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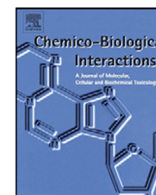

The effect of disease associated point mutations on 5β-reductase (AKR1D1) enzyme function

Rebekka Mindnich [a], Jason E. Drury [a], Trevor M. Penning [a,b,*]

[a] University of Pennsylvania, School of Medicine, Department of Pharmacology, Philadelphia, PA 19104-6084, USA
[b] Center of Excellence in Environmental Toxicology, Department of Pharmacology, University of Pennsylvania, Philadelphia, PA 19104-6084, USA

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ABSTRACT

The stereospecific 5β-reduction of Δ^4-3-ketosterols is very difficult to achieve chemically and introduces a 90° bend between ring A and B of the planar steroid. In mammals, the reaction is catalyzed by steroid 5β-reductase, a member of the aldo-keto reductase (AKR) family. The human enzyme, AKR1D1, plays an essential role in bile-acid biosynthesis since the 5β-configuration is required for the emulsifying properties of bile. Deficient 5β-reductase activity can lead to cholestasis and neo-natal liver failure and is often lethal if it remains untreated. In five patients with 5β-reductase deficiency, sequencing revealed individual, non-synonymous point mutations in the AKR1D1 gene: L106F, P133R, G223E, P198L and R261C. However, mapping these mutations to the AKR1D1 crystal structure failed to reveal any obvious involvement in substrate or cofactor binding or catalytic mechanism, and it remained unclear whether these mutations could be causal for the observed disease. We analyzed the positions of the reported mutations and found that they reside in highly conserved portions of AKR1D1 and hypothesized that they would likely lead to changes in protein folding, and hence enzyme activity. Attempts to purify the mutant enzymes for further characterization by over-expression in *Escherichia coli* yielded sufficient amounts of only one mutant (P133R). This enzyme exhibited reduced K_m and k_{cat} values with the bile acid intermediate Δ^4-cholesten-7α-ol-3-one as substrate reminiscent of uncompetitive inhibition. In addition, P133R displayed no change in cofactor affinity but was more thermolabile as judged by CD-spectroscopy. When all AKR1D1 mutants were expressed in HEK 293 cells, protein expression levels and enzyme activity were dramatically reduced. Furthermore, cycloheximide treatment revealed decreased stability of several of the mutants compared to wild type. Our data show, that all five mutations identified in patients with functional bile acid deficiency strongly affected AKR1D1 enzyme functionality and therefore may be causal for this disease.

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1. AKR1D1: a unique member of the AKR superfamily

Human steroid 5β-reductase (AKR1D1) is a member of the aldo-keto reductase (AKR) superfamily, enzymes that exert important metabolic functions in organisms of all phyla [1]. The superfamily comprises over 150 annotated members that share 20–99% sequence identity. The AKR nomenclature [2] distinguishes 16 families (AKR1-16) based on a minimum of 40% protein sequence similarity. Family members with 60% or more sequence identity are further grouped into subfamilies (e.g. AKR1A-E). Hence, AKR1D1 belongs to the AKR1 family and the AKR1D subfamily.

Despite the observed low sequence conservation between distant members due to high evolutionary diversity, all AKR enzymes share three important characteristics: (1) a conserved protein fold that results in a characteristic $(\alpha/\beta)_8$-barrel structure; (2) a conserved mode of cofactor and substrate binding; and (3) a tetrad of amino acids most important in catalysis: D50, Y55, K84 and H117 (based on the nomenclature of AKR1C9). In fact, although substrate specificity varies significantly among AKRs, including sugar aldehydes, ketosteroids, aflatoxin dialdehydes and polycyclic aromatic *trans*-dihydrodiols, most AKR enzymes catalyze the interconversion of aldehydes and ketones with primary and secondary alcohols. However, AKR1D enzymes are a notable exception. Instead of reducing an aldehyde or ketone group, they catalyze the reduction of double bonds in Δ^4-3-ketosterols in a stereo-specific manner [3,4], and consequently, these enzymes are also known as steroid 5β-reductases. Considering the difference between functionalities of these two enzymes, aldehyde/ketone reduction and double bond reduction, it is remarkable that a

* Corresponding author at: Center of Excellence in Environmental Toxicology, Department of Pharmacology, University of Pennsylvania, Philadelphia, PA 19104-6084, USA.
E-mail addresses: penning@mail.med.upenn.edu, penning@upenn.edu (T.M. Penning).

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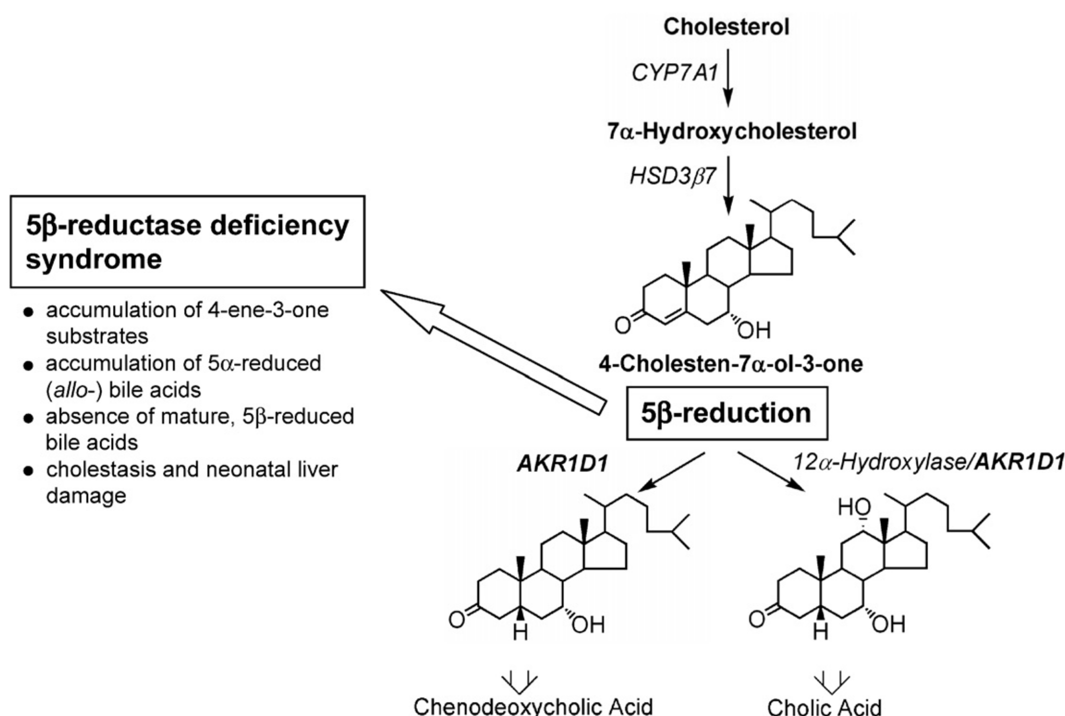

Fig. 1. Central role of 5β-reduction in bile acid biosynthesis. AKR1D1 is a key enzyme in bile acid biosynthesis that catalyzes the 5β-reduction of Δ^4-cholesten-7α-ol-3-one and Δ^4-cholestene-7α,12α-diol-3-one substrates that will ultimately result in the production of mature bile acids such as chenodeoxycholic and cholic acid. If 5β-reduction is inhibited through a functional defect in AKR1D1 or other factors, 5β-reductase deficiency syndrome is observed. Physiological consequences include cholestasis and liver damage and often result in death of the patient if untreated.

single amino acid substitution in the catalytic tetrad, from histidine to glutamate, is sufficient to achieve this switch from one function to another [5]. The recently determined crystal structure of AKR1D1 further elaborates the role of this residue where it is proposed to act as a superacid and in concert with the catalytic tyrosine facilitates hydride transfer to the Δ^4-ene [6]. Furthermore, strict conservation of this glutamate in all AKR1D proteins and almost complete absence from other AKR subfamilies highlights the importance of this substitution for 5β-reductase function.

2. The physiological role of AKR1D1 in bile acid biosynthesis

Steroid 5β-reductase activity is most abundant in the liver and led to the first isolation of the protein from rat liver [7]. More than 25 years later, the human gene, AKR1D1, was cloned based on a previously released rat 5β-reductase cDNA annotation [3,8]. Like its rat homolog, AKR1D1 is most abundant in liver and recombinant expression of the human protein in mammalian cells confirmed 5β-reductase activity towards steroids and bile acid intermediates [3,9,10].

In congruence with the enzyme's expression pattern and catalytic activity, AKR1D1 has been proposed to play a physiological role in steroid hormone metabolism as well as bile acid biosynthesis. In the latter, AKR1D1 catalyzes the central 5β-reduction step and reduces bile acid precursors such as Δ^4-cholesten-7α-ol-3-one and Δ^4-cholestene-7α,12α-diol-3-one to 5β-cholestan-7α-ol-3-one and 5β-cholestane-7α,12α-diol-3-one, respectively. The stereo-specific reduction of the sterol precursors is of physiological importance. While 5α-reduction leaves the overall structure in its planar shape, 5β-reduction introduces a 90° angle between the A and B ring. Sterols with this characteristic 5β-reduced structure occur in the bile acids of almost all mammals and this configuration is essential for the proper emulsification of dietary cholesterol, lipids, and essential nutrients and promotion of their absorption and digestion [11,12].

Evidence that AKR1D1 is the only human 5β-reductase and that its most important physiological role is in bile acid biosynthesis comes from patients with 5β-reductase deficiency, a metabolic syndrome first described by Setchell in 1988 [13]. The deficiency syndrome develops in infants within the first few weeks after birth and is characterized by reduced primary bile acid synthesis and accumulation of Δ^4-3-keto- and 5α-reduced (*allo*-) bile acids (Fig. 1). Furthermore, 5β-reduced steroids are absent from serum and urine. The effects of this rare metabolic disorder are severe and manifest in cholestasis and neo-natal liver damage probably caused by the accumulation of potentially cholestatic and hepatotoxic atypical bile acids. If untreated, 5β-reductase deficiency is often lethal. Loss of 5β-reductase activity could either result from a genetically determined enzyme defect or from secondary effects such as changes in protein/mRNA stability due to liver disease [14] or enzyme modification through chemical modification [15,16].

3. Disease associated AKR1D1 mutations affect conserved protein sites

In an effort to link putative genetic defects in AKR1D1 to the 5β-reductase deficiency syndrome, the genotype of AKR1D1 had been determined in several patients. Sequencing identified missense mutations leading to truncated, non-functional proteins in two patients [17,18], and five individual point mutations leading to non-synonymous amino acid substitutions in the AKR1D1 protein (L106F, P133R, P198L, G223E, and R261C) in three other patients [17–19]. Analysis of the mutations in the AKR1D1 crys-

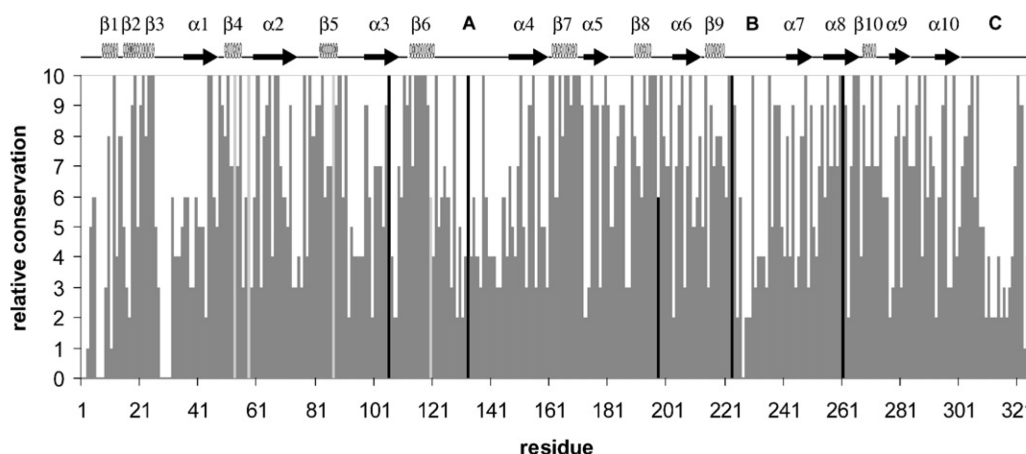

Fig. 2. Disease-associated mutations affect regions highly conserved in the AKR1 family. Multiple sequence alignment and calculation of conservation per site of AKR1 sequences annotated at the AKR website was performed with ClustalW and relative conservation visualized with JalView. Amino acid position in AKR1D1 is indicated on the *x*-axis; degree of conservation on the *y*-axis. Residues mutated in patients with 5β-reductase deficiency syndrome are indicated in black; the residues forming the catalytic tetrad are marked in light grey. Secondary structure elements of AKR1D1 are indicated above the plot. Notably, residues changed in the disease are highly conserved or are located in a highly conserved region of the protein.

tal structure however, did not reveal involvement of the affected residues in catalysis, or cofactor or substrate binding [6]. Therefore, it remained unclear whether the detected mutations could indeed cause the 5β-reductase deficiency phenotype observed in these patients.

Non-synonymous mutations not involved in enzyme function may still affect protein folding and stability if they are found within highly conserved residues or domains of a protein family. In this case, conservation reflects less the enzyme properties associated with catalysis but rather establishment of the common and characteristic protein fold of a family. These cues are the basis for instance in sequence based algorithms that identify conserved domains (e.g. CD search at NCBI) [20,21] or in homology modeling (examples for AKR1D1 include [22,23]). To test this hypothesis we examined sequence conservation within the AKR1 family (Fig. 2). We find that all the AKR1D1 mutations occur either in conserved residues or conserved domains. Of these, two mutations, L106F and R261C, reside in α-helices 3 and 8, respectively, and could affect protein stability. The remaining mutations are located in loop or turn regions of the protein. These mutations severely alter the physico-chemical properties of the residue at the affected site. In two cases, the small but rigid proline is replaced by either a large positively charged amino acid (P133R) or a similar small hydrophobic, but flexible side chain (P198L). In G223E, the α-hydrogen is replaced by a carboxylic acid side chain. It is notable, that two mutations, P133R and G223E, reside in loops A and B, respectively, which convey substrate recognition and binding properties. These findings support the notion that the observed mutations could indeed result in changes in protein structure and stability and ultimately lead to deficient 5β-reductase activity.

4. Disease associated AKR1D1 mutations affect protein integrity and enzyme activity

Further evidence of diminished structural integrity comes from our efforts to purify and enzymatically characterize the mutant AKR1D1 isoforms, and from monitoring expression levels and stability of these mutants when expressed in human cells. Recombinant protein expression in *Escherichia coli* yielded abundant protein in lysed cells [24]. However, subsequent efforts to isolate the enzymes either failed or yielded extremely low amounts of purified protein in comparison to the wild type enzyme (Table 1). Only the P133R mutant was obtained in sufficient purity and amounts that allowed further characterization. Determination of melt curves by CD spectroscopy revealed decreased thermal stability of the P133R mutant in the absence of cofactor. Melt curves were recorded following the absorbance at 222 nm while raising the temperature from 14 to 94 °C in increments of 2 °C. The T_m, the temperature where half of the protein was unfolded according to measurement of molar elipticity, was 41 °C for the mutant compared to 48 °C for the wild type protein. However, the presence of cofactor eliminated the observed difference [24]. Following expression of recombinant AKR1D1 in HEK 293 cells, levels of mutant proteins were significantly decreased compared to wild type (Fig. 3). For two of the mutations, L106F and R261C, protein expression levels were less than 1% of that of the wild type enzyme. In addition, cycloheximide treatment revealed decreased stability of mutant protein as expression levels declined compared to wild type AKR1D1 over a 24 h time course.

However, the mutations observed in patients with 5β-reductase deficiency not only affected expression levels and stability but also

Table 1
Purification of recombinant AKR1D1 mutant protein.

Ni-sepharose fraction of	Volume (mL)	Total protein (mg)	Total activity (nmol min^{-1})	Specific activity (nmol min^{-1} mg^{-1})	Purification factor	Yield (%)
WT	4.0	5.7	462	81	9.9	56
L106F[a]	14.5	3.3	0.2	0.05	1.25	7
P198L	3.9	0.4	ND	ND	–	0
R261C	4.1	0.5	ND	ND	–	0
P133R	5.0	1.5	36	24.4	9.47	16

[a] By SDS-PAGE analysis, sample is only 10% pure.

Table 2
Effect of the P133R mutation on K_m and k_{cat} of 5β-reduction.

	Wild type		P133R mutant	
	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)
Testosterone	7.1 ± 1.7	2.7 ± 1.2	2.7 ± 0.3	12.7 ± 2.2
Cortisone	9.9 ± 0.1	15.1 ± 0.3	0.6 ± 0.02	1.3 ± 0.1
4-Cholesten-7α-ol-3-one	1.7 ± 0.1	0.8 ± 0.2	0.2[a]	0.08[a]

Activity measurements were determined fluorimetrically.
[a] Estimated.

enzymatic activity. A study of the purified P133R mutant revealed drastic changes in kinetic constants with different steroid and bile acid intermediate substrates (Table 2). With Δ^4-cholesten-7α-ol-3-one as substrate the reaction proceeded with a highly reduced K_m and k_{cat} and kinetics were reminiscent of uncompetitive inhibition. Hence, the P133R mutation converts AKR1D1 from a low affinity, high capacity enzyme, to one with high affinity and low capacity. The mutant enzymes also showed severely decreased enzymatic activity when compared to wild type following expression in human HEK293 cells. The AKR1D1 mutants P198L and G223E did not show measurable 5β-reductase activity after 60 h following addition of substrate to the culture medium. The mutants L106F, P133R and R261C exhibited 5β-reductase activity that was only a fraction of that of wild type AKR1D1 (Fig. 4). Quite notably, L106F and R261C showed detectable enzyme activity despite extremely low expression levels indicating that these mutations did not necessarily affect enzyme activity and that low protein expression accounted for their reduced activity in the HEK-293 cells.

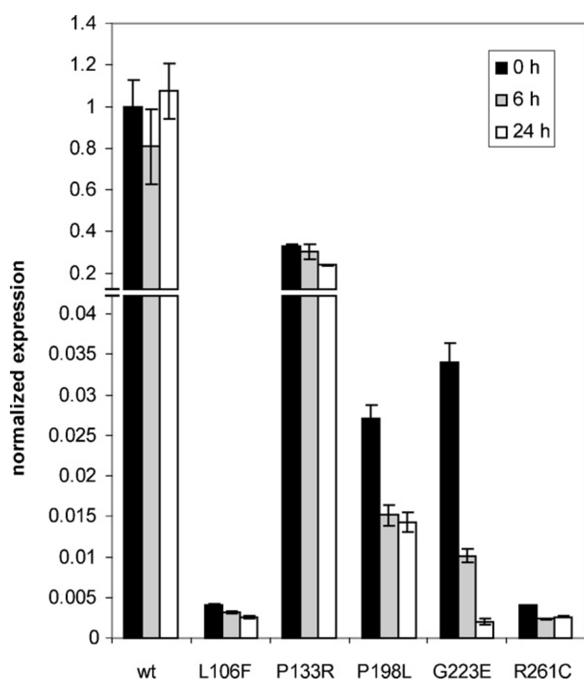

Fig. 3. AKR1D1 disease mutants showed reduced protein expression and stability versus wild type enzyme. Expression of wild type and mutant AKR1D1 in HEK 293 cells was observed by Western Blotting with a specific anti-AKR1D1 antibody. Expression levels were determined with densitometry and analysis with the Uniscan and imageJ software packages. Expression levels of the L106F and R261C mutants were so low that they could only be detected by a highly sensitive detection method (SuperSignal West Femto Kit (Thermo Scientific)). Time indicates time points after cycloheximide treatment.

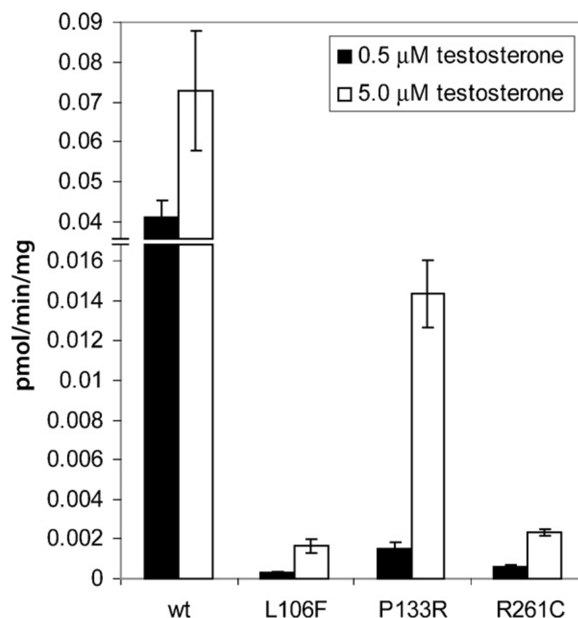

Fig. 4. AKR1D1 disease mutants had reduced or no enzyme activity when expressed in HEK-293 cells. Wild type and mutant AKR1D1 were expressed in HEK 293 cells and 5β-reductase activity was measured radiometrically by following the conversion of tritiated testosterone to 5β-DHT. Product verification and quantification was carried out by thin layer chromatography. Initial velocities were estimated from linear phase of product formation. In case of the mutants P198L and G223E no substrate conversion was detected within 60 h following addition of testosterone to the medium.

5. Conclusions

In the AKR family, AKR1D1 is the only enzyme that does not catalyze the interconversion of aldehyde and ketone groups with primary and secondary alcohols but instead catalyzes the reduction of double bonds in Δ^4-3-ketosteroids and sterols.

In contrast to the closely related AKR1C subfamily, only one functional AKR1D1 gene is present in all vertebrates except fish. Furthermore, compared to other AKR1 family members the number of SNPs and other genomic variations is low in AKR1D1 [25] and sequence conservation between distantly related 5β-reductase members is significantly higher than between 5α-reductase homologs [26]. This agrees well with the central physiological function of AKR1D1 in bile acid biosynthesis, an important metabolic pathway in all vertebrates. Defects in bile acid metabolism are rare (<0.1% of population) but can have severe consequences, as seen in patients with 5β-reductase deficiency. We showed that five mutations detected in these patients had either reduced protein expression and stability or enzyme activity, or both. As a result, net levels of physiologically active 5β-reductase would be drastically reduced. Although the disease-associated mutations did not seem to directly affect substrate binding or catalysis, they significantly altered physico-chemical properties of conserved residues or

areas within the protein thereby disrupting stability and enzymatic activity of AKR1D1.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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