Structure–phenotype correlations of human CYP21A2 mutations in congenital adrenal hyperplasia

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Mutations in the cytochrome p450 (CYP)21A2 gene, which encodes the enzyme steroid 21-hydroxylase, cause the majority of cases in congenital adrenal hyperplasia, an autosomal recessive disorder. To date, more than 100 CYP21A2 mutations have been reported. These mutations can be associated either with severe salt-wasting or simple virilizing phenotypes or with milder nonclassical phenotypes. Not all CYP21A2 mutations have, however, been characterized biochemically, and the clinical consequences of these mutations remain unknown. Using the crystal structure of its bovine homolog as a template, we have constructed a humanized model of CYP21A2 to provide comprehensive structural explanations for the clinical manifestations caused by each of the known disease-causing missense mutations in CYP21A2. Mutations that affect membrane anchoring, disrupt heme and/or substrate binding, or impair stability of CYP21A2 cause complete loss of function and salt-wasting disease. In contrast, mutations altering the transmembrane region or conserved hydrophobic patches cause up to a 98% reduction in enzyme activity and simple virilizing disease. Mild nonclassical disease can result from interference in oxidoreductase interactions, salt-bridge and hydrogen-bonding networks, and nonconserved hydrophobic clusters. A simple *in silico* evaluation of previously uncharacterized gene mutations could, thus, potentially help predict the often diverse phenotypes of a monogenic disorder.

structure–phenotype correlation | molecular modeling | CYP21A2 structural analysis

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder, with an overall incidence of 1:15,000 live births worldwide (1). Up to 95% of cases arise from mutations in the cytochrome p450 (CYP) 21A2 (*CYP21A2*) gene, which encodes the enzyme steroid 21-hydroxylase. Classical CAH is characterized by a complete or near-complete loss of enzyme activity, which leads to salt-wasting (SW) or simple virilizing (SV) CAH. SW CAH patients display impaired cortisol synthesis, signs of hyperandrogenemia, and features of aldosterone deficiency. In contrast, in SV CAH, patients have sufficient aldosterone to maintain sodium homeostasis. However, because of the shunting of cortisol precursors through the adrenal androgen biosynthetic pathway resulting in the formation of testosterone and dihydrotestosterone, patients display signs of hyperandrogenemia with impaired fertility. Female newborns in both SW and SV forms of CAH have ambiguous genitalia.

Nonclassical (NC) CAH is observed in 1 in 100 subjects in a heterogeneous population, for example, in New York City, with patients generally displaying a mild and variable phenotype because of a ~20–60% retention of 21-hydroxylase activity. Ethnic-specific frequency indicates that among Eastern European Jews, 1 in 27 subjects have NC CAH, making it the most frequent autosomal disorder in humans. The newborn females do not have ambiguous genitalia, whereas females postnatally often exhibit hirsutism, oligomenorrhea, and impaired fertility. In males, hyperandrogenism is difficult to detect and often only comes to attention in adulthood owing to short stature and impaired fertility (2).

CYP21 genes are a part of the RP-C4-CYP21-TNX module and are located within the major histocompatibility complex on

chromosome 6 (3). *CYP21A2* is present 30 kb downstream of its nonfunctional pseudogene *CYP21A1*. Importantly, however, the two genes share 98% nucleotide homology in the exonic regions and 96% nucleotide homology in the intronic regions (3). Common *CYP21A2* mutations arise from gene recombination with *CYP21A1*, gross gene deletions, and point mutations (4). However, about 5% of CAH patients do not harbor mutations arising from recombination. From our own work over the past three decades, and from the work of others, over 100 *CYP21A2* mutations have been cataloged. In most cases, genotypes and phenotypes are correlated (5). However, in rare instances, genotype–phenotype discordance has been reported (6).

We present data demonstrating that a molecular characterization of *CYP21A2* mutations can be used to predict clinical phenotype and disease severity based upon changes it brings in the structure of the enzyme, steroid 21 hydroxylase. Previously, molecular models of the human CYP21A2 protein structure have been constructed using bacterial CYP102A1 (7) and rabbit CYP2C5 (8) as templates. Structural analyses of CYP21A2 mutants have been performed using these two templates (9–15). Other templates, such as the human CYP2C8, were also used as homology models of CYP21A2 (16).

Recently, the bovine CYP21A2 crystal structure has become available (17). We have, thus, constructed a humanized model of CYP21A2 and mapped disease-causing mutations to investigate the structural role of all known mutations in *CYP21A2*. Here, we provide full structural explanations for clinical manifestations observed in patients of CAH with varied mutations. We also highlight structural criteria that might allow the prediction of clinical severity through such *in silico* modeling.

Results

Humanized Model of CYP21A2. Bovine and the human sequences share 79% sequence identity. The human model of CYP21A2 was constructed by aligning bovine and human sequences and extracting structural features from the bovine template (17). An alignment of six cytochrome P450 proteins involved in steroid biosynthesis was also carried out to identify conserved structural elements (Fig. S1).

The derived human CYP21A2 model exhibits the characteristic triangular prism shape, containing 16 helices and 9 β -sheets (Fig. S2). All conserved structural motifs and elements are canonical to steroid-synthesizing cytochrome P450 proteins. A single heme prosthetic molecule is positioned centrally and is surrounded by B'-C loop, I-helix, K-K' region, and a Cys-pocket. The structural elements that enclose heme in a closed binding cavity are the most conserved. There are 23 amino acid residues that lie

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causing SW CAH. Similarly, the gate of the distal substrate-binding site is flanked by G64, which, when mutated to a glutamate with a longer side chain, prevents substrate entry causing SW CAH.

Impairment of structural stability. The loss of hydrophobic interactions of CYP21A2 impairs protein stability and the organization of its secondary structures, resulting in an inactive enzyme and SW CAH. Because residue Y59 is located in a hydrophobic pocket formed by residues from the A-helix and the β 1- and β 2-sheets, its mutation to asparagine disrupts the hydrophobicity of this region resulting in loss of function (Fig. 1B). Hydrophobicity is also disrupted when V139, a D-helix conserved residue, upon its mutation to glutamate, fails to interact with residues V440 and L436 on the L-helix. Furthermore, charge repulsions between the side chain of mutated V139E and E437 of the E-helix render the protein unstable and inactive causing SW CAH (Fig. 1B). Similarly, residue P386, located in a very hydrophobic pocket on the β 7-sheet, when mutated to the polar and bulkier residue arginine, will cause the disruption of hydrophobicity and protein instability. Disruption of π - π stacking among the interacting residues W302, F306, and F404 by their mutation into nonaromatic residues, such as in W302R/S and F404S, prevents stable packing interactions (Fig. 1B), resulting in SW CAH.

Protein inactivation can also occur when conformational flexibility is impaired, mostly because of the introduction of bulkier residues. For example, the sharp fold between the E- and F-helices, where G178 is located, can only accommodate the smallest residue. Even the mutation of glycine to alanine will hinder the flexibility of this fold. Similarly, G375 is present on the turn of β 5- β 6 hairpin. Its mutation to a more rigid residue, serine, will likewise impair flexibility of the fold, rendering the protein unstable. The mutation T450P likewise reduces flexibility of β 8-sheet, which helps stabilize the very long C-terminal loop. Structural elements in CYP21A2 are also maintained in position by a set of hydrogen bonds between the C-terminal residue Q481, Q318 (J-helix), and backbone carbonyl oxygens of L446 and Q447 (L-helix). When Q481 is mutated to proline, destabilization of the structure renders the protein inactive (Fig. 1B). Other substitutions where leucine is mutated to proline, such as in L142P (D-helix), L166P (E-helix), L167P (E-helix), L261P (H-helix), and L446P (L-helix), also cause helical disruption and destabilization of secondary structures. This is because the respective leucine residues are positioned in the middle of the helices.

Finally, the mutation of residues that form salt-bridge interactions and assist in maintaining tertiary structural elements of CYP21A2 also lead to SW CAH (Fig. 1C and Table S2). R366 (β 4-sheet) forms a salt bridge with D111 (β' -C loop), and its mutation to cysteine will disrupt this salt-bridge interaction, causing protein inactivation (Fig. 1C). In contrast, R354 hydrogen bonds with the side chains of E351 and R408 (E-R-R triad) and with the backbone atoms of P401 and W405 (K' -L loop). Mutation of R354 will, therefore, disrupt a network of hydrogen bond interactions and destabilize the protein (Fig. 1C). R408C/L mutations would likewise destabilize structural elements because of the extensive loss of hydrogen bonds.

Mutations Causing SV CAH. SV CAH is observed in 25% of classical CAH cases and can be mapped to two sets of structural aberrations that cause up to a 98% reduction of CYP21A2 activity: alterations in the transmembrane region and changes to conserved hydrophobic patches.

The membrane anchoring helix must enter the hydrophobic endoplasmic reticular membrane to allow for CYP21A2 to be active. Mutation of a semiconserved helix residue, A15, to a polar threonine prevents optimal anchoring, thus resulting in a gross reduction but not abrogation of enzyme activity and causing SV CAH (compare with SW CAH, above). Proximal to the anchoring helix, is residue H38, which lies in the loop between the N terminus and the A-helix and hydrogen bonds with Y47. When mutated to a nonpolar leucine, hydrogen bonds are disrupted resulting in SV CAH.

Hydrophobic patches regulate inter- and intraprotein interactions (22), and their disruption in several mutations causes a near loss of enzyme activity and SV CAH. First, the highly conserved residue I77 sits in a hydrophobic pocket formed with residues from L71 (β 2-sheets), M81 (B-helix), A391 (β 4-sheet- K' -helix), L388 (K' -helix), L419 and C423 (loop between K' -L helices). Its replacement by a polar residue, such as threonine, disrupts the hydrophobic environment (Fig. 2A). Second, the conserved residue C169 makes hydrophobic interactions with the loop between E-F helices and F-helix. Its mutation to arginine alters the region's hydrophobicity. Third, the mutation of I172, which forms a hydrophobic pocket with M186 and M187 residues of F-helix, to asparagine causes a loss of the hydrophobic pocket. Fourth, hydrophobicity is also lost when interactions between V281, present on the I-helix, and the H-helix are disrupted in the V281G mutation. The helix is also rendered unstable due to the high conformational flexibility imparted by glycine. Finally, L363 and A434 lie very close to heme and contribute to the hydrophobic environment of the heme-binding site (compare with SW CAH). Although mutations L363W and A434V conserve this hydrophobicity, they cause steric clashes with the heme because

Fig. 2. Structural determinants of CYP21A2 mutations causing SV and NC CAH. (A) I77 and I317, which are surrounded by hydrophobic residues. Mutations of I77 or I317 (cyan sticks) result in disruption of hydrophobicity. Whereas mutation I77T results in SV CAH, L317M/V causes NC CAH (because valine and methionine are both hydrophobic residues that can be tolerated at this position). (B) Positively charged residues that have been implicated in the binding of oxidoreductase. Mutation of these residues causes NC CAH. All of these residues are present on the same face of CYP21A2. (C) Disruption of salt-bridge and hydrogen bonding interactions that result in localized destabilization of structure causes NC CAH. Salt-bridge interactions between R149-E162 (Ca), R479-E163 (Cb), R136-D407 (Cc), R435-E431 (Cd), R483-D322 (Ce), and hydrogen-bonding network between G35-H62 and G67 (Cf).

of replacement by a bulkier residue. Thus, a near-complete rather than a complete loss of enzyme activity ensues.

Other mutations that alter the charge on and/or the size of individual residues can result in steric clashes and conformational instability within certain parts of the CYP21A2 molecule. These relatively localized alterations can cause grossly reduced enzyme activity and SV CAH. For example, E320 is a highly conserved residue on a negatively charged Glu-Glu-Leu-Asp (EELD) motif (22, 23). In the E320K mutation, a change from acidic to basic residue alters the charge on the motif. Likewise, replacement of R408 with histidine, when the latter is not protonated, prevents normal hydrogen bonding with E351 and R354. Another residue P432 lies adjacent to the loop that contains invariant C428, which binds heme and P432 to optimally position the loop. Its mutation to leucine makes the structure more flexible and prevents cysteine from being presented to heme. In contrast, the mutation of G424 to serine imparts rigidity to the loop between K'- and L-helix. Examples of steric hindrance include the L300F and L308F mutations. Both residues are present in a very constricted space on I-helix, so that their mutation to a bulky phenylalanine causes localized destabilization of secondary structure. Finally, P463 mutation to leucine interferes with the conformation of the β 8- β 9 loop with the subsequent closure of substrate entrance channel.

Mutations Causing Nonclassical CAH. The following mutation-induced aberrations in protein structure significantly reduce CYP21A2 activity (2).

Oxidoreductase Interactions. The oxidation of steroidogenic substrates by CYP21A2 requires delivery of two electrons from P450 oxidoreductase (POR). Both charged and hydrophobic residues on the proximal surface of cytochrome P450 are involved in interaction with POR (24). The basic residues on the surface of CYP21A2, namely K121, R132, R341, R339, and R369, are known to interact with the acidic residues on POR (8, 17). All of these positively charged residues are also aligned to one face of the protein (Fig. 2B). When these residues are mutated (K121Q, R132C, R339H, R341W/P, and R369Q), the positively charged side chain of the interacting residue is lost. The strength of interaction with POR is affected, which results in mild, NC CAH.

Salt-bridge and hydrogen-bonding networks. CYP21A2 has many hydrogen bonds and salt bridges that stabilize the overall tertiary structure of the protein. Mutations of nonconserved residues participating in these interactions mainly cause NC CAH (Fig. 2C). For example, H62 hydrogen bonds with the backbone of G35 (adjacent to the N-terminal loop) and side chain of G67 (β 2-sheet). Substitution of histidine by hydrophobic leucine (H62L) may disrupt hydrogen bonding to reduce, but not eliminate, enzyme activity. Likewise, R149C, D407N, E431K, R435C, D322G, R479L, and R483Q/P mutations correspond to changes in residues that contribute in salt-bridge formation, including R149 (D-helix)-E162 (E-helix), R316 (J-helix)-D407 (K'-helix), E431 (L-helix)-R435 (L-helix), R479 (β 9)-E163 (E-helix), and R483 (C-terminal)-D322 (J-helix). When these residues are mutated, salt-bridge formation will be prevented resulting in a localized, as opposed to global, destabilization of tertiary structure (Table S2). Additionally, the introduction of a bulkier side chain than the native residue causes NC CAH. Thus, the mutations H119R, R124H, T168N, I194N, I230T, V249A, A265V, S301Y, A265C, V304M, and N387K show side-chain steric clashes with the neighboring residues.

Nonconserved hydrophobic clusters. The introduction of a single polar residue in a hydrophobic cluster will obliterate its hydrophobicity and destabilize the local environment. For example, I171 is located in a hydrophobic region, and its mutation to asparagine will disrupt the hydrophobic cluster. Similarly, P459 sits in a hydrophobic pocket, surrounded by V305 and L308 (I-helix), L458 (β 8- β 9 loop), and M473 and P475 (β 8- β 9 loop). P453 is likewise positioned in a hydrophobic pocket surrounded by L308 (I-helix), L452 (β 8-sheet), L458 (β 8- β 9 loop). Furthermore, A391 sits in a hydrophobic pocket, surrounded by L388, L419, and I77. Mutations A391T, P453S, and P459H will all disrupt

hydrophobicity of their respective regions. In contrast, L317 is present in a region with tight packing in a hydrophobic pocket surrounded by a leucine cluster formed by L307 (I-helix); I313 (J-helix); L321 (loop between J-K helices); and L344, L345, L442, and L446 (K-helix). Its mutation to a methionine or valine disrupts the optimal packing of side chains but does not alter the hydrophobic environment.

Other mild mutations. Certain mutations result in mild disease, because the mutated residues are either reasonably well tolerated or the disrupted interactions are compensated by other residues. These mutations, therefore, variably reduce, but do not grossly lower or eliminate, enzyme activity. For example, P30 is present near the transmembrane region and, along with P31 and P34, forms the hinge to orient protein away from membrane. Its mutation to leucine, a hydrophobic residue, is tolerated and only somewhat reduces enzyme activity. Residue V281 is present on the I-helix in a very constricted space. When mutated to leucine, the increase in chain length results in steric clashes that are relatively modest and, thus, cause mild NC CAH. Although the mutation Y47C disables hydrogen bonding with H38, the interaction is compensated by a weak His-Cys interaction, similar to that observed in glutamine amidotransferases (25). Mutation R224W is mild because tryptophan can still interact with the phosphate head group through its nitrogen atom (26). Although another mutation R233G/K may prevent R233 from binding to the 3-keto oxygen of the proximal 17OHP in the proper orientation, it does not influence protein activity significantly, resulting in minimal phenotype.

Structural Implications of Previously Uncharacterized Mutations.

In Fig. 3, we document structural aberrations in previously uncharacterized mutations on the basis of changes that each mutation brings to the humanized model of CYP21A2. The classification of these mutations is based on an exhaustive sequence and structural analysis of all CAH-causing mutations.

Discussion

Although newborn screening for CAH is becoming used increasingly, difficulties regarding prediction of clinical phenotype remain (1). We have used computational modeling to perform an exhaustive sequence-structure-clinical outcome analysis of known and previously unreported missense CYP21A2 mutations as they relate to the phenotype and severity of CAH. We show that this *in silico* approach can predict the extent of loss of function of the steroidogenic CYP21A2 enzyme. With certain mutations, varying degrees of loss of function have been confirmed biochemically by overexpressing the mutated enzyme *in vitro* (27–32), whereas, in other cases, the extent to which the enzyme is disrupted remains unknown.

We find that enzyme activity is completely lost *in silico*, resulting in SW CAH when mutations alter the heme-binding or substrate-binding pocket, disrupt membrane anchoring of the enzyme, or cause gross structural instability by interfering with protein scaffolding. This accounts for ~35% of residues around the heme- and ligand-binding sites. However, mutations that occur near the transmembrane domain or in conserved hydrophobic patches other than the heme- and substrate-binding region result in a near-complete loss of enzyme activity, with less severe, SV disease. In contrast, mutations disrupting nonconserved hydrophobic clusters, salt-bridge interactions, hydrogen bonding, or oxidoreductase interactions or those that are tolerated sterically or compensated by other residues, produce milder, NC disease. Of these, eight are present on the surface, whereas the rest lie within the core of the protein. We, thus, infer that there is overall concordance between the extent and type of structural disruption of CYP21A2 and the clinical phenotype and severity of CAH.

There are, however, several exceptions to this concordance. First, single-point mutations to polar or nonpolar residues can show distinct clinical presentations. For example, R356 (K-helix) is located near the heme-binding site and interacts with Q389 (K'-helix) and Q462 (β 8- β 9) to maintain secondary structural

Fig. 3. Previously unreported CYP21A2 mutations. Previously unreported missense mutations, namely S113Y/F, V305D, F306V, L307V, R316L, L321P, E351K, R366H, G381S, P386L, F404L, R408H, R426P, L433P, and R444P are shown. The location of the mutated residue and the impact each mutation has on protein structure and the predicted clinical outcomes are shown.

elements. Its mutation to hydrophobic tyrosine or proline leads to SW CAH, because hydrogen bonding is completely lost. However, when R356 mutates to glutamine, partial hydrogen bonding may still be preserved, so that the R356Q mutation exhibits SV CAH. Similarly R408C/L mutation causes SW CAH, whereas R408H leads to SV CAH. Likewise, because R408 makes extensive hydrogen-bonding network with E351 and R354 (E-R-R triad), different substitutions may yield distinct clinical phenotypes.

Second, although the analysis presented in this study is based on individual point mutations, compound mutations may produce severe SW disease, even if the individual mutation is relatively mild. For example, the three mutations I236N, V237E, and M239K in *CYP21A2* gene, which arise from homologous recombination between the *CYP21A1* pseudogene and *CYP21A2*, occur as a cluster in the same patient. When individually mutated to asparagine, I236 does not significantly disrupt structure. Similarly, although the M239K mutation causes charge repulsions between K239 and H235 side chains, it does not disrupt the structure significantly. However, the V237E is highly disruptive:

it modifies the hydrophobic region and alters conformation of the substrate-binding site, resulting in loss of function of the enzyme. Thus, compound mutations of these residues, namely I236N, V237E, and M239K, cause SW CAH.

Finally, because CAH is a recessive disorder, phenotype can vary between homozygous and heterozygous mutations. L166 is present in the middle of the E-helix. Similarly to L167P mutation, L166P can lead to SW, because proline in the middle of the helix destabilizes the structure. However, L166P has been observed as NC CAH in a patient with V281L mutation on the other allele. The clinical outcome in this case may be attributable to the V281L mutation, which is, in itself, reported to exhibit NC CAH phenotype. Thus, although our analysis predicts the severity of a given mutation in CAH, variations can be observed when compound and heterozygous mutations are under consideration.

Even with the above caveats, the results of our analysis of 113 missense mutations in terms of changes they bring to the structure of CYP21A2 is compatible with our observations in clinical practice. Indeed, many of the phenotypes of the mutation have been well ascertained by experienced clinicians.

Monogenic disorders, such as CAH, Gaucher, and many others, are often characterized by diverse clinical phenotypes, toward which there are oftentimes no ready explanations. Particularly with the advent of tools through which fetal DNA from maternal plasma can be sequenced, it is now possible to obtain genotype information very early during gestation. This *in silico* computational approach may help clinicians to attribute a phenotype, hence facilitating prenatal therapeutic decisions.

Methods

The amino acid sequence for human CYP21A2 was taken from UniProt (accession no. P08686). The sequence from the bovine CYP21A2 crystal structure [Protein Data Bank ID code 3QZ1 (17)] was extracted and aligned with the human sequence using ClustalX (33) (overall sequence similarity, 79%). The sequence conservation in the heme-binding cavity, between human and bovine, is 95%. The alignment revealed that the initial 28 residues at the N-terminal did not have a corresponding structural template and were, therefore, built separately.

Models of human CYP21A2 were generated using MODELLER version 9.10 (34, 35), and stereochemical properties evaluated using the program PROCHECK version 3.5.4 (36). The final model was chosen based on low-energy function and a low α rmsd overlap between the bovine template and the humanized model. The model was constructed with the spatial position of heme and ligands retained in their respective binding sites. This was based on the assumption that the ligands and cofactor interact in

a similar manner in the human as in the bovine crystal structure; this comparison replicates all conserved interactions from the template into the model. Several rounds of energy minimization were carried out to obtain a final low-energy conformation with no steric clashes between side chains. The final α rmsd between the bovine template and the humanized model was 0.22 Å (Fig. S4).

The first 28 residues corresponding to the N-terminal region including the membrane-anchoring α -helix were constructed after secondary structure prediction using PSIPRED (37). Residues 1–25 were modeled in a canonical α -helical conformation, whereas residues 26–28, which connect the anchoring transmembrane helix to the first residue in the model, were built in a random-coil conformation using MODELLER. The orientation and position of the model, with respect to the lipid bilayer, was obtained using the membrane self-assembly coarse-grained dynamics protocol by Cojocaru et al. (38). This permits unbiased orientation of the protein in the membrane, which is not achievable via atomistic simulations. At the end of 1 μ s, the coarse-grained model was converted to atomistic resolution using the reverse-conversion protocol (39) and further subjected to restraint-free molecular-dynamics simulations to test the stability of the model (SI Methods). The molecular graphics images were generated using PyMOL (www.pymol.org).

Mutations were identified from the Human Cytochrome P450 Allele Nomenclature database (www.cypalleles.ki.se). Redundancies were removed, and clinical conditions were cross-evaluated with respect to the original publications (Table S1).

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