of natural as well as synthetic mutations by site-directed mutagenesis experiments (9). The sequences of P450c21, P450c17, P450 2B4, P450 2C8 and P450 2C9 were aligned with ClustalW (10), displayed and edited using GeneDoc (<http://www.psc.edu/biomed/genedoc>). Molecular modeling of human P450c21 was performed by MODELLER (11, 12), which uses satisfaction of spatial restraints for comparative modeling of proteins. The model was based on the X-ray crystal structure of rabbit P450 2B4 in complex with 4-(4-chlorophenyl) imidazole (PDB id 1SUO) (8), which shares 27% sequence identity and 50% similarity with the human P450c21. Several preliminary models were built and the one with the most favorable energy restraints was chosen. Model was checked with the program WHAT IF (13) for detailed validation and reports of structural parameters and compared with the Whatcheck (14) report for the crystal structure of P450 2B4. A Ramachandran plot of the model was compared with the template structure for abnormalities (14–16). A model of human P450 oxidoreductase (POR) was also constructed to study the interaction of P450c21 with POR, its obligate electron donor (17). The human POR model was based on the X-ray crystal structure of rat POR (18) which shares 94% sequence identity with human POR. Energy minimizations of the model structures were carried out by GROMOS, models were examined and edited using DeepView (19) and images were generated using the ray tracing program POVray (www.povray.org). Final model

of P450c21 was compared with the template structure and root-mean-square distance (RMSD) values were calculated by DALI (20, 21).

Results

Identification of a new 962_963insA mutation in the CYP21A2 gene

Clinical and biochemical findings in our patient were consistent with the diagnosis of SW CAH. Therefore, genetic analysis of the CYP21A2 gene was performed. We found on one allele, a new insertion of an adenine between nucleotides 962 and 963 in exon 4 of the CYP21A2 gene (962_963insA) (Fig. 1). This insertion of an adenine created a frameshift, which led to a stop codon at amino acid 161 truncating the P450c21 protein. To our knowledge, this mutation has not previously been identified in a patient with 21OHase deficiency.

On the other allele, codon 318 was changed from CAG, encoding glutamine (Q), to TAG (X), a stop codon (Fig. 1). This nonsense mutation Q318X in exon 8 of the CYP21A2 gene was first reported in homozygote form in a patient presenting with severe SW CAH (3). Activity of the Q318X protein has been reported to be completely lost (3). As Q318X is present in the CYP21A1

pseudogene, this mutation in the CYP21A2 gene is thought to be formed through gene conversion during meiosis (22, 23). Thus, Q318X is a common mutation identified in patients with 21OHase deficiency from all over the world (24).

The novel 962_963insA mutation in our patient was inherited from the mother, while the Q318X mutation was inherited from the father; but both parents were heterozygous for their respective CYP21A2 mutation and, therefore, clinically healthy.

The new P450c21 protein model

We performed a structural alignment of the amino acid sequence of P450c21 with three mammalian P450 proteins, which share considerable sequence homology and are available in the PDB database. We also included human P450c17 in our alignment as it is a closely related steroidogenic enzyme for which some structure–function information is available (9). All major structural elements of P450 proteins aligned perfectly in all five proteins (Fig. 2). The model of P450c21 protein had a similar fold as other P450 structures and closely resembled the P450 2B4 structure as inferred by an RMSD value of 0.93 Å obtained by the program DALI (20, 21). A comparison of Ramachandran plots of both

Figure 1 CYP21A2 mutation analysis. (Top) Sequence analysis showing the novel 962_963insA mutation and the previously described Q318X mutation. (Middle) Pedigree of the CAH index patient (black circle). (Bottom) Gene diagram.

Figure 2 Multiple sequence alignment of P450c21 amino acid sequence with P450c17, P450 2B4, P450 2C8 and P450 2C9. Sequences of P450 2B4, 2C8 and 2C9 used in alignment were taken from the crystal structures of these proteins in the RSCB protein structure database (<http://www.rcsb.org/pdb/>) and include N-terminal modifications engineered to facilitate expression in bacteria and solubilization for purification and crystallization of these proteins. The PDB identification numbers for the structures are given in parentheses. Sequence alignment was performed with CLUSTAL W (10) and edited with GENEDOC. Amino acid residues involved in heme binding (Cys424), stabilization (Arg91, Arg124, His365 and Arg426) and mutations for which experimental evidence was not available are indicated with*. All numbering is according to P450c21 amino acid sequence. The dark-shaded regions represent the amino acids conserved across all five P450s in alignment and lighter shades represent lesser levels of similarity. Despite major differences in amino acid sequence among various P450 family members, the necessary structural features needed for heme and redox partner binding are well conserved.

structures showed that our model for P450c21 had good overall structural parameters and compared well with the crystal structure of P450 2B4 that was used as the template for our model (Table 1). All cytochrome P450 proteins have a heme ligand in the center attached covalently with a cysteine. This cysteine group is well conserved in all P450 proteins (Fig 2); Cys 428 acts as heme linker in P450c21, whereas Cys 436 has this role in P450 2B4 (7, 8). The heme group is stabilized by at least four amino acids forming coordinate linkages with the propionate side chains of the heme. Some of these amino acids (mostly arginines) are conserved among the members of a particular P450 family but, in general, arginine, histidine and tryptophan residues are involved in the stabilization of heme propionates groups in all P450 proteins. Some of these conserved arginines are also involved in the interaction

with POR and form a chain for electron transfer from POR to the heme ligand. In P450 2B4 these residues are Arg 98, Arg 125, His 369, Ser 430 and Arg 434. An arginine residue (Arg 133/Arg 125 in P450 2B4) characteristic of family 2 of P450s is found on the N-terminal helix that is involved in interaction with POR as well as stabilization of heme propionate. This arginine is well conserved in 2B4, 2C8 and 2C9 and Arg 133 is found to have this role in P450 2B4 open configuration, while Arg 125/Trp 121 stabilizes one of the heme propionates in the closed structure of P450 2B4 (8); in P450c21 this arginine group is absent, although another arginine at position 124 in P450c21 lies $<3 \text{ \AA}$ to the heme propionate and might have the same role as Arg 125/133 of 2B4. Other heme propionate ligands in P450c21 are Arg 91, His 365 and Arg 426 (Arg 98, His 369 and Arg 434 in 2B4)

Table 1 Validation parameters for the 3D model of P450c21 compared with the crystal structure of the template (P450 2B4). Model structures were evaluated with the program WHATIF (13, 16), crystal structure of P450 2B4 was evaluated by the WhatCheck report (14) and RMSD calculations were performed with DALI (20, 21).

	P450c21 model	P450 2B4 (1SUO)
DALI RMSD value	0.93 Å	—
Ramachandran Z score	0.424	0.440
RMS Z score for bond angles	0.983	0.836
RMS Z score for bond length	0.777	0.689
Improper dihedral RMS Z score	0.757	0.424

that are conserved across P450 s and other potential coordination linkages are likely to be Thr 295 and Leu 288.

Catalysis by P450 proteins is carried out through the central heme group, where oxygen binding takes place and P450 substrates are positioned to interact with heme iron for the hydroxylation reaction. A number of structural features of P450 proteins are needed to carry out a series of steps involving interaction of substrate to P450 and transfer of electrons from POR to P450 through positively charged groups (mostly arginines) on P450 (25), some of which are linked to heme (26). Mutant 161X results in a truncated P450c21 protein that does not have any of its structural features required for activity and will neither bind nor metabolize substrates (Fig. 3A). It lacks structural features for holding substrates and is unable to accept electrons from POR. Mutation of Q318X also results in a truncated protein which—although larger than 161X protein—lacks heme and substrate binding regions required for P450c21 activity (Fig 3B). This protein cannot form a complete P450 structure and will be inactive.

We analyzed some P450c21 mutants that have been identified in patients but have not been tested for their enzymatic activity in an *in vitro* system (Fig. 3D). Mutants G292S, L300F, R408C and G424S are reported in patients with classical forms of SW or SV CAH similar to other mutations with an identical phenotype which were tested to have <10% of normal activity (2,27–29). Two more mutations with unknown *in vitro* activity are S301Y and R341W for which clinical manifestation as NC, late-onset CAH suggests reduced enzymatic activity (28,30). All these mutations are in positions that are well conserved among all P450 structures that we analyzed (Fig. 2). Mutations G292S, L300F and S301Y are in the region of P450c21 that interacts with POR and is important for electron transport. A change in topology that affects POR interaction might affect enzymatic activity in case of G292S and L300F mutations. The serine at position

301 is unique in P450c21 and a change to tyrosine might result in a different charge pattern adversely affecting the electron transfer system, although such an effect is likely to be a milder one. The mutation R341W will result in altered interaction with POR and a defective electron transfer. This arginine is conserved among all P450 proteins and in P450c17; an R347H (corresponding to R341 in P450c21) mutation has been reported to affect only 17,20 lyase activity of P450c17 (31). From the analysis of the P450c21 model, it appears that the R341W mutation may have reduced activity. The mutant R408C is likely to interfere with proper folding of the protein by the formation of an intra-molecular disulfide bond with C428 that is required for heme binding or, alternatively ligating to a heme molecule itself that would create a sterically unfavorable environment for heme binding to C428 and would result in a partially inactive protein. Mutation G424 is very close to the heme binding pocket (<4 Å) and a change to serine is likely to interfere with coordination linkage of the heme propionate group to R426.

Discussion

Over 80 different mutations of the *CYP21A2* gene causing CAH have been described. Most mutations result from false recombinations between the normally active *CYP21A2* gene and the inactive *CYP21A1* pseudogene (24). False recombinations consist of either unequal crossing over during meiosis, which result in net deletions of *CYP21A2*, or apparent gene conversions transferring deleterious mutations that are normally present in the *CYP21A1* pseudogene to *CYP21A2*.

The patient with CAH described here suffered from severe deficiency of mineralocorticoids and glucocorticoids due to the absence of 21OHase activity. She was found to be compound heterozygous for the mutations Q318X and 962_963insA of the *CYP21A2* gene. Q318X is a common mutation of the *CYP21A2* gene formed by gene conversion between the *CYP21A1* pseudogene and the active *CYP21A2* gene causing a complete loss of 21OHase activity (3). Thus, Q318X is frequently found in patients with SW CAH (1).

In contrast, the 962_963insA mutation has not been described previously. Rarely, insertions cause 21OHase deficiency (Table 2) (28,32–35). Reviewing the literature, we found a total of four different insertions of a single nucleotide among all known *CYP21A2* mutations. The insertion of a thymine at position F306 in exon 7 seems to be the most common insertion (Table 2). This mutation may be found alone or in a cluster with other mutations but was never found in homozygous form (28,32,33). Clustering of the pseudogene-derived mutations in exons 7 and 8 (V281L-F306insT-Q318X-R356W) seems to be more frequent in the Dutch population (28). The 995_996insA

Figure 3 Molecular modeling of the human P450c21 protein. The 3D model of human P450c21 protein was constructed using the program MODELLER and was based on the crystal structure of mammalian P450 2B4 that shares 50% sequence similarity with P450c21 amino acid sequence. We used an inhibitor bound form of the P450 2B4 structure (1SUO) as a template for our model of P450c21. (A) Model of P450c21, amino acids 31-161 are depicted in green and the missing part of the P450c21 structure in 161X truncated protein is shown in red. The central heme ligand is in dark blue and cysteine 428 that links to heme iron is shown in yellow as CPK spheres. (B) Model of P450c21 with amino acids 31-318 in green and the missing part of the structure in 318X mutant depicted in red. The cysteine 428 that binds to the central iron of the heme the group is found on the missing part of the structure in the truncated protein. (C) Known nonsense mutations of P450c21 depicted in the protein model. These mutations create premature stop codons in the P450c21 sequence and result in a P450 protein without a heme binding site (Cys 428) and many of them also lack other structural features due to truncations as early as aa 74. None of these proteins can either bind nor metabolize its substrates. The P450c21 protein is depicted as a ribbon model. The central heme group is shown in blue, cysteine 428 in grey and nonsense mutations are shown as red CPK spheres. (D) Reported missense mutations of P450c21. A ribbons diagram model of P450c21 protein is shown with the central heme group (blue) and cysteine 428 (yellow) as CPK spheres. Mutations with <10% of normal activity are shown in red, 10–60% activity in blue and 100% activity in green; mutations with unknown *in vitro* activity are shown in yellow.

mutation in exon 4 was found in the compound heterozygous form in one female patient with SV CAH (28). The H28insC mutation in exon 1 was found homozygote in one Brazilian girl whose parents were consanguineous (35). This H28insC mutation leads to a stop codon at amino acid 78 and thus to a complete loss

of 21OHase activity. Similarly, the 64insT mutation in exon 1 was described in the homozygous form in a boy of non-consanguineous parents from a rural area in Spain and leads to a frameshift generating a stop codon in exon 2. In addition to these four single nucleotide insertions, insertion of 16 nt after codon V397 has been

Table 2 Genotype and phenotype correlations in patients with *CYP21A2* mutations including the insertion of a nucleotide.

Reference	Allele 1	Allele 2	Phenotype						Prader stage
			XY, males			XX, females			
			SW	SV	NC	SW	SV	NC	
34	64insT	64insT	1						
32	I172N-CL6-F306insT-Q318X	CL6				1			4
32	CL6-V281L-F306insT-Q318X-R356W	Q318X-R356W				1			4
32	Del	F306insT-Q318X-R356W				1			4
32	V281L	CL6-F306insT-V281L		1					
28	CL6	V281L-F306insT-Q318X-R356W				1			n.s.
28	I2G	F306insT	1						
28	I172N	V281L-F306insT-Q318X-R356W				1			n.s.
28	1123delC	V281L-F306insT-Q318X-R356W				2			n.s.
28	995_996insA	del/con					1		n.s.
33	10 bp del	F306insT				1			n.s.
35	H28insC	H28insC				1			3
*	Q318X	962_963insA				1			5

SW, classic salt wasting; SV, simple virilizing; NC, non-classic; CL6, cluster containing three mutations in exon 6 (I236N, V237E, and M239K); n.s., not specified. *Index patient described in this paper.

reported in a Chinese CAH patient (33), and two complex mutations combining insertions with deletions have been identified as C168delTGinsA (36) and R482delGGinsC (37). In general, all reported insertions created frameshifts, which resulted in truncated and thus functionally inactive P450c21 proteins. This is also true for the newly identified 962_963insA mutation, which leads to a stop codon at aa 161 of the P450c21 protein.

For structure–function studies we generated a new model for the P450c21 protein. Some earlier 3D models of P450c21 have been described but these were based on structures of bacterial P450s (38, 39). Mammalian type 2 P450 proteins that are localized in the endoplasmic reticulum are dependent on a separate protein known as NADPH POR for the supply of electrons (40). P450 reductase is a flavoprotein that transfers electrons from NADPH to the heme ligand of cytochrome P450s via FAD and FMN moieties that are part of POR. This interaction is achieved via arginine and lysine residues on P450s and aspartic and glutamic acid residues on POR. In contrast, bacterial type 2 P450 BM3, on which the previous models were based, has an in-built flavo-protein electron transfer domain (41, 42). Recent advances in crystallization and structure determination of P450 proteins has led to the availability of seven mammalian P450 structures in the PDB database, namely P450 2A6, 2B4, 2C5, 2D6, 2C8, 2C9 and 3A4. Our model is based on rabbit P450 2B4, a mammalian P450 protein whose structure has recently been solved (7, 8) and which shares better sequence homology with P450c21 than

bacterial P450 proteins. Moreover, P450 2B4 has been the subject of extensive site-directed mutagenesis studies that provided information not only about active site composition but also POR-P450 interaction (26). We have analyzed our model based on P450 2B4 and human P450c17, which is a closely related enzyme and participates in the catabolism of cholesterol metabolites such as P450c21. Our new model is based on a closely related mammalian P450 that has both open and substrate bound forms of the structure available. Recent structural studies of P450 proteins have demonstrated dynamic structural rearrangements in the P450 structures after substrate binding that also affects the way a P450 might interact with its electron donating partners (43). Structure–function prediction based on our model and sequence alignment allows us more accurately to predict the role of various amino acids in P450c21 protein that take part in heme binding and interaction with POR. This will also help in analyzing mutations that are reported to cause CAH, since we can predict the role of individual amino acids in P450c21 for its enzymatic activity with better accuracy.

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