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Stereospecific reduction of 5β-reduced steroids by human ketosteroid reductases of the AKR (aldo-keto reductase) superfamily: role of AKR1C1–AKR1C4 in the metabolism of testosterone and progesterone via the 5β-reductase pathway

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Active sex hormones such as testosterone and progesterone are metabolized to tetrahydrosteroids in the liver to terminate hormone action. One main metabolic pathway, the 5β-pathway, involves 5β-steroid reductase (AKR1D1, where AKR refers to the aldo-keto reductase superfamily), which catalyses the reduction of the 4-ene structure, and ketosteroid reductases (AKR1C1–AKR1C4), which catalyse the subsequent reduction of the 3-oxo group. The activities of the four human AKR1C enzymes on 5β-dihydrotestosterone, 5β-pregnane-3,20-dione and 20α-hydroxy-5β-pregnan-3-one, the intermediate 5β-dihydrosteroids on the 5β-pathway of testosterone and progesterone metabolism, were investigated. Product characterization by liquid chromatography–MS revealed that the reduction of the 3-oxo group of the three steroids predominantly favoured the formation of the corresponding 3α-hydroxy steroids. The stereochemistry was explained by molecular docking. Kinetic properties of the enzymes identified AKR1C4 as the major enzyme responsible for the hepatic formation of 5β-tetrahydrosteroid of testosterone, but indicated differential routes and roles of human AKR1C for the hepatic formation of 5β-tetrahydrosteroids of progesterone. Comparison of the kinetics of the AKR1C1–AKR1C4-catalysed reactions with those of AKR1D1 suggested that the three intermediate 5β-dihydrosteroids derived from testosterone and progesterone are unlikely to accumulate in liver, and that the identities and levels of 5β-reduced metabolites formed in peripheral tissues will be governed by the local expression of AKR1D1 and AKR1C1–AKR1C3.

Key words: dihydrosteroid, hydroxysteroid dehydrogenases, liquid-chromatography–MS (LC–MS), steroid metabolism, tetrahydrosteroid.

INTRODUCTION

The levels of active steroid hormones are tightly regulated through a balance of synthesis and metabolism. Liver is the primary site of metabolism for steroid hormones containing a Δ^4-3-one functionality, which include active hormones such as testosterone and progesterone. These active hormones are converted into THS (tetrahydrosteroids), which are conjugated in phase 2 reactions leading to elimination [1,2]. The metabolic sequence for the formation of THS involves the pathways shown in Scheme 1. In the first step, the 4-ene moiety of the steroid is reduced by 5α- or 5β-steroid reductases to form the respective DHS (dihydrosteroids). In the subsequent step, the 3-oxo group of DHS is reduced by ketosteroid reductases to form THS. 5α-Steroid reductases and the 5α-pathway have been a focus since the potent androgen 5α-DHT (5α-dihydrotestosterone, also called 17β-hydroxy-5α-androstan-3-one) is formed from testosterone [3,4]. In contrast, less is known about the 5β-pathway since 5β-steroid reductase, which is a member of the AKR (aldo-keto reductase) family (see http://www.med.upenn.edu/akr), named as AKR1D1, is historically believed to be only involved in steroid inactivation [5,6]. It is now recognized that the 5β-reduced steroids are not inactive. 5β-Pregnane-3,20-dione is a potent ligand for the PXR (pregnane X receptor) and the constitutive androstane receptor [7]. Activation of the hepatic PXR leads to induction of CYP3A4 (cytochrome P450 3A4), which is responsible for the metabolism of approximately 50 % of drugs consumed. Additionally, 5β-reduced pregnanes are important neuroactive steroids [8] and have been implicated in vasorelaxation [9] and human parturition [10,11].

Enzymes most implicated in the metabolism of 5α/β-DHS to the corresponding isomeric 3α/β,5α/β-THS are the NADPH-dependent HSDs (hydroxysteroid dehydrogenases) of the AKR1C subfamily [12,13]. The four human isoforms are: AKR1C1 (also known as 20α-HSD), AKR1C2 (also known as human type 3 3α-HSD), AKR1C3 (also known as human type 2 3α-HSD and type 5 17β-HSD) and AKR1C4 (also known as human type 1 3α-HSD). These enzymes are highly homologous in sequence (identity >86 %), but have different tissue expression properties. All isoforms are abundantly expressed in liver at comparable levels, but AKR1C4 is found only in liver, whereas AKR1C1–3 are differentially expressed in the lung, prostate, mammary gland and testis [13]. Individual human AKR1C enzymes catalyse ketosteroid reduction at the 3-, 17-, or 20-position to varying degrees depending on the substrate. Individual isoforms can reduce the same steroid with different

Abbreviations used: AKR, aldo-keto reductase; APCI, atmospheric pressure chemical ionization; HSD, hydroxysteroid dehydrogenase; DHS, dihydrosteroid(s); 5α/β-DHT, 5α/β-dihydrotestosterone; LC, liquid chromatography; MRM, multiple reaction monitoring; PXR, pregnane X receptor; THS, tetrahydrosteroid(s).

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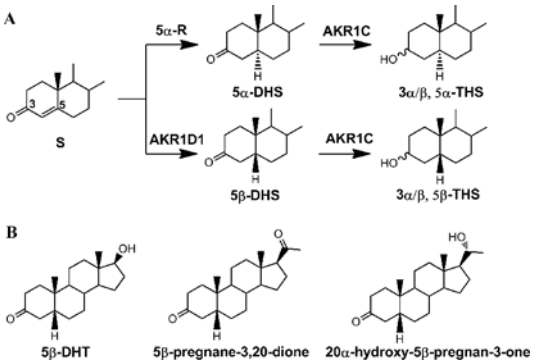


Scheme 1 Pathways of steroid metabolism leading to THS formation (A) and structures of the intermediate 5β-DHS in the pathways of testosterone and progesterone metabolism (B)

S, steroid.

stereochemical outcomes and the stereochemistry of the reaction is often steroid-dependent [4,14,15]. Although the four human AKR1C enzymes are implicated in reducing both 5α- and 5β-DHS, only their involvement in the reduction of 5α-DHT has been thoroughly investigated [4]. Incomplete kinetic data have been reported for the reduction of 5β-reduced steroids by human AKR1C enzymes [16–20], but there has not been any rigorous metabolite identification (i.e. by MS). Nor has there been any attempt to distinguish the role of individual human AKR1C isoforms on reducing 5β-DHS in testosterone and progesterone metabolism.

It is known that the major metabolites of testosterone include androstanediol (or 5α-androstane-3α,17β-diol, a 3α,5α-THS) and etiocholanolone (5β-androstane-3α,17β-diol, a 3α,5β-THS) [1]. Differential stereochemical and kinetic behaviour of human AKR1C enzymes established their different roles in the formation of 5α-THS during testosterone metabolism [4]. Specifically, with 5α-DHT, the intermediate steroid on the 5α-pathway, AKR1C1 acts as a reductive 3β-HSD to yield 5α-androstane-3β,17β-diol as the main product, AKR1C2 and AKR1C4 catalyse the efficient formation of 5α-androstane-3α,17β-diol, and AKR1C3 catalyses the slow formation of a mixture of 3α,5α- and 3β,5α-products. The liver-specific expression of AKR1C4 limits this enzyme to the clearance of 5α-DHT in that organ. However, AKR1C1 and AKR1C2 are believed to be responsible for the formation of 5α-androstane-3β,17β-diol [a pro-apoptotic ligand of ERβ (oestrogen receptor β)] and 5α-androstane-3α,17β-diol (an inactive androgen) respectively in androgen target tissues, such as the prostate.

A major metabolite of the potent progestin progesterone is pregnanediol (or 5β-pregnane-3α,20α-diol), which is a 3α,5β-THS, indicating the importance of the 5β-pathway in the metabolism of progesterone [1]. Progesterone metabolism via the 5β-pathway can occur similarly to testosterone metabolism, i.e. progesterone is reduced to 5β-pregnane-3,20-dione by AKR1D1, which in turn is reduced at the 3-oxo position by AKR1C1–4 (Scheme 1). However, since human AKR1C enzymes also have 20-ketoreductase activity, formation of 20-hydroxy steroids is also possible. Thus, alternatively, progesterone can be first reduced at the 20-oxo position to form 20α-hydroxyprogesterone, a reaction that is known to be catalysed by AKR1C1 [13]. 20α-Hydroxyprogesterone can then be reduced by AKR1D1 to form 20α-hydroxy-5β-pregnan-3-one, which in turn can be reduced by AKR1C1–4 enzymes to isomeric 5β-pregnane-3,20-diols. Thus, for progesterone, there are two possible intermediate 5β-DHS on the 5β-pathway of progesterone metabolism, i.e. 5β-pregnane-3,20-dione and 20α-hydroxy-5β-pregnan-3-one.

In the present study, we have characterized the stereochemical outcomes and kinetic properties of the reduction of three intermediate 5β-DHS, i.e. 5β-DHT, 5β-pregnane-3,20-dione and 20α-hydroxy-5β-pregnan-3-one, catalysed by AKR1C1–4 enzymes. We demonstrate that the 3-oxo reduction of these three 5β-reduced steroids by all of the human AKR1C enzymes stereospecifically favoured the formation of 3α,5β-THS. Our results support differential roles of human AKR1C enzymes in the hepatic metabolism of active testosterone and progesterone. In addition, the activities of AKR1C1–4 enzymes were compared with that of AKR1D1 in the preceding step of the pathway (i.e. the 5β-reduction). Results suggest that the intermediate 5β-DHS are unlikely to accumulate in the liver and enter the circulation. Local formation of bioactive 5β-reduced steroids would thus be dependent upon the presence of AKR1D1 when it co-exists with extrahepatic AKR1C1–3 isoforms.

EXPERIMENTAL

Materials

All steroids were obtained from Steraloids. Pyridine nucleotides were purchased from Roche Applied Science. All other reagents were purchased from Sigma–Aldrich and were of ACS (American Chemical Society) grade or better. Recombinant AKR1C1–4 enzymes were overexpressed and purified to homogeneity as described previously [21,22]. Under standard assay conditions, the specific activities of AKR1C1–3 were determined to be 1.8 μmol/min per mg for AKR1C1, 2.3 μmol/min per mg for AKR1C2 and 1.3 μmol/min per mg for AKR1C3 for the $NADP^+$-dependent oxidation of 0.2 mM 1-acenaphthenol, the specific activity of AKR1C4 was 0.3 μmol/min per mg for the NAD^+-dependent oxidation of 75 μM androsterone and the specific activity of AKR1D1 was 80 nmol/min per mg for the NADPH-dependent reduction of 10 μM testosterone [14,21,23].

LC (liquid chromatography)–MS analysis

Products for the reduction of ketosteroids catalysed by AKR1C1–4 enzymes were prepared as follows. Reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.0), 20 μM steroid, 0.5 mM NADPH and 4 % methanol in a total volume of 500 μl. The reaction was initiated by the addition of purified enzyme (7.3 μg of AKR1C1, 9.0 μg of AKR1C2, 12.3 μg of AKR1C3 or 5.6 μg of AKR1C4), and for the no-enzyme control buffer was substituted. Systems were incubated at 37 °C and incubation times were 20 min and 90 min for the enzymatic reactions and 90 min for non-enzymatic controls. Reaction mixtures were extracted twice with 1 ml of water-saturated ethyl acetate. The pooled organic extracts were vacuum-dried and the residues were re-dissolved in 200 μl of methanol.

Normal-phase chiral chromatography for LC–MS experiments was performed using a Waters Alliance 2690 HPLC system. Gradient elution was performed in the linear mode. A Chiralpak IA column (250 mm long × 4.6 mm diameter, internal diameter 5 μm; Daicel Chemical Industries) was employed with a flow rate of 1 ml/min. Solvent A was hexanes (Fisher, Optima grade) and solvent B was ethanol/hexanes (1:1, v/v). A linear gradient was used starting with 98.5 % solvent A to 50 % solvent A in 17 min and maintained for 5 min to elute all of the polar compounds and prevent overpressure. At 25 min the gradient was changed to 98.5 % solvent A and kept at 98.5 % to re-equilibrate the column to the initial conditions. The separation was performed at 30 °C.

MS was conducted using a Finnigan TSQ Quantum Ultra AM spectrometer (Thermo Fisher) equipped with an APCI (atmospheric pressure chemical ionization) source operated in the positive-ion mode. Full-scan analysis was conducted in Q3 only in the mass interval 150–400. For MRM (multiple reaction monitoring) analyses, unit resolution was maintained for both parent and product ions. Operating conditions were as follows: vaporizer temperature 450 °C, heated capillary temperature 300 °C with a discharge current of 6 μA applied to the corona needle, and nitrogen was used for the sheath gas, auxiliary gas and ion sweep gas, set at 25, 5 and 3 (in arbitrary units) respectively. CID (collision-induced dissiociation) was performed using argon as the collision gas at 1.5 mTorr in the second [r.f. (radio frequency)-only] quadrupole. An additional D.C. offset voltage was applied to the region of the second multipole ion guide (Q0) at 10 V to impart enough translational kinetic energy to the ions so that solvent adduct ions dissociate to form sample ions. Full scanning analyses were performed in the range of *m/z* 100 to *m/z* 600. Products of the reactions were identified on the basis of their LC retention times and mass spectra relative to those observed with the authentic standards. LC–APCI/MRM/MS analyses were conducted using *m/z* 291 ($[M+H]^+$)→273 ($[M+H–H_2O]^+$) (collision energy, 26 eV) for 5β-DHT; *m/z* 275 ($[M+H–H_2O]^+$)→259 ($[M+H–2H_2O]^+$) (collision energy, 26 eV) for 5β-androstane-3α/β,17β-diol; *m/z* 317 ($[M+H]^+$)→299 ($[M+H–H_2O]^+$) (collision energy, 23 eV) for 5β-pregnane-3,20-dione; *m/z* 301($[M+H–H_2O]^+$) →283 ($[M+H–H_2O]^+$) (collision energy, 23 eV) for 3α/β-hydroxy-5β-pregnan-20-one and 20α/β-hydroxy-5β-pregnan-3-one; and *m/z* 303 ($[M+H–H_2O]^+$)→285 ($[M+H–H_2O]^+$) (collision energy, 23 eV) for 5β-pregnane-3α/β,20α/β-diols.

TLC analysis

Samples were prepared in a similar fashion as for LC–MS analysis and applied to Partisil LK6D Silica TLC plates (Whatman). Chromatograms were developed in cyclohexane/ethyl acetate (5:13, v/v). Products were identified by co-migration with authentic standards applied to the same plate following visualization by spraying with acetic acid/sulfuric acid/anisaldehyde (100:2:1, by vol.) and heat.

Determination of steady-state kinetic parameters

Initial rates of the NADPH-dependent reduction of ketosteroids catalysed by AKR1C1–4 enzymes were measured with a Hitachi F-2500 fluorescence spectrophotometer by monitoring the change in fluorescence emission of NADPH. λ_{ex} and λ_{em} wavelengths were set at 340 nm and 450 nm respectively. Changes in fluorescence units were converted into nanomoles of cofactor by using standard curves of fluorescence emission against known NADPH concentrations. Typical reaction samples contained enzyme (1.4 μg of AKR1C1, 1.2 μg of AKR1C2, 1.6 μg of AKR1C3, 0.8 μg of AKR1C4 or 2.1 μg of AKR1D1), NADPH (saturating concentration 8 μM or 25 μM), various steroid concentrations (0.1 μM–50 μM), 100 mM potassium phosphate buffer (pH 7.0) and 4 % methanol in a total volume of 1 ml. Reaction mixtures were run at 25 °C. Analysis of kinetic data was carried out as described previously [14]. Data were fitted by non-linear least-squares regression to eqn (1):

$$v = \frac{k_{cat}[E][S]}{K_m + [S]} \quad (1)$$

or eqn (2):

$$v = \frac{k_{cat}[E]}{1 + (K_m/[S]) + [S]/K_i} \quad (2)$$

where v is the initial velocity, [E] and [S] are the total molar concentrations of the enzyme and steroid substrate respectively, k_{cat} (s^{-1}) is the turnover number, K_m (μM) is the apparent Michaelis–Menten constant for the steroid substrate and K_i (μM) is the apparent dissociation constant for the presumed inhibitory enzyme–substrate complex [24].

Molecular modelling

Conformations of 5α-DHT bound to AKR1C1 (PDB code 1MRQ) and AKR1C2 (PDB code 1IHI) found previously using the automated docking program AUTODOCK were used as the templates to model the binding of 5β-DHT [25]. The structure of 5β-DHT was retrieved from the co-ordinates of the ternary complex AKR1D1–$NADP^+$–5β-DHT (PDB code 3DOP). Two templates were used for the manual docking of 5β-DHT. Template 1 is the productive docking position of 5α-DHT in AKR1C2 for the formation of the 3α-reduced product, i.e. the β-face of the steroid is presented to NADPH. Template 2 is the productive docking position of 5α-DHT in AKR1C1 for the formation of the 3β-reduced product, i.e. the α-face of the steroid is presented to NADPH. Docking of 5β-DHT was performed as follows. With each template, 5β-DHT was manually docked in position by overlaying the 3-oxo group of 5β-DHT to that of the template. The docking of 5β-DHT on to the template was examined with the steroids in a similar facial orientation and also in a reversed facial orientation. Positions of 5β-DHT were then adjusted to avoid steric clashing with the surrounding residues if possible without significantly changing the position and orientation of the 3-oxo group. The four docked models were evaluated on the basis of distances to surrounding residues as either 'allowed' or 'disallowed' (<2 Å; 1 Å = 0.1 nm).

RESULTS

Reduction of 5β-DHT catalysed by AKR1C1–4

The 3-oxo reduction of 5β-DHT can yield two possible products, 5β-androstane-3α,17β-diol and 5β-androstane-3β,17β-diol. Both synthetic standards are available and could be well separated by TLC (results not shown) and LC–MS methods (Figure 1). The NADPH-dependent reductions of 5β-DHT catalysed by AKR1C1–4 were analysed. All reactions were run to completion, where the relative intensities of the 5β-DHT peak in samples containing AKR1C1–4 were less than 2 % of the 5β-DHT peak in the non-enzymatic control sample. In all samples containing AKR1C enzymes, 5β-androstane-3α,17β-diol was found to be the predominant product. Only with AKR1C2 did the reaction yield a low but significant amount of 5β-androstane-3β,17β-diol (11.4 % of the total products) (Table 1). In contrast, reaction samples with AKR1C1, AKR1C3 and AKR1C4 contained less than 1.5 % of the total products as 5β-androstane-3β,17β-diol. No significant epimerase activity was observed with AKR1C3 and AKR1C4, where epimerase activity was seen during the reduction of 5α-DHT and 5α-DHT-17β-sulfate by these enyzmes [4,14].

Individual human AKR1C enzymes are known to have different kinetic properties with the same steroid substrate [4,13–15]. This was also true with 5β-DHT. k_{cat} and K_m values for the reduction of 5β-DHT catalysed by AKR1C1–4 isoforms were determined by continuous spectrofluorimetric assays where the

Table 1 Product distribution for the reduction of 5β-DHT and 5β-pregnane-3,20-dione catalysed by human AKR1C enzymes

For 5β-DHT reduction, 3α- and 3β- products were 5β-androstane-3α,17β-diol and 5β-androstane-3β,17β-diol. There was no significant difference in product distribution between samples from 20 min and 90 min incubations. For 5β-pregnane-3,20-dione reduction, 3α- and 3α,20α- products were 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3α,20α-diol. Values are shown as percentage of total.

	Substrate . . .	5β-DHT		5β-pregnane-3,20-dione	
Enzyme	Product. . .	3α-	3β-	3α-	3α,20α-
AKR1C1		98.5	1.5	14.2	85.8
AKR1C2		88.6	11.4	66.2	33.8
AKR1C3		99.6	0.4	76.6	23.4
AKR1C4		99.3	0.7	22.4	77.6

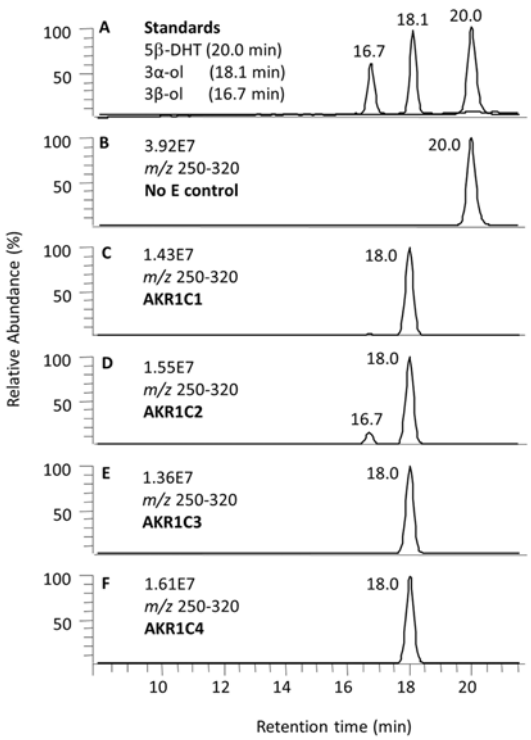


Figure 1 Product characterization by LC–MS for the reduction of 5β-DHT catalysed by human AKR1C isoforms

(**A**) Ion chromatograms (*m/z* 250–320) of authentic standards. 3α-ol, 5β-androstane-3α,17β-diol; 3β-ol, 5β-androstane-3β,17β-diol. (**B**–**F**) Corresponding ion chromatograms of reaction samples containing no enzyme (**B**) and AKR1C1–4 (**C**–**F**). Samples were prepared as described in the Experimental section. The disappearance of substrate in each reaction sample containing enzyme indicated that the reaction had reached the end point.

depletion of NADPH was monitored (see the Supplementary Figure S1 at http://www.BiochemJ.org/bj/437/bj4370053add.htm for representative results). Thus, for the reduction of 5β-DHT catalysed by AKR1C2, the activity measured represented the total activity, since a mixture of stereoisomeric products were formed. Results are shown in Table 2 (see Supplementary Figure S1 for representative results). AKR1C4 was found to be the most efficient enzyme for the reduction of 5β-DHT with the highest k_{cat} and the lowest K_m values. The catalytic activity of AKR1C1 displayed a k_{cat} value that was approximately three times lower and a K_m that was 26 times higher compared with AKR1C4. The kinetic behaviours of AKR1C2 and AKR1C3 were similar for the reduction of 5β-DHT, both showing a low catalytic efficiency and moderate substrate inhibition.

Reduction of 5β-pregnane-3,20-dione and 20α-hydroxy-5β-pregnan-3-one catalysed by AKR1C1–4

5β-Pregnane-3,20-dione and 20α-hydroxy-5β-pregnan-3-one are the intermediate 5β-DHS on the 5β-pathway of progesterone metabolism, which are subject to metabolic transformation by AKR1C enzymes for the formation of 5β-THS.

For 5β-pregnane-3,20-dione, both the 3- and 20-oxo groups can potentially be reduced by AKR1C enzymes. There are eight possible products for the reaction, which include four monohydroxy products (3α-hydroxy-5β-pregnan-20-one, 3β-hydroxy-5β-pregnan-20-one, 20α-hydroxy-5β-pregnan-3-one and 20β-hydroxy-5β-pregnan-3-one) and four dihydroxy products (5β-pregnane-3α,20α-diol, 5β-pregnane-3β,20α-diol, 5β-pregnane-3α,20β-diol and 5β-pregnane-3β,20β-diol). Synthetic standards for all possible products were available for product authentication by TLC and LC–MS. In LC–MS analysis, 5β-pregnane-3α,20β-diol and 5β-pregnane-3β,20β-diol could not be separated. However, lack of separation of these two standards by LC–MS had no effect on the results of the experiment, since no product peaks around their retention times were detected in the reaction samples. The retention time of 5β-pregnane-3α,20α-diol was close to 20β-hydroxy-5β-pregnan-3-one in LC–MS analysis; however, they could be well separated with TLC.

It was found by LC–MS analysis and TLC that, for the reduction of 5β-pregnane-3,20-dione catalysed by AKR1C enzymes, 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3α,20α-diol were detected as the main products at the end point of the reaction (Figure 2). The distribution between the two products for each AKR1C1–4 enzyme varies, with 5β-pregnane-3α,20α-diol as the major product for the AKR1C1 and AKR1C4 reactions and 3α-hydroxy-5β-pregnan-20-one as the major product for AKR1C2 and AKR1C3 (Table 1). Interestingly, significant transient formation of 20α-hydroxy-5β-pregnan-3-one was observed at earlier time points of the 5β-pregnane-3,20-dione reaction catalysed by AKR1C1 (Supplementary Figure S2 at http://www.BiochemJ.org/bj/437/bj4370053add.htm). Thus, in the reaction catalysed by AKR1C1, two monohydroxy products, 3α-hydroxy-5β-pregnan-20-one and 20α-hydroxy-5β-pregnan-3-one, were formed in comparable amounts, each of which was subsequently further converted into 5β-pregnane-3α,20α-diol. In contrast, only one monohydroxy product, 3α-hydroxy-5β-pregnan-20-one, was observed in the reaction catalysed by AKR1C2–4. The distribution between the the monohydroxy and dihydroxy products suggests that formation of the dihydroxy product was much slower for AKR1C2 and AKR1C3 than for AKR1C4.

For the reduction of 20α-hydroxy-5β-pregnan-3-one catalysed by AKR1C1–4 enzymes, there are two possible products: 5β-pregnane-3α,20α-diol and 5β-pregnane-3β,20α-diol. Product identification by LC–MS and TLC showed only the formation of 5β-pregnane-3α,20α-diol for the reaction catalysed by all four AKR1C enzymes.

Kinetic properties for the reductions of 5β-pregnane-3,20-dione and 20α-hydroxy-5β-pregnan-3-one catalysed by each AKR1C1–4 enzyme are shown in Table 2 (also see Supplementary Figure S1). With 5β-pregnane-3,20-dione as the substrate, AKR1C4 was moderately more efficient: its catalytic efficiency was 1.5-, 2.4- and 5.4-fold higher than those of AKR1C1,

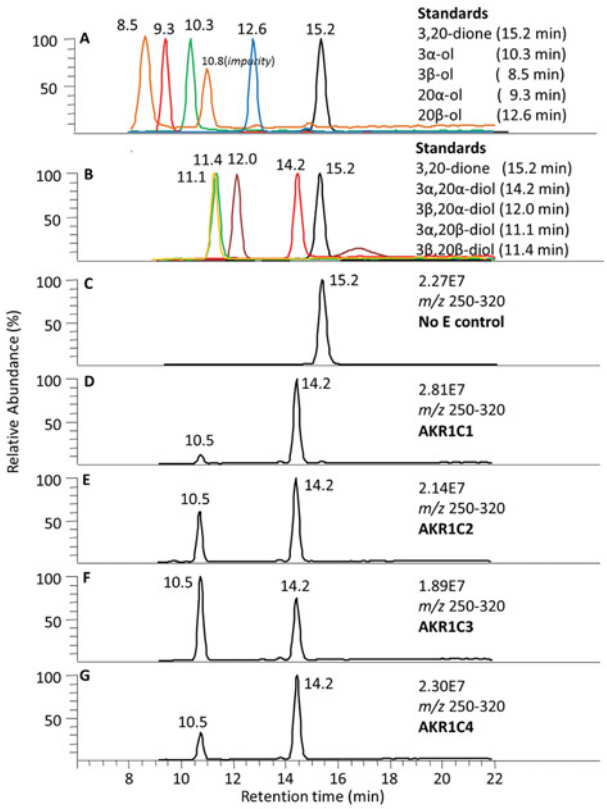


Figure 2 Product characterizations by LC–MS for the reduction of 5β-pregnane-3,20-dione catalysed by human AKR1C isoforms

(**A**) and (**B**) Ion chromatograms (*m/z* 250–320) of authentic standards. 3,20-dione, 5β-pregnane-3,20-dione; 3α-ol, 3α-hydroxy-5β-pregnan-20-one; 3β-ol, 3β-hydroxy-5β-pregnan-20-one; 20α-ol, 20α-hydroxy-5β-pregnan-3-one; 20β-ol, 20β-hydroxy-5β-pregnan-3-one; 3α,20α-diol, 5β-pregnane-3α,20α-diol; 3β,20α-diol, 5β-pregnane-3β,20α-diol; 3α,20β-diol, 5β-pregnane-3α,20β-diol; 3β,20β-diol, 5β-pregnane-3β,20β-diol. (**C**–**G**) Ion chromatograms of reaction samples containing no enzyme (**C**) and AKR1C1–4 (**D**–**G**). Samples were prepared as described in the Experimental section. The disappearance of substrate in each reaction sample containing enzyme indicated that the reaction had reached the end point.

AKR1C2 and AKR1C3 respectively. This difference in catalytic efficiency stemmed mostly from different k_{cat} values, since similar K_m values were obtained for AKR1C1–4. With the exception of AKR1C4, low degrees of substrate inhibition ($K_i/K_m > 16$) were observed for the reduction of 5β-pregnane-3,20-dione catalysed by AKR1C1–3. With 20α-hydroxy-5β-pregnan-3-one as the substrate, the differences in catalytic efficiency between AKR1C4 and AKR1C1–3 were more pronounced such that the catalytic efficiency of AKR1C4 was 8.9-, 12- and 15-fold higher than those of AKR1C1, AKR1C2 andAKR1C3 respectively. Low degrees of substrate inhibition ($K_i/K_m > 15$) were also observed for the reduction of 20α-hydroxy-5β-pregnan-3-one catalysed by AKR1C1, AKR1C3 and AKR1C4, but not for that by AKR1C2.

Kinetic properties for the reduction of 3α-hydroxy-5β-pregnan-20-one were also examined to compare the 20-oxo reduction activity of AKR1C enzymes with the 3-oxo reduction activity of 5β-steroids (Table 2). As expected, AKR1C1 displayed the highest k_{cat} value. Activities of AKR1C2 and AKR1C3 for this reaction were low. Although the value of k_{cat} for AKR1C4 was 4-fold lower than that for AKR1C1, a relatively smaller K_m of AKR1C4 resulted in a slightly better catalytic efficiency for the enzyme than AKR1C1.

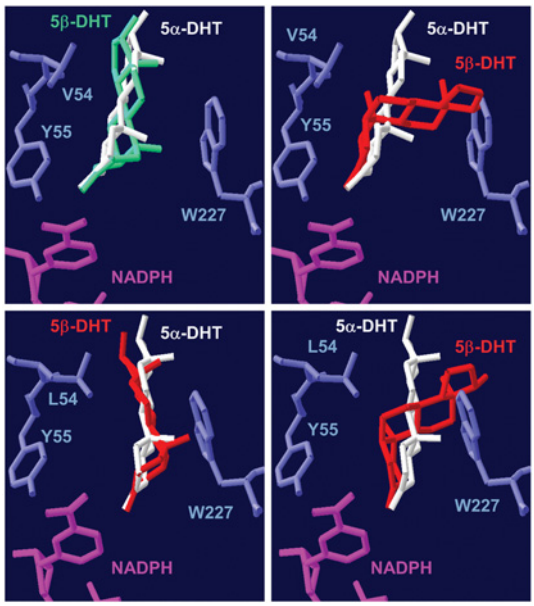


Figure 3 Docking of 5β-DHT into AKR1C active sites

Details of the model building are given in the Experimental section. Residues in the enzyme active sites are shown in blue. The cofactor is shown in purple, and the 5α-DHT template is shown in white. Docked 5β-DHT is shown in green (sterically allowed position) or red (sterically disallowed position). In the upper panels, the 5α-DHT template was positioned for 3α-reduction in AKR1C2. Upper left-hand panel, 5β-DHT docked on the template position so that the facial orientation of the A-ring was the same. Upper right-hand panel, 5β-DHT docked on the template position so that the facial orientation of the A-ring was inverted. In the lower panels, the 5α-DHT template was positioned for 3β-reduction in AKR1C1. Lower left-hand panel, 5β-DHT docked on the template position so that the facial orientation of the A-ring was the same. Lower right-hand panel, 5β-DHT docked on the template position so that the facial orientation of the A-ring was inverted. The Figures were prepared using Swiss-PDBViewer.

Reduction of testosterone, progesterone and 20α-hydroxyprogesterone catalysed by AKR1D1

Kinetic properties for the reduction of testosterone, progesterone and 20α-hydroxyprogesterone catalysed by AKR1D1 were examined (Table 2). The common feature of these reactions was the observation of potent substrate inhibition. For both testosterone and 20α-hyroxyprogesterone, K_i values were determined to be significantly lower than their respective K_m values. Thus the observed reaction rates were significantly lower than their estimated V_{max} values. In the reaction of progesterone, strong substrate inhibition was also observed. The data point of the lowest progesterone concentration displayed the highest reaction rate, so the kinetic parameters for the reaction could not be accurately determined. The rate constant reported was calculated on the basis of highest observed rate.

Molecular modelling of 5β-DHT binding in AKR1C1–4

Using an automated docking method we have previously found 'productive' docked positions of 5α-DHT in AKR1C1 and AKR1C2 to account for the stereospecific formation of 3β- and 3α-reduced products by the two enzymes respectively [25]. These productive positions of 5α-DHT were used as templates to model 5β-DHT binding. 5β-DHT was manually docked in the productive position of 5α-DHT in AKR1C2 using a similar facial orientation. The resulting model placed the 3-oxo group in position for a reaction in which the β-face of the steroid faces the 4-pro-*R*-hydride of the cofactor (Figure 3, upper left-hand panel). This model was deemed as 'allowed' since no steric

Table 2 Kinetic parameters for the reactions catalysed by AKR in the 5β-pathway for the metabolism of testosterone and progesterone

Steady-state parameters were determined using a fluorimetric assay. Values are given as means ± S.D. (n>2). n.a., not available.

Substrate (reaction)	Enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1} \cdot M^{-1}$) × 10^6	K_i (μM)
5β-DHT (3-oxo reduction)	AKR1C1	1.7 ± 0.1	5.2 ± 0.6	0.33	–
	AKR1C2	0.9 ± 0.1	0.8 ± 0.1	1.1	4.0 ± 0.2
	AKR1C3	1.2 ± 0.2	1.4 ± 0.3	0.8	5.5 ± 1.0
	AKR1C4	4.7 ± 0.3	0.2 ± 0.05	23.5	–
5β-Pregnane-3,20-dione (3- and 20-oxo reduction)	AKR1C1	4.3 ± 0.2	0.4 ± 0.1	10.8	24 ± 3
	AKR1C2	2.0 ± 0.2	0.3 ± 0.1	6.7	4.7 ± 1.2
	AKR1C3	0.9 ± 0.3	0.3 ± 0.1	3.0	5.7 ± 1.3
	AKR1C4	4.9 ± 0.1	0.3 ± 0.06	16.3	–
20α-Hydroxy-5β-pregnan-3-one (3-oxo reduction)	AKR1C1	2.1 ± 0.1	1.3 ± 0.1	1.6	28.5 ± 0.9
	AKR1C2	0.6 ± 0.1	0.5 ± 0.1	1.2	–
	AKR1C3	1.6 ± 0.3	1.7 ± 0.2	0.94	26 ± 3
	AKR1C4	4.3 ± 0.6	0.3 ± 0.1	14.3	8.2 ± 2.5
3α-Hydroxy-5β-pregnan-20-one (20-oxo reduction)	AKR1C1	4.0 ± 0.2	1.4 ± 0.2	2.9	–
	AKR1C2	0.9 ± 0.1	1.2 ± 0.4	0.75	22 ± 10
	AKR1C3	0.4 ± 0.04	1.3 ± 0.2	0.31	–
	AKR1C4	1.5 ± 0.05	0.4 ± 0.07	3.8	–
Testosterone (5β-reduction)	AKR1D1	4.7 ± 2.1	1.2 ± 0.7	3.9	0.4 ± 0.2
Progesterone (5β-reduction)*	AKR1D1	(0.21 ± 0.03)	(< 0.1)	n.a.	n.a.
20α-Hydroxyprogesterone (5β-reduction)	AKR1D1	12.3 ± 3.1	1.4 ± 0.4	8.8	0.06 ± 0.02

*For the reduction of progesterone catalysed by AKR1D1, the observed maximum rate constant was reported.

conflict with surrounding residues were observed. Using the same template, the facial orientation of the 5β-DHT was inverted to make the α-face of the steroid presented to the cofactor for 3β-HSD reaction (Figure 3, upper right-hand panel). However, the resulting model is clearly disallowed since the bent structure in 5β-DHT caused the steroid nucleus to clash with the side chain of Trp^{227}. Similar docking experiments were performed using the productive position of 5α-DHT in AKR1C1 as the template (the steroid was positioned for the formation of 3β-reduced product) (Figure 3, lower panels). 5β-DHT could not be accommodated in the position of this template in either facial orientation. Our docking experiments revealed that the steroid-binding channel of all human AKR1C enzymes is narrow between residues 54 and 227, and cannot accommodate a 5β-steroid in positions that lead to a 3β-HSD reaction. Of the four isoforms, only AKR1C2 has a valine residue at position 54, whereas the others have the slightly bulky Leu^{54}. This would explain why 5β-androstane-3β,17β-diol was observed as a minor product only with AKR1C2.

DISCUSSION

Active sex hormones such as testosterone (a potent androgen) and progesterone (a potent progestin) are extensively metabolized to THS in liver for the termination of hormone action. This is achieved by the sequential reduction of the 4-ene moiety and the 3-oxo group (Scheme 1). The four human AKR1C enzymes are implicated to perform reactions following either 5α-reductase or AKR1D1 to form 5α- and 5β-THS products respectively. Historically, 5β-reduced steroids have been mostly regarded as inactive steroids and thus received less attention than their 5α-reduced counterparts. The formation of 5β-reduced steroids is inarguably catabolically important since it is believed to account for two-thirds of the mass of steroids that are inactivated [26]. In the present study, the role of individual AKR1C1–4 enzymes on the 5β-pathways of testosterone and progesterone metabolism was elucidated.

Stereospecificity of 3-oxoreduction of 5β-DHS catalysed by human AKR1C isoforms

The 3-oxo reduction of 5β-DHT, 5β-pregnane-3,20-dione and 5β-pregnan-20α-3-one catalysed by all four human AKR1C enzymes yielded respective steroid products in the 3α-configuration as the sole or predominant product. This stereospecificity is in stark contrast with the previous observation that for the 3-oxo reduction of 5α-DHT and the hormone-replacement therapeutic tibolone (a Δ^5-3-one steroid) the distribution between 3α- and 3β-reduced products can vary significantly with each enzyme–substrate combination [4,15]. A comparison between 5α-DHT and 5β-DHT as substrate for AKR1C1–4 enzymes highlights the effect of the configuration of A/B-ring junction on stereochemical outcome of 3-oxo reduction. When 5α-DHT was used as the substrate, the reactions catalysed by AKR1C1, 3 and 4 enzymes yielded a mixture of 3α- and 3β-hydroxy androstanediols in comparable amounts (the ratios between the two products were 1:2.9, 1.6:1 and 3.6:1 respectively), whereas the reaction catalysed by AKR1C2 generated the 3α-reduced steroid as the predominant product (the 3α/3β ratio was 20:1). In contrast, when 5β-DHT was used as the substrate, the reactions catalysed by all four isoforms predominantly formed the 3α-reduced steroid (the 3α/3β ratios were 66:1, 7.8:1, 271:1 and 137:1 respectively). Again the AKR1C2 reaction was an exception in that a small but significant amount of 3β-product was now observed. These results confirmed that the structure of the A-ring significantly affects the stereochemical outcome of the reaction.

Molecular modelling of the binding of 5β-DHT provided the structural basis for the effect of A-ring structure on stereochemistry. 5β-Reduced steroids are structurally distinct in that the 5β-configuration causes a 90° bend in the A/B-ring junction. It was found that a steroid with a bent structure can only be accommodated without steric clashing when its β-face is presented to the cofactor so that only the 3α-axial alcohol will form. This restriction is largely caused by the narrow channel formed between the side chains of residues 54 and 227 on the

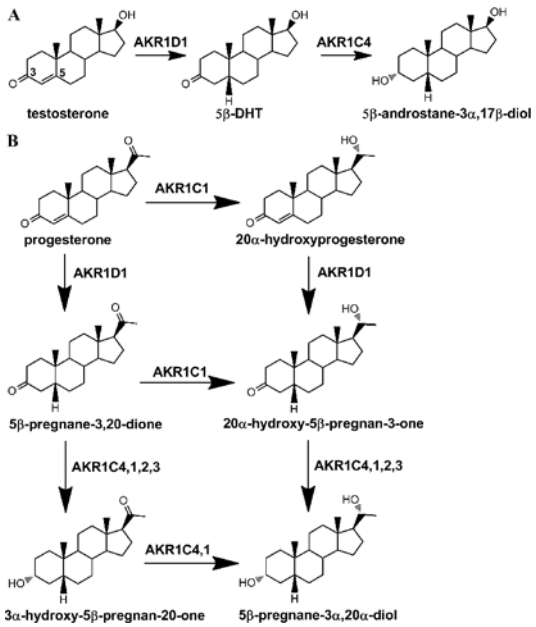
opposite sides of the steroid-binding pocket. The steroid is less restricted in AKR1C2 since the enzyme has a less bulky side chain at position 54 compared with other isoforms.

Substrate preferences of AKR1C1–4

All four human AKR1C enzymes are able to catalyse ketosteroid reduction at 3-, 17-, and 20-positions of steroids [13]. AKR1C1 is considered mainly as a 20-ketoreductase on the basis of its preference to reduce progesterone, whereas AKR1C2 and AKR1C4 are efficient 3-oxosteroid reductases and AKR1C3 acts as a 17-ketosteroid reductase. Our results in the present study indicate that these assignments can change on the basis of the substrate examined. When 5α- and 5β-DHT are compared as substrates, with the exception of AKR1C2, human AKR1C enzymes in general displayed higher turnover numbers with the 5β-reduced steroid substrates. Increased reactivity with 5β-reduced steroids at the 3-position resulted in comparable reactivity for reduction at the 3-position and the 20-position for AKR1C1. AKR1C2, however, is a poor enzyme with 5β-reduced steroids for reaction at either the 3- or 20-positions, due to its decreased activity for 3-oxo reduction. Interestingly, AKR1C3 displayed better turnover numbers for 3-ketoreduction (5β-DHT and 20α-hydroxy-5β-pregnan-3-one) than that for 20-ketoreduction (3α-hydroxy-5β-pregnan-20-one), and these turnover numbers were also higher than that for its known 17-ketoreduction activity [13]. AKR1C4 retains its strong preference for reaction at the 3-position with 5β-reduced substrates as seen with 5α-reduced substrates. These substrate preferences suggest different roles of individual AKR1C1–4 enzymes in systemic (liver) and peripheral steroid hormone metabolism.

Implications for 5β-THS formation in liver

AKR1D1 is known to be predominantly expressed in liver [27] and is now shown to efficiently catalyse the reduction of testosterone, progesterone and 20α-hydroxyprogesterone. Strikingly, all three reactions displayed potent substrate inhibition. With testosterone and 20α-hydroxyprogesterone, the values of K_i, estimated by fitting the data to the kinetic equation with a substrate inhibition term, were significantly less than their respective values of K_m. As a result of the potent substrate inhibition, the value of k_{cat} predicted by data analysis could not be effectively reached in these reactions. In fact, the observed maximum rate constants were 5- and 10-fold lower than their predicted k_{cat} values for testosterone and 20α-hydroxyprogesterone respectively. With progesterone, values of k_{cat} and K_m could not be accurately determined; the available data indicated strong substrate inhibition that resulted in low observed reaction rates. When the kinetics of the step catalysed by AKR1D1 were compared with the subsequent step catalysed by AKR1C1–4, the catalytic efficiencies of AKR1D1 were found to be much lower than those of AKR1C4, which displayed the highest catalytic efficiency among human AKR1C isoforms for 3-oxo reduction with the 5β-reduced substrates. This would suggest that the formation of 5β-DHS (the reaction catalysed by AKR1D1) occurs less efficiently than the disappearance of 5β-DHS (the reaction catalysed by AKR1C1–4 enzymes). Taken together, it appears that the intermediate 5β-DHS metabolites are prevented from accumulating in the liver by strong substrate inhibition in the AKR1D1 reaction and by the fast reaction catalysed by AKR1C4 leading to their metabolism to 5β-THS. This is consistent with the observation of very low levels of 5β-pregnane-3,20-dione in circulation [28]. Since the build-up of 5β-pregnane-3,20-dione is predicted not to occur in the liver, a different ligand may be responsible for the activation of PXR in this organ.



Scheme 2 Proposed roles of AKR enzymes on the 5β-pathways of (A) testosterone and (B) progesterone metabolism

Expression of human AKR1C isoforms in fresh liver biopsies were found to follow the rank order of AKR1C4>AKR1C1>>AKR1C2>AKR1C3 [29]. In addition, AKR1C4 has displayed the highest catalytically efficiency for 3-oxo reduction with almost all steroid substrates studied previously [4,13,14]. This is also the case with 5β-DHS studied in the present work, although the difference is more pronounced with 5β-DHT and 20α-hydroxy-5β-pregnan-3-one, but less so with 5β-pregnane-3,20-dione. Thus, being the most abundant and catalytically efficient enzyme, AKR1C4 is set to be the most important 3-ketoreductase for the formation of 3α,5α/β-THS in this organ. More specifically for the 5β-metabolic pathways, AKR1C4 is clearly the principal enzyme responsible for the formation of 3α,5β-THS of testosterone, whereas all AKR1C1–4 isoforms are likely to contribute to the formation of 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3α,20α-diol from 5β-pregnane-3,20-dione and the formation of 5β-pregnane-3α,20α -diol from 20α-hydroxy-5β-pregnan-3-one (Scheme 2).

Implications for peripheral formation of 5β-reduced steroids

In liver, 5β-DHS are unlikely to accumulate to high levels and enter the circulation, since they would be rapidly metabolized by AKR1C4. However, 5β-DHS may accumulate in tissues in which AKR1D1 and AKR1C1–3 are co-expressed since these extrahepatic human AKR1C isoforms displayed lower catalytic efficiencies for 5β-THS formation. The expression levels of AKR1D1 and AKR1C1–3 in a given peripheral tissue will control the identity and level of the 5β-reduced steroid metabolites formed. 5β-Reduced steroids are increasingly being recognized as active compounds with important regulating functions [8,9,11,30,31]. It is most likely that the active 5β-steroids are formed locally by AKR1D1 and AKR1C1–3 enzymes. Indeed, AKR1D1 has been found to be highly expressed in the placenta and myometrium where 5β-pregnane-3,20-dione has been implied to act as a tocolytic hormone and prevent

parturition [11]. 5β-Pregnane-3,20-dione can decrease uterine sensitivity to the uterotonic peptide hormone oxytocin by binding directly to the uterine oxytocin receptor [30], and also can act through PXR in regulating uterine contractility [31].

It is well established that progesterone and its metabolites are important neurosteroids. 3α-hydroxy-5β-pregnan-20-one and its 5α-isomer 3α-hydroxy-5α-pregnan-20-one are highly potent positive modulators of the $GABA_A$ receptor and exert differential effects on the $GABA_C$ receptor (where GABA is γ-aminobutyric acid) [8,32]. It is believed that biosynthesis of these steroids occurs in the central nervous system. Although it is not known whether AKR1D1 is expressed in human brain, expression and activities of 5α-reductase and AKR1C1–3 in human brain have been demonstrated previously [33,34]. However, 5β-reductase and its products have been found in the quail brain, and progesterone was found to be metabolized to 5β-pregnane-3,20-dione and 3α-hydroxy-5β-pregnan-20-one in the brains of 1-day-old chicks [35,36]. Thus it is highly probable that thses two 5β-pregnane steroids are also formed locally in human brain by AKR1D1 and AKR1C1–3.

AKR expression can be regulated by factors such as hormonal status and oxidative stress [37,38], which may explain the changes in circulating levels of progesterone metabolites during female menstrual cycle and pregnancy [28,39]. Dysregulation in AKR expression may also contribute to the abnormal plasma concentrations of neurosteroids observed in diseases such as the premenstrual dysphoric disorder [40], depression disorder [41] and chronic fatigue syndrome [42].

AUTHOR CONTRIBUTION

Yi Jin and Trevor Penning developed the overall experimental strategy and wrote the paper. Yi Jin performed the majority of the experiments. Clementina Mesaros and Ian Blair performed LC–MS analysis.

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SUPPLEMENTARY ONLINE DATA

Stereospecific reduction of 5β-reduced steroids by human ketosteroid reductases of the AKR (aldo-keto reductase) superfamily: role of AKR1C1–AKR1C4 in the metabolism of testosterone and progesterone via the 5β-reductase pathway

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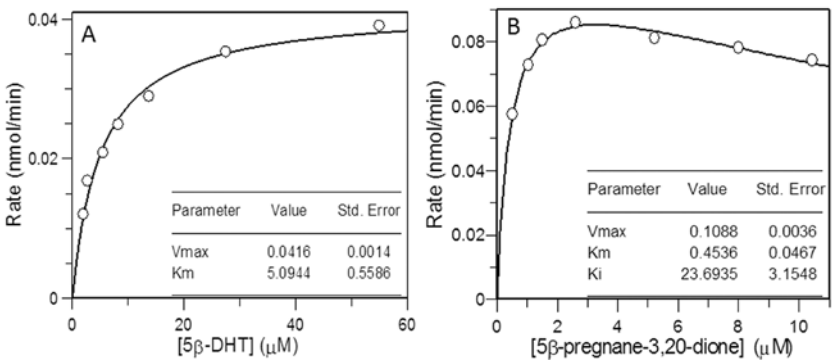

Figure S1 Representative kinetic data for the reaction catalysed by AKR1 enzymes

(**A**) Reduction of 5β-DHT catalysed by AKR1C1, and (**B**) reduction of 5β-pregane-3,20-dione catalysed by AKR1C1. Initial velocities of reactions were measured fluorimetrically at 25°C and data were analysed as described in the Experimental section of the main paper. Results are mean values of incubations performed in duplicate or triplicate. Non-linear fitting analysis of the data using the program GraFit yielded the curves and estimates of V_{max}, K_m, K_i and associated S.E.M. (Std. Error) values.

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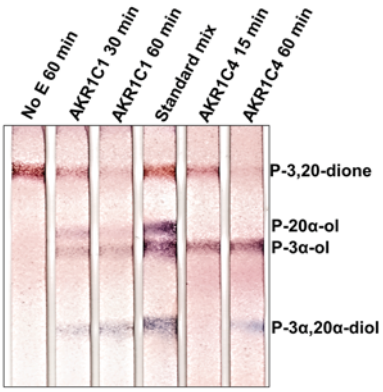

Figure S2 TLC chromatograms of the reduction of 5β-pregnane-3,20-dione catalysed by AKR1C1 and AKR1C4

Comparable amounts of 3α-hydroxy-5β-pregnan-20-one and 20α-hydroxy-5β-pregnan-3-one were formed from a 30 min reaction of 5β-pregnane-3,20-dione catalysed by AKR1C1, each of which was subsequently further converted into 5β-pregnane-3α,20α-diol. In contrast, only one monohydroxy product 3α-hydroxy-5β-pregnan-20-one was observed in the 15 min reaction of 5β-pregnane-3,20-dione catalysed by AKR1C4. Formation of 5β-pregnane-3α,20α-diol was also observed in the 60 min incubation of AKR1C4. Experiments were performed as described in the Experimental section of the main paper. Reactions were conducted in 1 ml systems containing 100 mM potassium phosphate (pH 7.0), 0.4 mM NADPH, 39.3 μM steroid and purified enzyme (4.6 μg of AKR1C1 or 5.6 μg of AKR1C4). Buffer was used for the no-enzyme (No E) control. P-3,20-dione, 5β-pregnane-3,20-dione; P-20α-ol, 20α-hydroxy-5β-pregnan-3-one; P-3α-ol, 3α-hydroxy-5β-pregnan-20-one; P-3α, 20α-diol, 5β-pregnane-3α,20α-diol.

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