Human and Murine Steroid 5β-Reductases (AKR1D1 and AKR1D4): Insights into the Role of the Catalytic Glutamic Acid

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Abstract

Mammalian steroid 5β-reductases belong to the Aldo-Keto Reductase 1D sub-family and are essential for the formation of A-ring 5β-reduced steroids. Steroid 5β-reduction is required for the biosynthesis of bile-acids and the metabolism of all steroid hormones that contain a Δ^4-3-ketosteroid functionally to yield the 5β-reduced metabolites. In mammalian AKR1D enzymes the conserved catalytic tetrad found in all AKRs (Y55, H117, K84 and D50) has changed in that the conserved H117 is replaced with a glutamic acid (E120). E120 may act as a "superacid" to facilitate enolization of the Δ^4-ketosteroid. In addition, the absence of the bulky imidazole side chain of histidine in E120 permits the steroid to penetrate deeper into the active site so that hydride transfer can occur to the steroid C5 position. In murine steroid 5β-reductase AKR1D4, we find that there is a long-form, with an 18 amino-acid extension at the N-terminus (AKR1D4L) and a short-form (AKR1D4S), where the latter is recognized as AKR1D4 by the major data-bases. Both enzymes were purified to homogeneity and product profiling was performed. With progesterone and cortisol, AKR1D4L and AKR1D4S catalyzed smooth conversion to the 5β-dihydrosteroids. However, with Δ^4-androstene-3,17-dione as substrate, a mixture of products was observed which included, 5β-androstane-3,17-dione (expected) but 3α-hydroxy-5β-androstan-17-one was also formed. The latter compound was distinguished from its isomeric 3β-hydroxy-5β-androstan-17-one by forming picolinic acid derivatives followed by LC-MS. These data show that AKR1D4L and AKR1D4S also act as 3α-hydroxysteroid dehydrogenases when presented with Δ^4-androstene-3,17-dione and suggest that E120 alters the position the steroid to enable a correct trajectory for hydride transfer and may not act as a "superacid".

Keywords: aldo-keto reductase, steroid 5β-reductase, hydroxysteroid dehydrogenase

1. Introduction

Aldo-keto reductases (AKRs) of the 1D subfamily act as steroid-double bond reductases (5β-reductases) [1] and are essential to produce bile-acid precursors and metabolize Δ^4-3-ketosteroids to their corresponding 5β-reduced metabolites [2-4]. Many of these 5β-reduced metabolites have physiological functions in their own right, for example 5β-dihydroprogesterone is a tocolytic and helps prevent premature parturition [5] , while the 5β-cholanic acids act as ligands for the farnesoid-X receptor [6, 7].

Other AKRs function as carbonyl reductases [1]. As it is chemically more difficult to reduce a double-bond than a carbonyl functionality [8, 9] it was noted that all AKR1D enzymes have an altered conserved catalytic tetrad whereby the catalytic histidine is replaced by a glutamic acid [1]. It was reasoned that substitution of H117 in rat 3α-hydroxysteroid dehydrogenase (AKR1C9) for a glutamic acid would introduce 5β-reductase activity into this enzyme [10]. The AKR1C9 H117E mutant abolished the dehydrogenase activity and introduced the double bond reductase activity as predicted [10]. However, the reason for the loss of the dehydrogenase activity was not so clear.

Crystal structures of the human AKR1D1•$NADP^+$•Steroid complexes provided evidence for a dual role for E120 [11]. First, E120 was found to exist in the fully protonated *anti*-conformation suggesting that it could act as a superacid to aid in the enolization of the Δ^4-ketosteroid substrate, Figure 1. Second, the replacement of the bulky side chain of histidine with glutamic acid allowed the steroid substrate to penetrate more deeply into the active site. This penetration permits hydride transfer to the C5 position of the steroid rather than direct hydride transfer to the C3 position of a 3-ketosteroid, thus providing an explanation for the loss of the dehydrogenase activity in the AKR1C9-H117E mutant [12, 13]. Consistent with this notion the AKR1D1-E120H mutant was found to act as a 3β-hydroxysteroid dehydrogenase with an enhancement of k_{cat}/K_m for the dehydrogenase activity over the wild type enzyme of 10^6. This point mutation was described as an example of “perfect enzyme engineering” [14].

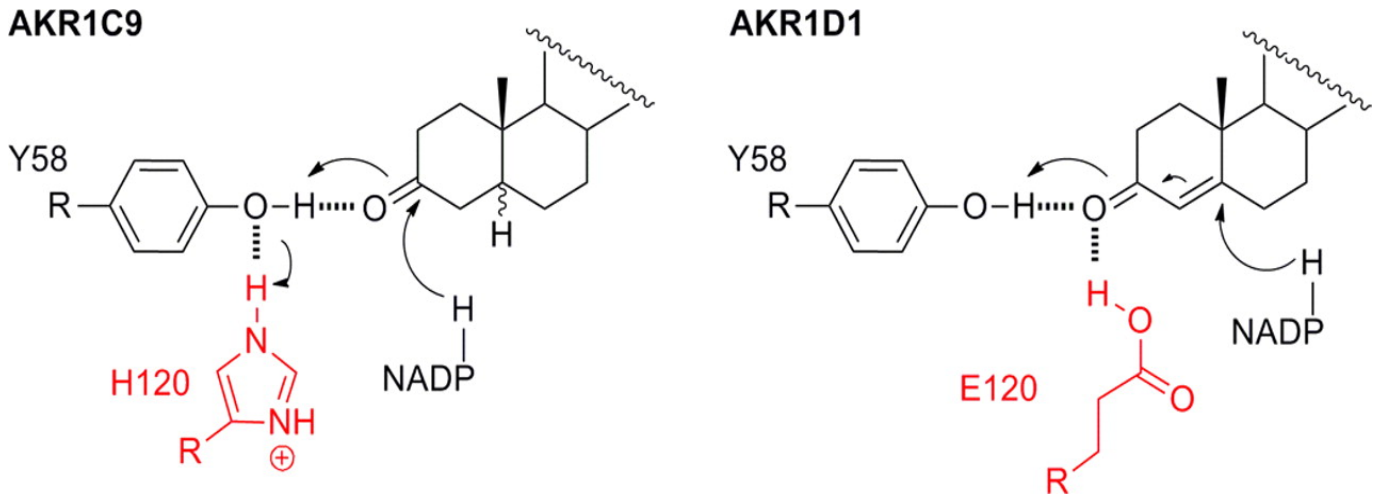


Figure 1. Mechanism of 3-Ketosteroid Reduction versus Steroid Double-Bond Reduction Catalyzed by AKR1 family members. Hydride transfer to C3 with a proton relay to H117 (left); hydride transfer to C5 with E120 acting as a superacid (right)

Interest exists in murine steroid 5β-reductase (AKR1D4) since it is a paralog of the human enzyme, AKR1D1. The AKR nomenclature avoids designating the murine enzyme as akr1d1, and instead gives each enzyme its own unique name to avoid implying that AKR enzymes have the same function across species [15]. Studies in trying to relate human and murine AKRs of the 1C subfamily have shown that it is not possible to assign them has having identical functions and tissue distribution [16].

Despite these issues the development of knockout murine models may help determine the role of steroid 5β-reducatse and 5β-dihydrosteroids in physiology and pathophysiology. In this report, we describe the characterization of a long and short form of AKR1D4L and AKR1D4S respectively. We find that when these enzymes are incubated with Δ^4-androstene-3,17-dione both the expected product 5β-androstane-3,17-dione and 3α-hydroxy-5β-androstan-17-one are produced. The formation of a 3α-hydroxysteroid in the presence of an E120 residue suggests

that this residue does not act as a superacid and that its role is to determine positioning of the steroid substrate at the active site.

1. Methods

1. 1. Materials: [1,2,6,7–^{3}H(N)]-Δ^{4}-androstene-3,17-dione (4-AD) (Specific Radioactivity 95.1 Ci/mole-Perkin Elmer); [1,2,6,7-^{3}H(N)]-cortisol (Specific Radioactivity 78.3 Ci/mmol -Perkin Elmer) and [4-^{14}C]-progesterone Specific Radioactivity 55 mCi/mmole- were obtained from American Radiolabeled Chemicals. Unlabeled steroids were purchased from Steraloids. NADPH-disodium salt was purchased from Roche.

1. 2. Enzyme Purification: AKR1D4L and AKR1D4S cDNAs were obtained by RT-PCR from murine liver poly(A)$^{+}$-RNA and were subcloned into a pET28a vector at the *NdeI* and *EcoRI* sites which provided a 6-His-Tag and a Thrombin cleavage site. The primers for AKR1D4L were: Forward: (*NdeI*)) 5-aaaagcc cat ATG TGC CTC TGC CCT GTT CAG-3' and Reverse: (*EcoRI*) 5'aaaagcc gaattc ATT AGT ATT CGT CAT GAA ATG GGT ATT C-3'; and the primers for AKR1D4S were Forward: 5'- CCT GGT GCC GCG CGG CAG CCA TAT GAA CCT CAG CGC TGC ACA CC-3' and Reverse: 5'-GGT GTG CAG CGC TGA GGT TCA TAT GGC TGC CGC GCG GCA CCA GG-3'. Both enzymes were purified to homogeneity using sequential chromatography on DEAE and His-Trap FF chromatography by FPLC, see Fig. 2.

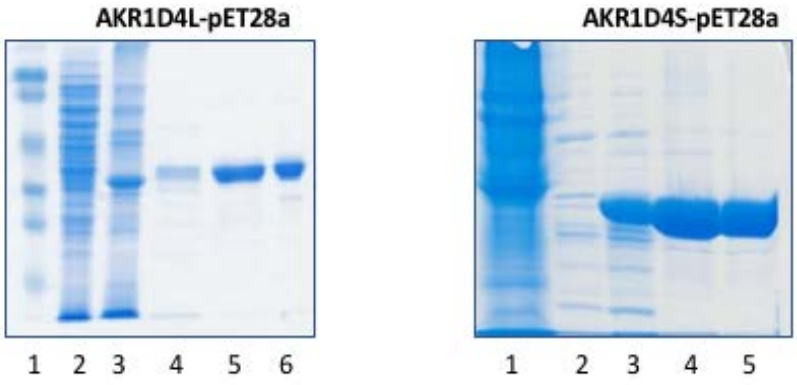


Figure 2. Purification of AKR1D4L and AKR1D4S From pET28a Expression Vectors. AKR1D4L (left), lane 1 mol. wt. markers; lane 2, *E. coli* lysate; lane 3, *E. coli* lysate precipitate, lane 4, combined DEAE; and lane 5 & 6 HisTrap eluant, AKR1D4S (right) lane1 1, *E*. coli lysate, lane 2 HisTrap flow through, lanes 3-5 AKR1D4 purified fractions from HisTrap.

1. 2. Determination of Steady State Kinetic Constants. Systems 1 mL contained varied concentration of steroid substrate, 7. 2 μM NADPH, 4% acetonitrile in 100 mM potassium phosphate pH 7.0. Reactions were initiated by the addition of enzyme and the consumption of NADPH was monitored fluorimetrically at 37 ^{o}C for 3 min. Excitation was at 340 nm and emission was followed at 460 nm. Relative fluorescence units were converted into nmoles NADPH consumed using calibration curves. Plots of v versus [S] were fitted to the hyperbolic function of the Michealis-Menton equation iteratively to produce estimates of k_{cat} and K_m and their associated standard errors using GraphPad Prism Version 7 (GraphPad Software, La Jolla, CA) to calculate V_{max}, K_m, and k_{cat}. Enzyme assays were run in the presence of 4% acetonitrile as co-solvent to maintain solubility of the steroid substrate. It is not possible to determine the effect of the co-solvent on enzyme activity since to our knowledge there is no water soluble substrate for the enzyme.

1. 3. Product Profiling by Thin-Layer Chromatography. Assay mixtures (200 µL) contained 0.2 mM NADPH, 4% acetonitrile, 0.07 µM ^{3}H-Δ^{4}-AD, 2 µM Δ^{4}-AD, and either 5 µg/mL AKR1D4L or 5 µg/mL AKR1D4S in 100 mM potassium phosphate pH 7.0, and incubated at 37 ^{o}C for 3 h. Alternatively, assay mixtures contained 10 µM cortisol, 0.07 µM [^{3}H] cortisol, 5 µg/mL of

AKR1D4S and AKR1D4L or 10 μM progesterone and 0.91 μM [^{14}C]-progesterone. The reactions were quenched at 60, 90, 120 min with 1 mL ethyl acetate. The mixture was allowed to separate at -20°C for 1 hour followed by a thawing period and the layers separated using centrifugation at 1000 rpm for 20 min. The extraction was repeated three times. The organic fractions were combined in a borosilicate tube and dried by Savant Electro SpeedVac Concentrator. The residue was dissolved in 100 μL acetonitrile and spotted onto a TLC plate. The TLC plate was then developed with 80:20 toluene: acetone twice. The TLC plate was read using a radioactivity plate reader and the peaks integrated as a percent of the total radioactivity on the plate. Product profiling by the radiochromatographic method was used to produce time courses and for initial product identity by co-chromatography with synthetic standards.

1. 4. Product Profiling by LC-MS/MS. Steroids (2.86 μg) including those from control reactions (no enzyme) were dissolved in 2.86 mL HPLC grade ethanol to yield solutions of 1 ng/μL, which were then diluted to 200 pg/μL. In addition, the following standards (1 ng) 5β-dihydrotestosterone, testosterone, 3α-hydroxy-5β-androstan-17-one and 3β-hydroxy-5β-androstan-17-one were prepared. The picolinic acid derivatization solution (1 mL) contained: 50 mg picolinic acid; 20 mg dimethylamino-pyridine; 40 mg nitromethylbenzoic anhydride. The derivatization solution (100 μL) was added to each sample followed by the addition of 40 μL triethylamine. The reactions were incubated at room temperature for 90 min with gentle shaking. The derivatized samples were purified through a C18 solid-phase extraction column and the eluted samples were dried by speed vacuum and stored at -20 °C for mass spec analysis. LC-MS/MS analysis for product identity was performed as described [17]. LC-MS/MS methods were used to distinguish between 3α-hydroxy-5β-androstane-17-one and 3β-hydroxy-5β-androstane17-one.

1.5. Detection of AKR1D4L and AKR1D4S Transcripts in Murine Liver.

Total RNA was extracted from liver tissue from nine male mice using Tri-Reagent (Sigma-Aldrich, Dorset, UK). The concentration was determined spectrophotometrically at OD260 on a Nanodrop spectrophotometer (Thermo Scientific, Hemel Hempstead, UK). Reverse transcription was performed in a 20 µl volume; 1 µg of total RNA was incubated with 1 x RT Buffer, 100mM dNTP Mix, 10x RT Random Primers, 50 U/ µl MultiScribe Reverse Transcriptase and 20U/µl RNase Inhibitor. The reaction was carried out at 25 ^{o}C for 10 min, 37 ^{o}C for 120 min and terminated by heating to 85 ^{o}C for 5 min.

Quantitative PCR was conducted on a QuantStudio 7 Realtime PCR System (Applied Biosystems, Warrington, UK). Reactions were performed on 384-well plates in a 6 µl volume of 1 x KlearKall Master Mix with standard ROX (LGC Genomics, Middlesex, UK) using a 1/40 dilution of cDNA. TaqMan assays (FAM labelled) and all reagents were purchased from Applied Biosystems (Applied Biosystems, Foster City, US). The reaction conditions were as follows: 95 ^{o}C for 3 min followed by 40 cycles of 95 ^{o}C for 3 sec and 60 ^{o}C for 20 sec. Primers and probes were supplied by AppliedBiosystems as 'assays on demand': AKR1D4 a premade assay detecting the short and long isoforms, AKR1D4L a custom designed assay detecting only the long form, and the housekeeping genes 18S and HPRT were provided as preoptimised controls. Data were obtained as Ct values (cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values. All reactions were normalised against the geometric mean of 18S and HPRT [(Ct of the target gene) – (Ct of the reference gene)]. Data were expressed as arbitrary units using the following transformation: [arbitrary units (AU) = 1,000 × ($2^{-\Delta ct}$)] or as a ratio of AKR1D4 to AKR1D4L. This assay did not allow the detection of only the AKR1D4S form since these primers detect both the long and short form.

3. Results

3. 1. Purification and Characterization of AKR1D4L and AKR1D4S. AKR1D4L and ARR1D4S cDNAs were subcloned into pET28a prokaryotic expression vectors. AKR1D4L differs from AKR1D4S since it has an eighteen amino acid N-terminal extension. AKR1D4S is the sequence for murine found in the major data-bases, (see, Reference Sequence XP_006505888; and UNIPROT Q8VCX1 (AK1D1_MOUSE) Figure 3. AKR1D4L and AKR1D4S were purified to homogeneity has his-tag-proteins. Steady state parameters (k_{cat}, K_m and k_{cat}/K_m) were generated for the 5β-reduction of Δ^4-AD and cortisone respectively. These parameters were essentially identical for AKR1D4L and AKR1D4S forms. The values for the 5β-reduction of

cortisone were also similar to those generated for AKR1D1 (human 5β-reductase) [18, 19] but the values were 10-times lower for the reduction Δ^4-AD, and this was mainly due to an increase in K_m, see Table 1.

3. 2. Product Characterization Δ^4-Androstene-3,17-dione Reduction. We used [^{3}H]-Δ^4-AD in discontinuous assays in which substrate and product were separated by radiochromatography, we expected to see only one product 5β-androstane-3,17-dione formed by the AKR1D4 enzymes. To our surprise we saw an additional product peak which was tentatively assigned as 3α/β-hydroxy-5β-androstan-17-one, Figure 4. To identify this product, we prepared picolinate

																	ATG	TGC
																	M	C
CTC	TGC	CCT	GTT	CAG	AAT	CAC	CCC	AGA	AGC	TTC	AGA	TTC	TTC	TCT	ACG	ATG	AAC	
L	C	P	V	Q	N	H	P	R	S	F	R	F	F	S	T	M	N	
CTC	AGC	GCT	GCA	CAC	CAC	CAA	ATA	TCC	CTA	AGT	GAT	GGG	AAC	AAC	ATT	CCA	CTC	
L	S	A	A	H	H	Q	I	S	L	S	D	G	N	N	I	P	L	
ATT	GGG	CTT	GGA	ACC	TAC	TCA	GAT	CCT	AGA	CCG	GTC	CCT	GGG	AAG	ACC	TAT	GTG	
I	G	L	G	T	Y	S	D	P	R	P	V	P	G	K	T	Y	V	
GCA	GTG	AAG	ACA	GCT	ATT	GAT	GAG	GGG	TAC	CGG	CAC	ATT	GAT	GGC	GCC	TAT	GTT	
A	V	K	T	A	I	D	E	G	Y	R	H	I	D	G	A	Y	V	
TAC	CAC	AAT	GAG	CAT	GAA	GTC	GGT	GAG	GCC	ATC	AGA	GAA	AAG	ATA	GCA	GAA	GGG	
Y	H	N	E	H	E	V	G	E	A	I	R	E	K	I	A	E	G	
AAG	GTA	AAG	AGG	GAA	GAG	ATC	TTC	TAC	TGT	GGA	AAG	TTA	TGG	AAT	ACA	GAG	CAT	
K	V	K	R	E	E	I	F	Y	C	G	K	L	W	N	T	E	H	
GTC	CCA	TCA	ATG	GTC	CTC	CCA	GCC	CTG	GAA	AGG	ACG	CTA	AAG	GCC	CTC	AAA	CTA	
V	P	S	M	V	L	P	A	L	E	R	T	L	K	A	L	K	L	
GAT	TAC	ATA	GAC	CTT	TAT	ATT	ATC	GAA	CTG	CCC	ATG	GCC	TTT	AAG	CCT	GGA	AAG	
D	Y	I	D	L	Y	I	I	E	L	P	M	A	F	K	P	O	K	
GAA	ATT	TAC	CCT	AGA	GAT	GAA	AAT	GGC	CGA	ATT	ATA	TAT	GAC	AAA	ACA	AAT	CTG	
E	I	Y	P	R	D	E	N	G	R	I	I	Y	D	K	T	N	L	
TGT	GCC	ACG	TGG	GAG	GCA	TTG	GAA	GCT	TGC	AAA	GAT	GCT	GGC	TTG	GTG	AAA	TCC	
C	A	T	W	E	A	L	E	A	C	K	D	A	G	L	V	K	S	
CTT	GGG	GTG	TCT	AAT	TTT	AAC	CGC	AGG	CAG	CTG	GAG	CTC	ATC	TTG	AAC	AAG	CCA	
L	G	V	S	N	F	N	R	R	Q	L	E	L	I	L	N	K	P	
GGA	CTC	AAG	TAC	AAA	CCA	GTC	ACT	AAC	CAG	GTG	GAG	TGC	CAC	CCG	TAT	TTC	ACC	
G	L	K	Y	K	P	V	T	N	Q	V	E	C	H	P	Y	F	T	
CAG	ACA	AAA	CTC	TTG	AAA	TTC	TGC	CAG	CAG	CAT	GAC	ATC	GTC	ATT	GTC	GCA	CAT	
Q	T	K	L	L	K	F	C	Q	Q	H	D	I	V	I	V	A	H	
AGC	CCC	TTA	GGG	ACC	TGC	CGC	AAC	CCA	TCA	TGG	GTG	AAC	GTC	TCT	TCT	CCA	CCC	
S	P	L	G	T	C	R	N	P	S	W	V	N	V	S	S	P	P	
TTG	TTA	AAT	GAC	GAA	CTC	CTA	ACC	TCA	CTG	GGG	AAA	AAG	TAC	AAT	AAG	ACA	CAA	
L	L	N	D	E	L	L	T	S	L	G	K	K	Y	N	K	T	Q	
GCT	CAA	ATT	GTG	TTG	CGT	TTC	AAC	ATC	CAG	CGA	GGG	ATA	GTT	GTC	ATT	CCT	AAA	
A	Q	I	V	L	R	F	N	I	Q	R	G	I	V	V	I	P	K	
AGC	TTT	ACC	CCC	GAA	AGG	ATC	AAA	GAA	AAC	TTT	CAG	ATC	TTT	GAC	TTT	TCT	CTC	
S	F	T	P	E	R	I	K	E	N	F	Q	I	F	D	F	S	L	
ACG	GAA	GAA	GAA	ATG	AAG	GAC	ATT	GAT	GCC	TTG	AAT	AAA	AAT	GTC	CGC	TAT	GTG	
T	E	E	E	M	K	D	I	D	A	L	N	K	N	V	R	Y	V	
GAG	TTG	CTC	ATG	TGG	AGT	GAC	CAT	CCT	GAA	TAC	CCA	TTT	CAT	GAC	GAA	TAC	tga	
E	L	L	M	W	S	D	H	P	E	Y	P	F	H	D	E	Y	-Stop	

Figure 3. Sequence of AKR1D4L and AKR1D4S Showing the 18 Amino-Acid N-terminal Extension in AKR1D4L (in red).

Table 1. Comparison of Steady-State Kinetic Constants for AKR1D1 with AKR1D4L and AKR1D4S

Enzyme	Substrate	K_m (μM)*	k_{cat} (min^{-1})*	k_{cat}/K_m (min^{-1} mM^{-1})
AKR1D1 (human)	Cortisone	15.1 ± 0.3	11.7 ± 0.1	780
AKR1D4L (murine)	Cortisone	7.1 ± 0.89	4.5 ± 0.2	630
AKR1D4S (murine)	Cortisone	11.4 ± 1.7	7.6 ± 0.5	620
AKR1D1 (human)	Δ^4-androstene-3,17-dione	0.9 ± 0.2	6.0 ± 0.8	672
AKR1D4L (murine)	Δ^4-androstene-3,17-dione	5.7 ± 2.3	3.2 ± 0.4	56
AKR1D4S (murine)	Δ^4-androstene-3,17-dione	8.0 ± 1.5	5.1 ± 0.4	62

*Values ± SE

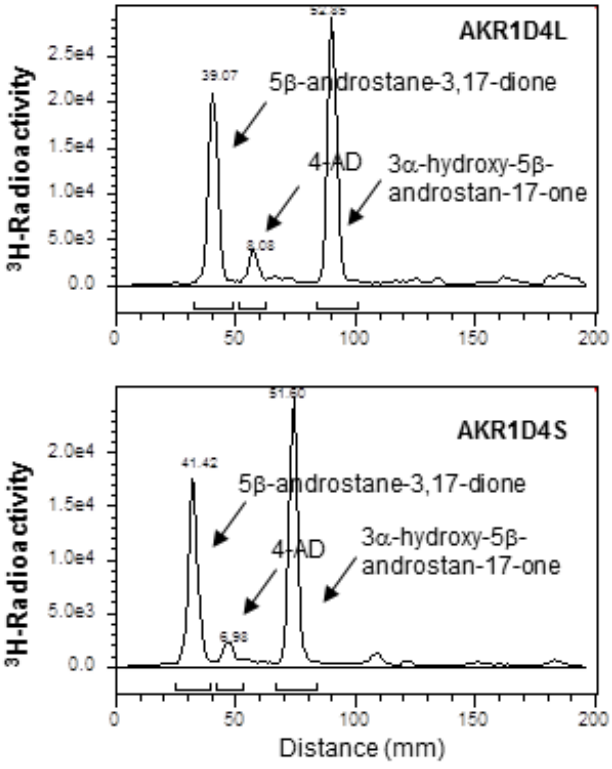


Figure 4. Radiochromatography of [^{3}H]-Δ^4-Androstene-3,17-Dione Reduction Products Formed by AKR1D4L and AKR1D4S. Overtime there is complete conversion to the major product 3α/β-hydroxy-5β-androstan-17-one (see Materials and Methods for details).

esters of both the 3α-hydroxy-5β-androstan-17-one and 3β-hydroxy-5β–androstan-17-one as standards. The choice of the picolinate group was to aid the detection and separation of the isomers by HPLC-UV, and because these are pre-ionized derivatives under acidic conditions, to aid their detection by LC-ESI-MS/MS [17]. We found that the two picolinate ester isomers were easily separated by HPLC and the standards identified 3α-hydroxy-5β-androstan-17-one as the final product of the AKR1D4L and AKR1D4S catalyzed reduction of Δ^4-AD. Identity was based upon retention time and ion transition *m/z* shown in mass spectrometric chromatograms acquired by a selected reaction monitoring (SRM) mass spectrometric method (e.g. ion transition *m/z*: 396 ($M+H^+$) → 124 ($PA+H^+$) for 3α/β-hydroxy-5β-androstan-17-one picolinate), Figure 5. Thus, these reactions clearly showed that AKR1D4L and AKR1D4S had 3α-hydroxysteroid dehydrogenase activity.

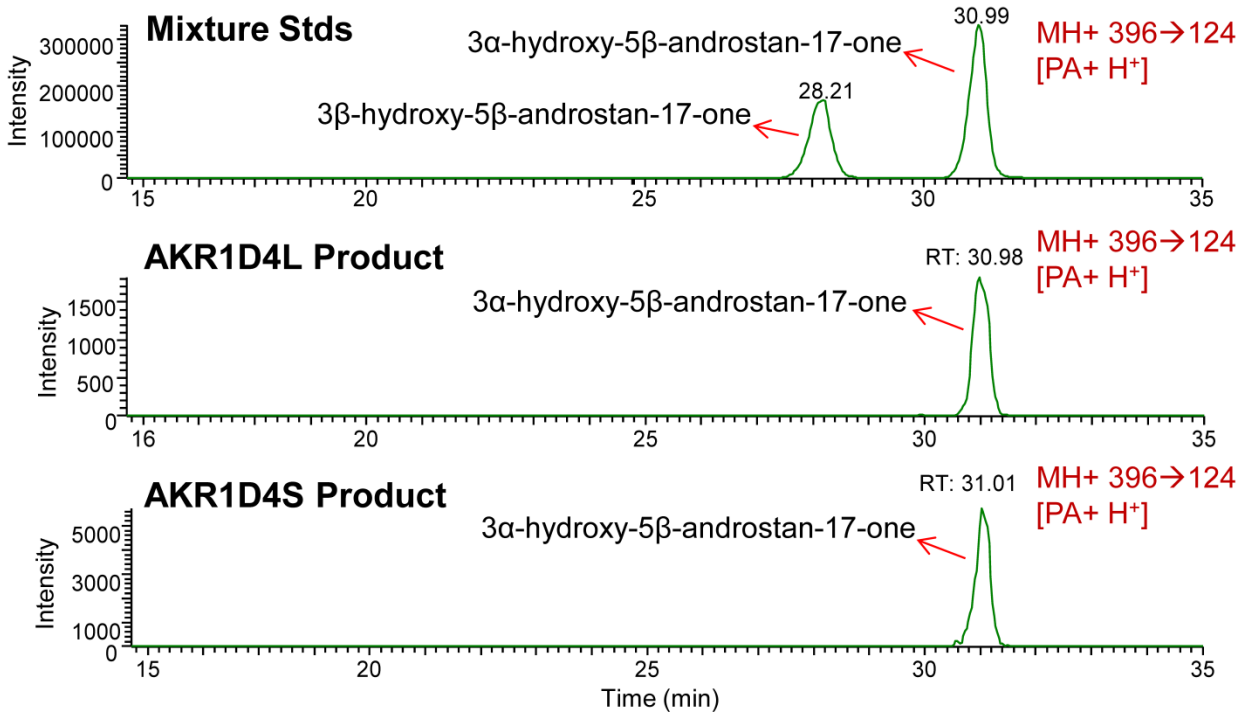


Figure 5. Identification of 3α-Hydroxy-5β-androstane-17-one as the Reduction Product of Δ^4-AD Catalyzed by AKR1D4L and AKR1D4S by LC-ESI-MS. Following incubation of Δ^4-AD steroid products were extracted and hydroxysteroids were esterified with picolinic acid. The picolinate derivatives were then subjected to LC-ESI-MS/MS. The mass transition shown refers to the loss of the picolinate.

The possibility remained that 5β-androstane-3,17-dione could also be reduced at the 17-ketone position to produce a series of 17β-hydroxysteroids, However, thin-layer chromatography indicates that 5β-dihydrostestosterone is easily separated from 3α-hydroxy-5β-androstane-17-one and 3β-hydroxy-5β-androstane-17-one and was not formed. In addition, 5β-androstane-3α,17β-diol and 5β-androstane-3β,17β-diol are more polar than the either 3α-hydroxy-5β-androstane-17-one and 3β-hydroxy-5β-androstane-17-one, and thus bis-reduction on both the 3-ketone and 17-ketone groups of 5β-androstane-3,17-dione did not occur (see Supplemental Material, Fig. S1).

3. 3. Time Course of 3α-hydroxy-5β-androstan-17-one Formation and Reduction of 5β-Androstane-3,17-dione as Substrate. Using discontinuous assays with [^{3}H]-Δ^4-AD as substrate, we noticed its rapid consumption and immediate formation of 5β-androstane-3,17-dione as product. Over time we observed evidence for a precursor-product relationship for the conversion of 5β-androstane-3,17-dione to 3α-hydroxy-5β-androstane-17-one., Figure 6.

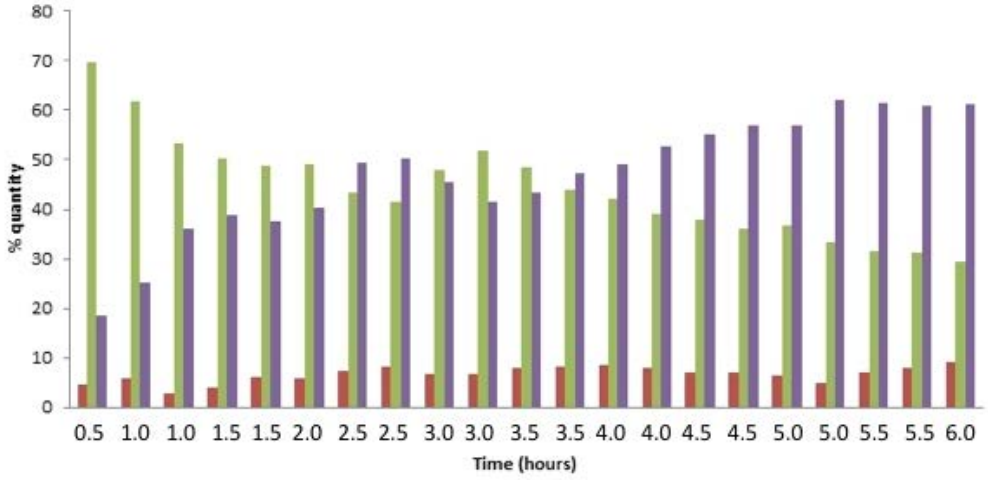


Figure 6. Time Course of for the Formation of 5β-Androstane-3,17-Dione and 3α-Hydroxy-5β-androstane-17-one Following the Reduction [^{3}H]-Δ^4-Androstene-3,17-Dione Catalyzed by AKR1D4L. Rapid conversion of Δ^4-AD (red bar) to 5β-androstane-3,17-dione (green bar) is observed followed by subsequent formation of 3α-hydroxy-5β-androstane-17-one (purple bar).

Steroid amounts are quantified as percent of radioactivity in each peak on the radiochromatogram. Similar results were obtained with AKR1D4S (see Materials and Methods for details).

We also conducted steady state kinetic analysis of the 3α-hydroxysteroid dehydrogenase reaction using 5β-androstane-3,17-dione as substrate. Formation of 3α-hydroxy-5β-androstan-17-one occurred somewhat more slowly than that observed in the conversion of Δ^4-AD to 5β–androstane-3,17-dione. This was supported by the k_{cat}/K_m values for the 3α–hydroxysteroid dehydrogenase reaction which were 5-times lower than for the 5β–reduction reaction. Similar k_{cat}/K_m values for the reduction of 5β-androstane-3,17-dione to yield 3α-hydroxy-5β–androstan-17-one were noted for AKR1D4L and AKR14DS forms, see Table 2. The formation of 3α-hydroxy-5β–androstan-17-one was confirmed by thin-layer chromtaography.

Table 2. Steady State Kinetic Constants for the Reduction of 5β-Androstane-3,17-dione Catalyzed by AKR1D4L and AKR1D4S

Enzyme	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
AKR1D4L	5β-Androstane-3,17-dione	56.8 ± 9.1	0.82 ± 0.04	14.0
AKR1D4S	5β-Androstane-3,17-dione	45.4 ± 4.9	0.49 ± 0.015	11.1

3. 4. AKR1D4 5β–reduction of progesterone and cortisol. We sought to determine whether the 3α-hydroxysteoid dehydrogenase assigned to AKR1D4L and AKR1D4S could be observed with C21 steroids e.g. progesterone and cortisol. Using [^{14}C]-progesterone and [^{3}H]-cortisol as substrates in discontinuous assays we conducted product profiling by radiochromatography. With both AKR1D4L and AKR1D4S we observed only the formation of 5β-dihydroprogesterone and 5β-dihydrocortisol, Figure 7. Thus the 3α-hydroxysteroid dehydrogenase activity of AKR1D4L and AKR1D4S was limited to C19-steroids.

3. 5. Tissue Distribution of AKR1D4L and AKR1D4S. Using customized ptimers for quantitative PCR we were able to show that in nine different murine liver samples the ratio of total AKR1D4 transcripts : AKR1D4L was approximately 2.5 fold (2.42 ± 0.25, n= 9) indicating the the AKR1D4S form was more abundant. Mouse ENCODE transcriptomic data reporst AKR1D4S expression in adult murine liver, kidney and adrenal.

DISCUSSION

We report the characterization of two murine steroid 5β-reductases (AKR1D4L and AKR1D4S). AKR1D4L differs from AKR1D4S in that it contains an 18 amino-acid extension at its N-terminus. Examination of this sequence for consensus leader sequences that may determine its subcellular localization were negative. Purification of these two enzymes to homogeneity and assignment of their steady state kinetic constants revealed no significance difference in their 5β-reductase activity and similar kinetic constants to those observed with the human AKR1D1 were obtained.

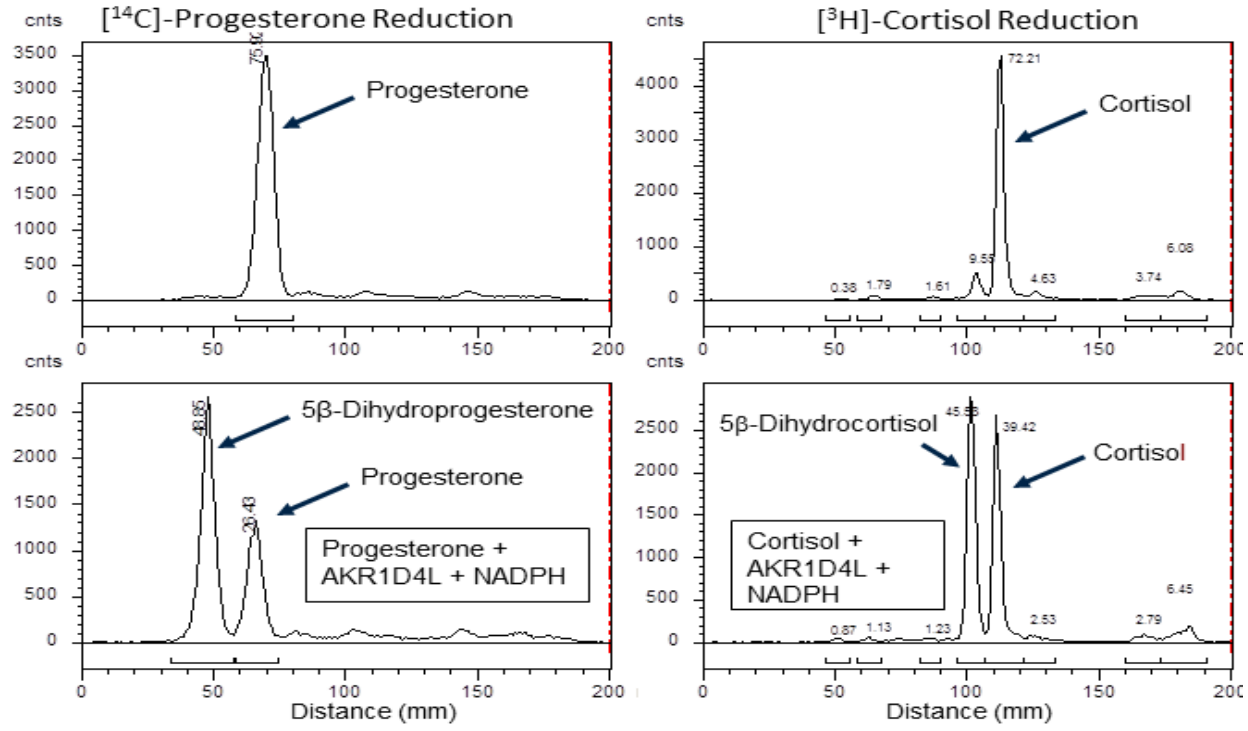


Figure 7. Radiochromatography of [^{14}C]-Progesterone and [^{3}H]-Cortisol Reduction Catalyzed by AKR1D4L. Either 10 μM [^{14}C]-Progesterone or 10 μM [^{3}H]-Cortisol were incubated with recombinant AKR1D4L in the presence of NADPH overnight for 24 h. Steroid products were extracted, dried and subjected to radiochromatography and identified by co-elution to synthetic standards; cnts = radioactive peak height; and distance = migration on the TLC plate. Top panel shows migration of synthetic standards

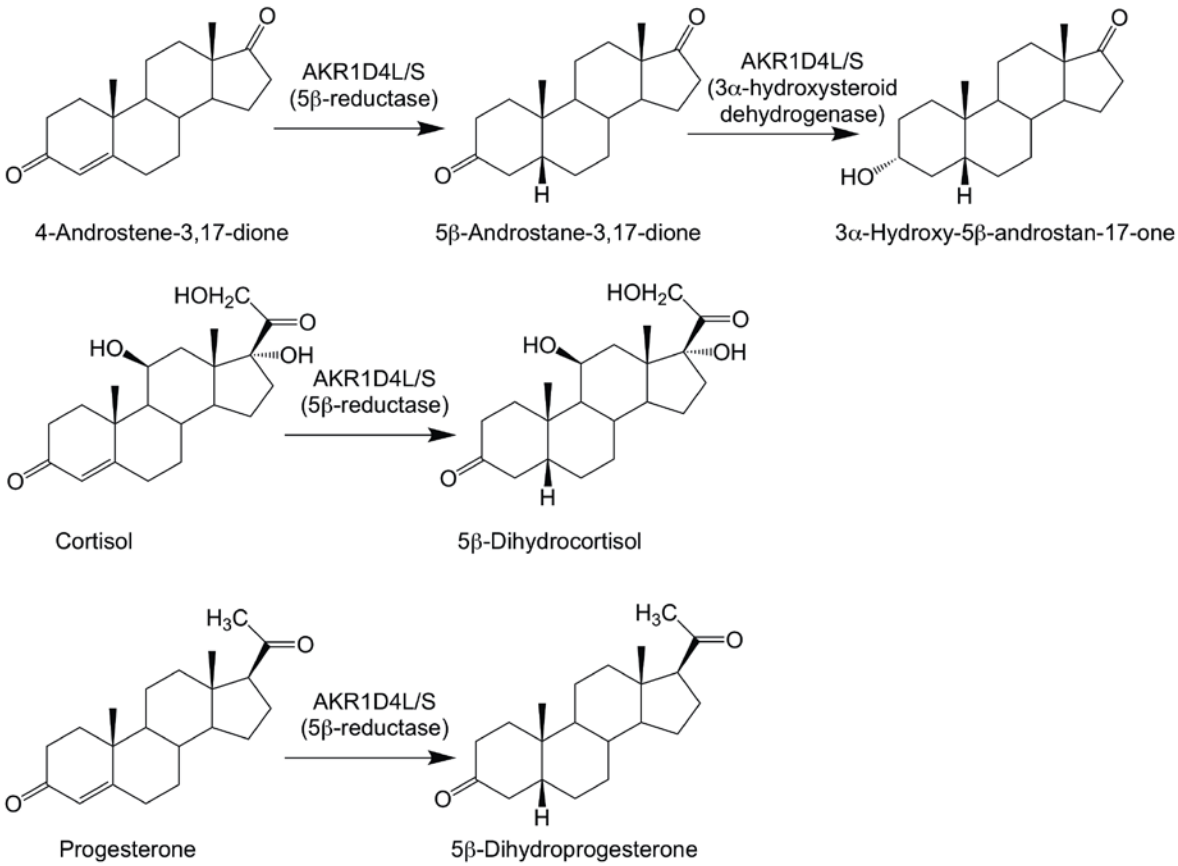
The conserved catalytic E120 in AKR1D enzymes was proposed to permit penetration of Δ^4-3-ketosteroids into the AKR1D active site and act as superacid based on the crystal structure of AKR1D1•$NADP^+$•Δ^4-3-ketosteroid complexes [11, 13]. The AKR1D1-E120H mutant showed almost perfect conversion of 5β-reductase to a 3β-hydroxysteroid dehydrogenase with an increase in catalytic efficiency for the dehydrogenase reaction of 10^6 [14]. The 3β-hydroxysteroid dehydrogenase reaction was also confirmed in the oxidation reduction using epi-androsterone as a substrate to form 5α-androstane-3,17-dione. Crystal structures of the AKR1D1–E120H•$NADP^+$•Epiandrosterone complex supported the concept that the presence of H120 blocked the penetration of steroid into the active site. And, thus reduction or oxidation at the C3 position was favored [14].

We now show that AKR1D4L and AKR1D4S display 3α-hydroxysteroid dehydrogenase on a C19 steroid, Δ^4-AD without mutation of E120 to histidine. This would suggest that E120 affects the correct positioning of the steroid for hydride transfer as proposed by Faucher et. al. [20] and may not have to act as a superacid. Long-range interactions likely favor the penetration of steroid into the AKR1D4 active sites since the 3α-hydroxysteroid dehydrogenase activity is not observed with either progesterone or cortisol both of which have side chains at C17. It is not possible to model these interactions in the absence of s crystal structure of a ternary complex for AKR1D4 since experience shows that the C17 side chain interacts with flexible loops at the back of the barrel structure.

There are notable differences between the 3β-hydroxysteroid dehydrogenase activity of AKR1D1-E120H and the 3α-hydoxysteroid dehydrogenase activity of AKR1D4L and AKR1D4S enzymes. The former activity is only observed with planar steroids with A/B *trans*-ring junctions but the later activity is observed with bent steroids with A/B *cis*-ring junctions. Based on these

differences we have avoided conducting molecular modeling studies to explain these differences. No crystal structure exists of AKR1D4L or AKR1D4S and thus a homology model would have to be built using an AKR1D1 structure. To dock steroids into such a model to explain preference for 3α-hydroxysteroid dehydrogenase activity for bent steroids in AKR1D4 over 3β-hydroxysteroid dehydrogenase activity in AKR1D1.E120H may be a daunting task. Crystal structures of AKR1C enzymes support the binding of steroids in many binding poses [21].

From a physiological perspective it is difficult to speculate on the impact of 3α-hydroxysteroid dehydrogenase in AKR1D4L and AKR1D4S on the metabolism of androgens to tetrahydrosteroids. It is clear that the reduction proceeds through the 5β-reduced steroid intermediate and the sequential reduction to a 5β-tetrahydrosteroid is not observed for the C21 steroids, cortisol or progesterone, Figure 8. Whether mice need a separate 3α-hydroxysteroid dehydrogenase to convert 5β-androstane-3,17-dione to 3α-hydroxy-5β-androstan-17-one is not clear. We suspect that the phenotype of mice which are AKR1D4 null or deficient may be difficult to interpret if there is unexpected effect on the metabolism of androgens.



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389

390 Figure 8. Sequential Reduction of 4-Androstene-3,17-dione to 3α-Hydroxy-5β-androstan-17-one
391 Catalyzed by AKR1D4L/S and Production of 5β-Dihydrosteroids of Cortisol and Progesterone
392 Catalyzed by AKR1D4L/S.
393

394 Interestingly, 5β-androstane-3,17-dione and 3α-hydroxy-5β-androstan-17-one are potent

395 inducers of erythropoiesis [22] in the chicken embryo and if they have the same function in

396 mammals, AKR1D4 deficient mice may be more prone to anemia.

397

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