Characterization of Disease-related 5β-Reductase (AKR1D1) Mutations Reveals Their Potential to Cause Bile Acid Deficiency

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Bile acid deficiency is a serious syndrome in newborns that can result in death if untreated. 5β-Reductase deficiency is one form of bile acid deficiency and is characterized by dramatically decreased levels of physiologically active 5β-reduced bile acids. AKR1D1 (aldo-keto reductase 1D1) is the only known human enzyme that catalyzes the reduction of the Δ^4-3-ketosteroid 5β-reductase and catalyzes the reduction of the Δ^4-3-ketosteroid to form the A/B cis ring structure, utilizing NADPH as a cofactor (1). The Δ^4-3-ketosteroid functionality is common to all steroid hormones except the estrogens, and the C4-C5 double bond can be further reduced in a stereo-specific manner. 5α-Reduction of testosterone to 5α-dihydrotestosterone results in increased androgen receptor activation (2, 3), whereas 5β-reduction of progesterone to 5β-pregnane-3,20-dione results in activation of the pregnane X receptor (4) and constitutive active/androstane receptor (5). In bile acid biosynthesis, AKR1D1 reduces Δ^4-cholesterol-7α-ol-3-one and Δ^4-cholesten-7α,12α-diol-3-one to their respective 5β-dihydrosteroid forms (6). The resulting 5β-reduced structure contains a 90° bend in the steroid scaffold that is believed to generate the essential emulsification characteristics of the resultant human bile acids.

Human 5β-reductase deficiency (OMIM 604741) was first diagnosed by Setchell et al. (7) in siblings with neonatal hepatitis and cholestasis. Since then, more than 20 cases have been reported, characterized by reduced primary bile acid biosynthesis and accumulation of hepatotoxic Δ^4-3-oxo- and 5α-reduced (allo-) bile acids (8–11). The deficiency can be treated with primary bile acids (12, 13), which normalize liver morphology and return liver function to normal. This treatment serves two functions. First there is feedback repression of the 7α-hydroxylase (CYP7A1), the key regulatory and rate-limiting step in primary bile acid biosynthesis, and this prevents the accumulation of the deleterious Δ^4-3-one precursors and allo-bile acids (14). Second, the natural bile acids administered lead to normal emulsification of fat and absorption of fat-soluble vitamins. Several point mutations in AKR1D1 (L106F, P133R, P198L, G223E, and R261C) have been detected in patients with bile acid deficiency; however, the effects of the observed mutations on enzyme structure-function and whether they are causal in the observed phenotype have remained unclear (8, 10, 15).

Recently, knowledge about AKR1D1 enzyme function increased with the elucidation of its crystal structure in complex with cofactor and different steroid substrates and products (16, 17). These studies revealed that AKR1D1 had an (α/β)h-barrel structure with three large loops (A, B, and C) at the back of the barrel and contained similar cofactor and steroid substrate binding sites compared with other AKR1C enzymes. These enzymes, AKR1C1–AKR1C4, act as 3-, 17-, and 20-ke-tosteroid reductases and have been thoroughly characterized (18–22). The amino acids of the aldo-keto reductase catalytic tetrad, consisting of Asp^50, Tyr^55, Lys^84, and His^117 (numbering according to rat AKR1C9 (3α-hydroxysteroid dehydrogenase)) are highly conserved in AKR1D1. However, substitution of histidine by glutamic acid translates into a functional switch...
Steroid 5β-Reductase Disease Mutants

from ketosteroid reduction (in AKR1C enzymes) to double bond reduction (in AKR1D1) (23).

Examination of the AKR1D1 crystal structure allowed us to map the position of the reported point mutations associated with bile acid deficiency (Fig. 1A). We found that all mutations except for P133R reside in areas highly conserved across AKR1D1 homologs in other mammalian species. However, none of the positions are in direct contact with the catalytic tetrad, cofactor, or substrate binding sites, and therefore it remains unclear whether they could indeed be causal in the reported cases of bile acid deficiency.

In this study, we examined the effects of the observed point mutations on enzyme function following their introduction into wild type AKR1D1. We report the expression, purification, and characterization of AKR1D1-P133R and compare it with the wild type enzyme. Furthermore, although enzymes with the point mutations L106F, P198L, G223E, and R261C could not be purified, we were able to study these enzymes following expression in mammalian cells. Our results indicate that all four point mutations had severe effects on one or more of the following: steady state kinetic parameters, enzyme stability, and amount of soluble protein expressed in cells. In summary, although none of the observed mutations affects catalysis in AKR1D1 directly, all drastically reduced 5β-reductase activity in a biological context. Hence, all AKR1D1 mutations reported in patients with bile acid deficiency and characterized in this study have the potential to cause the observed phenotype of 5β-reductase deficiency.

EXPERIMENTAL PROCEDURES

Materials—The vector pET-16b was purchased from Novagen. The GeneAmp RNA PCR Core kit was purchased from PerkinElmer Life Sciences. Escherichia coli strain C41 (DE3) was provided by Dr. J. E. Walker (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK). The QuikChange II site-directed mutagenesis kit was purchased from Stratagene. Restriction endonucleases were purchased from New England Biolabs. Synthetic oligonucleotides were obtained from Invitrogen. NADPH was obtained from Roche Applied Science. Steroids were purchased from Steraloids, Inc. [4-14C]Testosterone (50 mCi/mmole) was obtained from PerkinElmer Life Sciences. Nickel-Sepharose 6 Fast Flow was purchased from Amersham Biosciences. The bovine serum albumin protein standard was purchased from Sigma. Bradford concentration was measured using the Bradford assay, according to the manufacturer’s instructions, using bovine serum albumin as a standard.

Measurement of Steady State Kinetic Parameters by Spectrofluorimetric Assay—The $k_{cat}$ and $K_m$ values for testosterone and cortisone were determined fluorimetrically as described before (24) with steroid substrate concentration ranging from 0.60 to 40 μM. Kinetic analyses of initial velocities obtained were performed by fitting the data using the program GraFit to the Henri-Michaelis-Menten equation,

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \quad \text{(Eq. 1)}$$

where $v$ is the initial velocity of the reaction, $[S]$ is the molar concentration of the substrate, and $K_m$ is the Michaelis-Menten constant for the substrate. Dividing $V_{max}$ by the molar concentration of the enzyme gave $k_{cat}$. When substrate inhibition was observed, initial velocity data were fit in a similar manner to the following equation,

$$v = \frac{V_{max} \times [S]}{K_m + [S] + [S]^2/K_i} \quad \text{(Eq. 2)}$$

where variables were the same as in Equation 1 with the inclusion of $K_i$, which is the dissociation constant for substrate from the E-NADP$^+$ complex.

Fluorescence Titration with NADPH—The dissociation constant ($K_D$) of the enzyme for NADPH was determined by monitoring the quenching of the intrinsic protein fluorescence upon the addition of cofactor as described (25) with the following changes. Titrations were performed in 10 mM potassium phosphate (pH 6.0) in a 1.4-mI volume at 37 °C using 0.11 μM...
Circular Dichroism Experiments—Circular dichroism spectropolarimetry was performed with wild type (4.8 mM potassium phosphate (pH 7.0) and 1 mM EDTA. Samples and 190-nm wavelength at 22 °C in millidegrees on a Jasco J810 spectropolarimeter in a 0.1-cm cell. A background spectrum was maintained in 10-cm culture dishes at 37 °C and 5% CO2 and then assayed fluorimetrically.

Heat Stability Experiments—To determine the thermal stability of the enzymes, wild type and AKR1D1-P133R were heated for 10 min at various increments from 25 to 50 °C in 10 mM potassium phosphate (pH 7.0). Protein spectra were recorded between a 260- and 190-nm wavelength at 22 °C in millidegrees on a Jasco J810 spectropolarimeter in a 0.1-cm cell. A background spectrum was obtained with only buffer. Following the spectral scans, melt curves were performed on each of the samples at 222 nm by raising the temperature from 0 to 90 °C in increments of 2 °C. A second protein spectrum was recorded for each sample after the melt curve was complete.

Cycloheximide Treatment—Cells were either harvested for RNA expression and analysis of enzyme activity or subjected to cycloheximide treatment.

Reverse Transcription-PCR—RNA was extracted from cells using the RNeasy minikit from Qiagen according to the manufacturer’s protocol. To obtain cDNA, 1 μg of RNA was reverse transcribed with random hexamer primers by use of the GeneAmp RNA PCR core kit (Applied Biosystems). Quality of the cDNA was monitored by PCR amplification of GAPDH (primers 5’-dCAT CTC TGC CCC CTC TGC TGA-3’ and 5’-dGGA TGA CCT TGC CCA CAG CCT-3’). Endogenous AKR1D1 was amplified with a forward primer matching a sequence in exon 7 (5’-dGGG GTG GTT GTC ATT CCT AA-3’) and a reverse primer matching a sequence in the 3’-untranslated region of the gene (5’-dGAC TAC CCA TTG CAC CGT CT-3’). For exogenous AKR1D1 detection, the same forward primer was employed together with a reverse primer that anneals in the cloning vector 3’ of the insert (5’-dAAC TAG AAG GCA CAG TCG AG-3’).

Polyclonal AKR1D1 Antibody—A rabbit polyclonal antiserum raised against full-length purified His-tagged AKR1D1 was produced by ProSci, Inc. (Poway, CA). Antiserum preparation from the first bleed showed the lowest cross-reactivity for the detection of other human aldo-keto reductases and was subjected to further purification to improve specificity against AKR1D1. For this, 50–150 μg of human AKR1A1 and AKR1C1 to AKR1C4 were blotted onto a strip of nitrocellulose membrane. This membrane was incubated with a 1:50 dilution of the antiserum in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) for 1 h at 4 °C. Diluted antiserum was removed, the membrane was stripped for 20 min at 42 °C and washed three times 10 min each with TBS at room temperature, and the diluted antiserum from before was reapplied. This cycle was repeated seven times, and the precleared polyclonal α-AKR1D1 antibody was diluted 1:50 with TBS and then mixed 1:1 with glycerol and 0.02% sodium azide (final concentration) before aliquoting and storage at −20 °C. Enhanced specificity of this antibody for AKR1D1 versus a panel of human AKRs is documented in supplemental Fig. S1.

Western Blot—Cell pellets were washed once in Dulbecco’s phosphate-buffered saline and then lysed by incubation in lysis buffer (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA, 0.5 mM EGTA, 20 mM β-glycerophosphate, 100 μM sodium orthovanadate, 1× proteinase inhibitor mix (Roche Applied Science)) on ice for 30 min with intermittent agitation. Lysates were centrifuged, the soluble fraction was removed, and the protein concentration was measured by a Bradford assay. Eighty micrograms of total protein per sample well were resolved by SDS–PAGE and blotted onto a nitrocellulose membrane. For Western blot development, the membrane was blocked in 5% dry milk in TBST, the polyclonal anti-AKR1D1 antibody was diluted to 1:2000 in 3% dry milk in TBST, the secondary antibody (monoclonal mouse anti-rabbit horseradish peroxidase conjugate, Santa Cruz Biotechnology, Inc.) was diluted to 1:10,000 in 3% dry milk in TBST, and the signals were visualized using the ECL technique (GE Healthcare). To detect the mutants that were poorly expressed, the secondary antibody was diluted 1:100,000 in 3% dry milk in TBST, and the signals were visualized using the SuperSignal West Femto Kit (Thermo Scientific). For the subsequent detection of β-actin, blots were stripped by incubating twice for 10 min in 42 °C Western stripping buffer (Bio-Rad) and washing three times for 10 min each in TBST, and the procedure was then completed as described above. The primary antibody

The abbreviations used are: TBS, Tris-buffered saline; WT, wild type.
Steroid 5β-Reductase Disease Mutants

monoclonal mouse anti-β-actin was applied in a 1:1000 dilution in 3% dry milk in TBST, and the secondary antibody sheep anti-mouse hors eradish peroxidase conjugate was diluted 1:5000 in 3% dry milk in TBST. Detection was achieved by use of the ECL technique (GE Healthcare).

Detection of 5β-Reductase Activity in HEK 293 Cells—Cells were seeded and transiently transfected as described above. At 46 h after transfection, medium was replaced with 2 ml of Dulbecco’s modified Eagle’s medium containing 1% heat-inactivated fetal calf serum, GlutaMAX™, and 1% penicillin, streptomycin per well. Two hours later, testosterone was added in 10 μl of DMSO to yield final concentrations ranging from 0.5 to 20 μM per well. Final concentrations below 10 μM contained 20 nCi, and higher final concentrations contained 30 nCi of 14C-labeled testosterone, respectively. At time points, 200 μl of medium was removed and extracted twice with 2.5 volumes of cold water-saturated ethyl acetate, and the combined organic phase was dried down. Recovery of radioactive material following extraction was >90%.

Product Identification by Thin Layer Radiochromatography—Dried samples were resuspended in ethyl acetate, applied to Partisil LK6D Silica TLC (thin layer chromatography) plates (Whatman International Ltd.), and developed twice in toluene/acetone (80:20 v/v). Radiochromatograms were scanned with an automatic TLC-linear analyzer (Bioscan Imaging Scanner System 200-IBM with AutoChanger 3000, Bioscan (Washington, D. C.)), and the relative percentage of peaks was compared and quantified as nmol of product formed using the specific radioactivity of the isotope assuming that each steroid was determined steady state kinetic constants for the wild type and the AKR1D1-P133R mutant. The specific activity of the homogenous recombinant mutant enzyme with 10 μM testosterone was 24 nmol min⁻¹ mg⁻¹ and was 3-fold lower than wild type enzyme. The catalytic efficiency toward testosterone (0.21 min⁻¹ μM⁻¹) was found to be 12-fold less as reflected by an increase in K_m (12.7 μM) and a decrease in k_cat (2.7 min⁻¹) (Table 1). Interestingly, the substrate inhibition of wild type enzyme by testosterone was not observed with the P133R mutant. By contrast, Δ⁴-3-ketosteroids with longer C17 side chains (e.g. cortisone and Δ⁴-cholen-7α-ol-3-one) showed large decreases in k_cat and K_m. The k_cat of cortisone was decreased 16-fold (0.6 min⁻¹), and K_m was decreased 12-fold (1.3 μM). The same pattern was observed with Δ⁴-cholen-7α-ol-3-one; however, it was not possible to accurately measure the K_m for this substrate because the enzyme was essentially saturated at the lowest concentrations of substrate at which a reaction rate could be reliably measured (Fig. 2). At saturation, k_cat for Δ⁴-cholen-7α-ol-3-one was depressed 7-fold. By contrast, K_m is significantly lower than 0.8 μM, the value for wild type enzyme. Thus, the effect of this mutation was a change from a low affinity, high capacity enzyme to a high affinity, low capacity enzyme.

NADPH Binding and K_d Determination—We investigated whether the P133R mutation affects cofactor binding. AKR1D1 contains 5 tryptophan residues and has intrinsic fluorescence when excited at 295 nm. Incremental addition of NADPH quenched the fluorescence emission signal at 340 nm and generated an energy transfer band at 460 nm (Fig. 3A). This energy transfer band probably results from the interaction of the nicotinamide ring with Trp69 based on identical experiments with AKR1C2 and structural homology considerations (27). Plots of ΔF/ΔF_max versus [NADPH] were fitted to the Morrison equation to obtain the dissociation constant (K_d) of NADPH. Wild

![FIGURE 1. Location of the disease-related mutants in the AKR1D1 crystal structure.](Image)

**TABLE 1** Comparison of kinetic constants of wild type AKR1D1 and the AKR1D1-P133R mutant

| Enzyme - Substrate | k_cat (min⁻¹)* | K_m (μM) | K_i (μM) | k_cat / K_m (min⁻¹ μM⁻¹) |
|-------------------|---------------|----------|----------|------------------------|
| WT - Testosterone | 7.1 ± 1.7      | 15.1 ± 0.3 | 2.63     |
| P133R - Testosterone | 2.7 ± 0.3     | 12.7 ± 2.2 | 0.21     |
| WT - Cortisone    | 9.9 ± 0.1      | 15.1 ± 0.3 | NA       |
| P133R - Cortisone | 0.6 ± 0.02     | 1.3 ± 0.1 | NA       |**

* All activity measurements were determined fluorometrically.
type AKR1D1 displayed a $K_d$ of $43 \pm 16$ nM, whereas the P133R mutant was found to bind NADPH with slightly less affinity ($K_d = 69 \pm 5$ nM) (Fig. 3B). However, our enzyme assays contained saturating concentrations of cofactor, and therefore these small differences in $K_d$ are unlikely to account for the large decrease in catalytic efficiency. Interestingly, in the absence of cofactor, AKR1D1-P133R had greater fluorescence emission (20,500 arbitrary fluorescence units/µg of enzyme) at its $\lambda_{\text{max}}$ (343 nm) than wild type (4,700 arbitrary fluorescence units/µg) at its $\lambda_{\text{max}}$ (337 nm).

Comparison of Overall Structural and Thermal Stability—CD spectra were recorded for wild type and the AKR1D1-P133R mutant, in the presence and absence of NADPH (Fig. 4A) and showed deflections characteristic of a protein formed of both $\beta$-strands and $\alpha$-helices. Interestingly, the spectra of wild type and AKR1D1-P133R did not superimpose, reflecting some differences in $\beta$-strand and $\alpha$-helical content. To examine the differences between the two proteins further, we performed CD melt curves. AKR1D1-P133R had a $T_m$ of 41 °C, which was lower than wild type AKR1D1 with a $T_m$ of 48.5 °C (Fig. 4B). However, in the presence of cofactor, the differences in $T_m$ were attenuated. Activity measurements of wild type and AKR1D1-P133R following heating at incremental temperatures showed a 50% decrease in enzyme activity at 46.5 and 42 °C, respectively (Fig. 4C). At 46.5 °C, less than 5–10% of the activity of the P133R mutant remained, whereas >60% of the activity remained in wild type enzyme. These temperatures corresponded with the $T_m$ determined in the CD melt experiment without cofactor.

AKR1D1 Expression and Protein Stability in HEK293 Cells—Due to the difficulty to obtain purified natural mutants of AKR1D1, we conducted transient transfection studies. HEK 293 cells do not express endogenous AKR1D1 but express mRNA of wild type and mutant AKR1D1 enzymes to similar degrees following transfection (Fig. 5). However, protein levels were not uniform. Wild type AKR1D1 showed the highest amount of expressed protein, followed by AKR1D1-P133R, whereas low levels of expression were observed for all other mutants and were close to the detection limit of the polyclonal antibody. AKR1D1 protein bands were semiquantified by use of densitometry and compared with a standard curve obtained with recombinant AKR1D1. Wild type AKR1D1 was present at about 0.1% of soluble protein in the cell lysate. The relative expression levels of the wild type and mutant AKR1D1 were 1 (WT):0.33 (P133R):0.034 (G223E):0.027 (P198L):0.004 (L106F and R261C).

To investigate whether the low expression levels of mutant AKR1D1 were caused by increased degradation rates, we monitored wild type and mutant AKR1D1 protein levels over time following cycloheximide treatment (Fig. 6). Within 24 h after cycloheximide administration, wild type AKR1D1 levels did not decrease significantly, pointing to a fairly stable protein with a low degradation rate. AKR1D1-P133R and AKR1D1-P198L showed a mild reduction in expression level. AKR1D1-G223E

![FIGURE 2. Comparison of velocity versus substrate plots for wild type AKR1D1 and the P133R mutant using $\Delta^\omega$-cholesten-7α-ol-3-one as substrate. ■, AKR1D1; ▲, P133R. Assays were performed using standard fluorimetric assay conditions. For the wild type enzyme, the experiment was replicated twice, and for the P133R mutant, the experiment was replicated once. Measurements were done in duplicate, and variation was less than 10%. One representative experiment is shown.](image)

![FIGURE 3. Titration of the fluorescence emission spectra of AKR1D1 with increasing [NADPH]. A, emission spectrum of recombinant AKR1D1 excited at 295 nm following the addition of increasing concentrations of NADPH from 0 to 4 µM. A decrease in emission is seen at 340 nm, and an increase in emission is seen at 460 nm. B, plot of $\Delta F/\Delta F_{\text{max}}$ versus [NADPH], wild type (■), and P133R (▲).](image)
decreased significantly within the first 6 h and was no longer detectable 24 h following cycloheximide treatment. AKR1D1-L106F and AKR1D1-R261C were expressed very poorly and degraded within 6 h after cycloheximide treatment. Thus, disease-related mutants of AKR1D1 were associated with poor protein expression and/or stability when expressed in mammalian cells.

5β-Reduction of Testosterone by Wild Type and Mutant AKR1D1 in HEK 293 Cells—Cells transiently transfected with wild type and mutant AKR1D1 were also assayed for 5β-reductase activity using testosterone as substrate. Three of the five mutants displayed detectable but low 5β-reductase activity when expressed in HEK 293 cells. The observed activities in the presence of 0.5 and 5 μM testosterone in relation to the wild type enzyme are shown in Fig. 7. AKR1D1-P133R activity was significantly reduced compared with wild type enzyme, whereas 5β-reductase activity of the AKR1D1-R261C and AKR1D1-L106F mutants was at the limit of detection of the assay and barely observable in incubation times shorter than 24 h. We did not observe AKR1D1-P198L- or AKR1D1-G223E-mediated 5β-reduction even when incubation times were extended to 60 h (data not shown). Although HEK 293 cells did not display any 5β-reduction of testosterone on their own, testosterone metabolism in incubation times longer than 24 h led to accumulation of downstream products, including 5α-dihydrotestosterone, 3α-androstanediol, Δ4-androsten-3,17-dione, and 5α-androstanedione, which severely hampered quantification of residual 5β-reduction associated with the AKR1D1-R261C and AKR1D1-L106F mutants. Therefore, kinetic parameters were only estimated for wild type AKR1D1 (Fig. 7C) and AKR1D1-P133R (Fig. 7D). Measurement of enzyme activity in the HEK 293 cells revealed many of the same
Characteristics as observed for the purified enzymes. HEK 293-expressed wild type AKR1D1 exhibited a $K_m$ of about 4 $\mu$M with testosterone but displayed significant substrate inhibition with a $K_i$ value in the low micromolar range. In contrast, AKR1D1-P133R did not show any substrate inhibition and displayed non-saturable kinetics with a more than 10-fold increase in $K_m$ compared with the wild type enzyme.

**DISCUSSION**

**Characteristics of Observed AKR1D1 Mutations**—Major characteristics of 5β-reductase (AKR1D1) deficiency are an accumulation of the bile acid intermediates (3-oxo-$\Delta^4$ sterols), occurrence of $\Delta^4$-reduced side products (allo-bile acids), and lack of downstream end products (functional bile acids, such as cholic and deoxycholic acid) (7). However, besides defects in AKR1D1 itself, secondary causes that disrupt liver function and bile acid metabolism cannot be ruled out in the presentation of this phenotype (9, 11, 12, 28). In seven patients with 5β-reductase deficiency symptoms, mutations that alter the AKR1D1 amino acid sequence have been identified. Two mutations severely truncate the protein (10, 15), and five mutations result in an amino acid substitution (L106F, P133R, P198L, G223E, and R261C) (8, 10, 15). Although the truncated proteins are considered too short to remain functionally active (29), it is less clear whether the observed point mutations could disrupt homogeneity from E. coli and had detectable activity. The L106F, P198L, and R261C mutants gave very low yields of homogenous protein and/or displayed no detectable activity and accumulated in inclusion bodies, suggesting that in prokaryotic systems, these enzymes are unstable. As a result, the remaining mutants were characterized by mammalian cell expression.

Using the purified AKR1D1-P133R, the mutant was found to have depressed $K_{cat}$ and $V_{max}$ values with bile acid precursors. From these changes in kinetic parameters, it is predicted that bile acid precursors (e.g. $\Delta^4$-3-oxo bile acids) would accumulate and/or be directed to form hepatotoxic allo-bile acids, which is the clinical presentation observed. This change is unexpected in the P133R mutant because, based on structures of AKRs in the Protein Data Bank, this residue is not located in a functional region of the protein. Our studies showed an increase in thermal lability, as judged by CD melt curves and thermal denaturation. The attenuation of thermal lability by low micromolar concentrations of NADPH also suggests that this is not the reason for lower enzyme activity because these concentrations of NADPH are likely to be present in the cell and keep the enzyme in its NADPH-bound stable form. Instead, the enzyme behaves as if an uncompetitive inhibitor is present. It is noteworthy that the P133R mutant abolishes the substrate inhibition observed with testosterone, suggesting that this change...
Steroid 5β-Reductase Disease Mutants

prevents steroid subunit binding in the unproductive binding mode. A ribbon diagram shows the position of this residue in the structure (Fig. 1A), and its role in the non-productive binding of testosterone responsible for substrate inhibition in wild-type enzyme is shown as a perspective diagram in Fig. 1B. The loss of substrate inhibition observed in this mutant could either be due to direct movement of Tyr132 so that it is unable to hydrogen-bond to testosterone as previously observed (14) or due to a change in the tertiary structure of the enzyme. CD spectroscopy scans of wild type and P133R showed apparent differences in overall β-strand and α-helical content, whereas the P133R mutant red-shifted the tryptophan emission from 337 to 343 nm. This bathochromic shift would represent the tryptophans becoming more solvent-accessible (21) and would also be reflected in a greater emission (as observed).

L106F, P198L, G223E, and R261C Mutants—The remaining mutants were transiently expressed in HEK 293 cells for characterization. Transcript levels of wild type AKR1D1 and all five mutants were similarly expressed when measured by reverse transcription-PCR. Wild type AKR1D1 and P133R were expressed well and showed kinetic properties similarly to the homogeneous recombinant enzymes, suggesting that their properties are not affected by this expression system. By contrast, two mutants, L106F and R261C, were expressed more than 100 times lower than wild type AKR1D1 but showed 5β-reductase activity at the detection limit of the assay indicating that 5β-reductase deficiency syndromes here may not be due to defects in catalytic properties but by reduced expression of amounts of active protein. The P198L and G223E mutants may decrease physiological 5β-reductase activity due to a combination of effects on enzyme activity as well as expression levels. These mutants were more highly expressed than the L106F and R261C mutants but displayed no detectable 5β-reductase activity.

Relationship of Genotype to Phenotype—It is noteworthy that a single intact copy of the AKR1D1 gene may be sufficient to ensure proper downstream metabolism from the 3-oxo-Δ4 bile acid intermediates and prevent the development of bile acid deficiency symptoms. In all seven cases of a genetic 5β-reductase defect, heterozygous parents or siblings of the patients are healthy and had no reported liver defects. In one case, the patient heterozygous for the G223E mutation remained healthy without treatment after initial treatment with ursodeoxycholate was discontinued (15). The compound heterozygous twins reported by Gonzales et al. (8) carried mutations that still retained a significantly active (P133R) mutant and a marginally active (R261C) mutant that together may have contributed to milder symptoms that were successfully treated by cholic acid supplementation. By contrast, two of three reported cases with homozygous mutations in AKR1D1 suffered from severe complications that eventually necessitated liver transplantations (10).

Adaptation to Bile Acid Deficiency—The patient with the homozygous mutation in AKR1D1, P198L, responded well to bile acid supplementation treatment and remained healthy under treatment. Surprisingly, a later study by Palermo et al. (30) on this patient revealed that despite discontinued bile acid supplementation, the girl was healthy. Furthermore, despite strongly reduced levels of 5β-reduced steroids in serum the patient did not exhibit any evidence for a clinical condition associated with dysfunctional steroid metabolism. Although we could not demonstrate residual 5β-reductase activity for the P198L mutant, the presence of low but sufficient enzyme activity to yield low levels of 5β-reduced bile acid precursors may be present in this patient. Furthermore, the presence of a yet unidentified enzyme with 5β-reductase activity in addition to AKR1D1 cannot be ruled out.

The record of this patient also indicates that 5β-reductase activity may be most crucial during early childhood when the infant is initially confronted with establishing the uptake of nutrients from ingested food and development and regulation of bile acid metabolism. In the fetus and in newborns prior to infancy, 3-oxo-Δ4 bile acid intermediate levels are high and similar to the levels observed in the patients (31). However, within the first months of early childhood, these levels dropped significantly, indicating up-regulation of bile acid synthesis and offset of 5β-reductase activity. Inability to achieve this regulation, for example due to deficient 5β-reductase activity, may then result in cholestasis and bile acid deficiency syndromes that require supplemental treatment.

In summary, our data together with the history of affected patients indicate that the observed mutations in AKR1D1 can account for bile acid deficiency and liver damage due to reduced physiological 5β-reductase activity. Bile acid supplementation seems beneficial for these patients to surmount these complications in early life, but adaptive responses in later life indicate that supplementation may not be required.

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