Human 3α-hydroxysteroid dehydrogenase isoforms (AKR1C1–AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones

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The kinetic parameters, steroid substrate specificity and identities of reaction products were determined for four homogeneous recombinant human 3α-hydroxysteroid dehydrogenase (3α-HSD) isoforms of the aldo-keto reductase (AKR) superfamily. The enzymes correspond to type 1 3α-HSD (AKR1C4), type 2 3α(17β)-HSD (AKR1C3), type 3 3α-HSD (AKR1C2) and 20α(3α)-HSD (AKR1C1), and share at least 84% amino acid sequence identity. All enzymes acted as NAD(P)(H)-dependent 3-, 17- and 20-ketosteroid reductases and as 3α-, 17β- and 20α-hydroxysteroid oxidases. The functional plasticity of these isoforms highlights their ability to modulate the levels of active androgens, oestrogens and progestins. Salient features were that AKR1C4 was the most catalytically efficient, with k_{cat}/K_m values for substrates that exceeded those obtained with other isoforms by 10–30-fold. In the reduction direction, all isoforms inactivated 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one; 5α-DHT) to yield 5α-androstane-3α,17β-diol (3α-androstanediol). However, only AKR1C3 reduced Δ^4-androstene-3,17-dione to produce significant amounts of testosterone. All isoforms reduced oestrone to 17β-oestradiol, and progesterone to 20α-hydroxypregn-4-ene-3,20-dione (20α-hydroxyprogesterone). In the oxidation direction, only AKR1C2 converted 3α-androstanediol to the active hormone 5α-DHT. AKR1C3 and AKR1C4 oxidized testosterone to Δ^4-androstene-3,17-dione. All isoforms oxidized 17β-oestradiol to oestrone, and 20α-hydroxyprogesterone to progesterone. Discrete tissue distribution of these AKR1C enzymes was observed using isoform-specific reverse transcriptase-PCR. AKR1C4 was virtually liver-specific and its high k_{cat}/K_m allows this enzyme to form 5α/5β-tetrahydrosteroids robustly. AKR1C3 was most prominent in the prostate and mammary glands. The ability of AKR1C3 to interconvert testosterone with Δ^4-androstene-3,17-dione, but to inactivate 5α-DHT, is consistent with this enzyme eliminating active androgens from the prostate. In the mammary gland, AKR1C3 will convert Δ^4-androstene-3,17-dione to testosterone (a substrate aromatizable to 17β-oestradiol), oestrone to 17β-oestradiol, and progesterone to 20α-hydroxyprogesterone, and this concerted reductive activity may yield a pro-oesterogenic state. AKR1C3 is also the dominant form in the uterus and is responsible for the synthesis of 3α-androstanediol which has been implicated as a parturition hormone. The major isoforms in the brain, capable of synthesizing anxiolytic steroids, are AKR1C1 and AKR1C2. These studies are in stark contrast with those in rat where only a single AKR with positional- and stereospecificity for 3α-hydroxysteroids exists.

Key words: 5α-dihydrotestosterone, anxiolytic steroids, steroid hormones, steroid receptors.

INTRODUCTION

Human 3α-hydroxysteroid dehydrogenases (3α-HSDs; EC 1.1.1.213, formerly EC 1.1.1.50; renamed due to A-face specificity) play central roles in steroid hormone metabolism and action. In the liver, 3α-HSDs work in concert with 3-oxo-5α-steroid-4-dehydrogenase (5α-reductase; EC 1.3.99.5) and 5β-reductase to convert 5α/5β-dihydrosteroids into 5α/5β-tetrahydrosteroids [1,2]. In this manner they catalyse the second step in the metabolism of all steroid hormones that contain a Δ^4-3-ketosteroid functionality and serve to protect against circulating steroid hormone excess [1–3]. Hepatic 3α-HSD also plays a critical step in the synthesis of bile acids and is responsible for the production of 5β-chloestane-3α,7α-diol, which is a committed precursor of bile acids [4].

In steroid target tissues, the production of 5α/5β-tetrahydrosteroids catalysed by 3α-HSD is not without consequence. In the human prostate, 3α-HSD can regulate the occupancy of

Abbreviations used: AKR, aldo-keto reductase; allopregnanolone, 3α-hydroxy-5α-pregnan-20-one; 3α-androstanediol, 5α-androstane-3α,17β-diol; androsterone, 3α-hydroxy-5α-androstan-17-one; 5α-DHT, 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); 5α-dihydroprogesterone, 5α-pregnane-3,20-dione; GABA, γ-aminobutyric acid; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 5α-reductase, 3-oxo-5α-steroid-4-dehydrogenase; 20α-hydroxyprogesterone, 20α-hydroxy-pregn-4-ene-3,20-dione; 3′-UTR, 3′-untranslated region; RT, reverse transcriptase; poly(A)$^+$ RNA, polyadenylated RNA.

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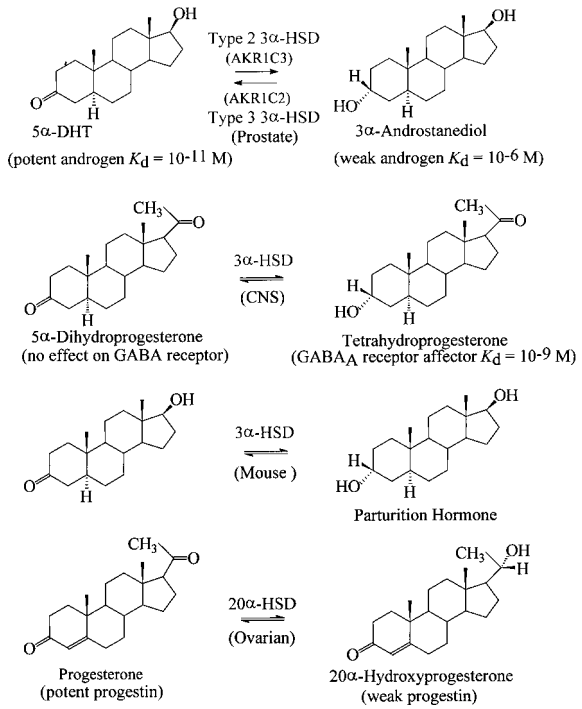


Scheme 1 Biologically important reactions catalysed by mammalian 3α- and 20α-HSD members of the AKR superfamily

Table 1 Human 3α-hydroxysteroid dehydrogenase isoforms

Percentage sequence identity relative to the published sequence for type 2 3α(17β)-HSD [18]. *The predicted amino acid sequence differed only at amino acids 75 and 175. Mutation of these residues to those in the original sequence failed to alter enzyme properties [24].

Enzyme	Nomenclature	Other names	Percentage sequence identity
Type 1 3α-HSD	AKR1C4	Chlordecone reductase/dihydrodiol dehydrogenase 4	84.1%
Type 2 3α(17β)-HSD	AKR1C3	Type 5 17β-HSD dihydrodiol dehydrogenase X	99.4%*
Type 3 3α-HSD	AKR1C2	Bile-acid binding protein dihydrodiol dehydrogenase 2	87.9%
20α(3α)-HSD	AKR1C1	Dihydrodiol dehydrogenase 1	86.0%

the androgen receptor. It catalyses the reduction of 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one; 5α-DHT), a potent androgen (with $K_d = 10^{-11}$ M for the androgen receptor) to 5α-androstane-3α,17β-diol (3α-androstanediol), a weak androgen (with $K_d = 10^{-6}$ M for the androgen receptor) [5] and is positioned to regulate normal and abnormal androgen-dependent growth of this gland (Scheme 1) [6–8]. By contrast, in the central nervous system, 3α-HSD can regulate the occupancy of the γ-aminobutyric acid (GABA)$_A$ receptor by converting 5α-dihydroprogesterone into 3α-hydroxy-5α-pregnan-20-one (allopregnanolone), a potent allosteric effector of the GABA$_A$ receptor ($K_d = 10^{-9}$ M) [9–11]. In the presence of GABA, allopregnanolone will potentiate GABA$_A$-mediated chloride conductance. As a result 3α-HSD is responsible for the production of anxiolytic steroids, and decreased activity in this pathway has been implicated in the symptoms of pre-menstrual syndrome [12]. Thus 3α-HSD isoforms regulate the occupancy of both a nuclear receptor (androgen receptor) and a membrane-bound chloride-ion gated channel (GABA$_A$ receptor) and may have profound effects on receptor function.

Further interest in 3α-HSD exists because of inactivation studies on the murine 5α-reductase type 1 gene. When this gene was inactivated a parturition defect was observed in null mice that could not be corrected by the administration of 5α-DHT, but was corrected by the administration of the downstream metabolite 3α-androstanediol [13]. The absence of the 5α-reductase type 1 gene leads to a defect in progesterone metabolism, and the lack of cervical ripening that ensures is responsible for the parturition defect [14]. To account for the effects of 3α-androstanediol, a product of the 3α-HSD reaction, it has been hypothesized that this steroid may antagonize the effects of progesterone in the cervix by working via an orphan nuclear receptor [14].

Human 3α-HSDs likely to be involved in these key reactions are members of the aldo-keto reductase (AKR) gene superfamily [15–20]. AKRs are monomeric 37 kDa proteins, are NAD(P)(H)-dependent, and share a common $(\alpha/\beta)_8$-barrel structural motif [21,22]. While the rat appears to express only one known 3α-HSD isoform (AKR1C9), at least four human isoforms exist. These are known as type 1 3α-HSD (AKR1C4) [17,18], type 2 3α(17β)-HSD (AKR1C3) [18,19], type 3 3α-HSD (AKR1C2) [15–17,20] and 20α(3α)-HSD (AKR1C1) [16] (see Table 1). The human isoforms share at least 84% amino acid sequence identity, and remarkably AKR1C1 and AKR1C2 differ by only seven amino acids [17,20]. The former is predominantly a 20α-HSD, and this change in positional specificity implies that it may play an important role in regulating progesterone action.

Progress has been made in characterizing each of the human 3α-HSD isoforms with respect to their perceived roles in steroid metabolism in the liver and steroid hormone target tissues. AKR1C4 and AKR1C3 were originally cloned from human liver [18] and implicated in hepatic steroid hormone metabolism. In contrast, AKR1C2 is identical with bile acid binding protein [16], and is implicated in bile acid synthesis and transport [15,16]. However, the relative abundance of these isoforms in any tissue has not been determined.

In attempts to characterize the 3α-HSD isoforms that regulate androgen receptor occupancy in the prostate, AKR1C3 was cloned from a human prostate cDNA library. The recombinant enzyme reduced 5α-DHT to 3α-androstanediol, but the enzyme was incapable of performing the reverse reaction [19]. Instead, its associated 17β-HSD activity oxidized 3α-androstanediol to yield 3α-hydroxy-5α-androstan-17-one (androsterone) and ultimately 5α-androstane-3,17-dione was produced [19]. Due to these reactions and its heightened expression in prostate epithelial cells it was proposed that the type 2 3α(17β)-HSD protects the prostate androgen receptor from androgen excess [19]. Others showed that AKR1C3 was identical with type 5 17β-HSD [23,24] and that it may function to produce testosterone from Δ^4-androstene-3,17-dione in an intracrine manner. AKR1C2 was also cloned from a human prostate cDNA library, and when transiently expressed in human embryonal kidney cells converted 3α-androstanediol into 5α-DHT [20]. Thus in human prostate at least two related 3α-HSD isoforms of the AKR superfamily exist and may function either to eliminate 5α-DHT (AKR1C3) or form 5α-DHT (AKR1C2).

Recently, rat 3α-HSD (AKR1C9) as well as recombinant AKR1C2 were shown to have their K_m values for 5α-dihydro-

progesterone decreased by 10–30-fold by the selective serotonin (5-hydroxytryptamine) re-uptake inhibitor fluoxetine. It has been suggested that fluoxetine exerts its beneficial effects in premenstrual syndrome by allosterically regulating 3α-HSD [25]. The same study showed a wide distribution of AKR1C3 and AKR1C2 mRNA in discrete brain regions.

The biological importance of the human 3α-HSD isoforms was the impetus for the present study. We report a comprehensive analysis of the kinetic properties and substrate specificity of each of the four homogeneous recombinant human 3α-HSD isoforms that belong to the AKR superfamily. The product of each reaction has been identified. We show that all isoforms can interconvert active androgens, oestrogens and progestins with their cognate inactive metabolites. The high sequence identity that exists between the human 3α-HSD cDNAs negates using probes for either the open-reading frame or 3′-untranslated region (UTR) to determine their tissue or cellular distribution. Thus previous reports on the distribution of 3α-HSD isoforms in the prostate and brain may be suspect. We have coupled our enzymic analysis with a quantitative reverse transcriptase (RT)-PCR assay, which permits the detection of each isoform in human tissues including prostate, mammary gland, uterus and brain. By combining these approaches new information about the tissue-specific properties of these enzymes has been revealed.

The nomenclature of the AKR superfamily was recommended by the 8th International Symposium on Enzymology & Molecular Biology of Carbonyl Metabolism, Deadwood, SD 29 June–3 July 1996; also visit the AKR superfamily homepage at: www.med.upenn.edu/akr [21].

MATERIALS AND METHODS

Materials

[4-^{14}C]5α-DHT (53.6 mCi/mmol), [4-^{14}C]oestrone (56.6 mCi/mmol), [4-^{14}C]progesterone (50.8 mCi/mmol), [4-^{14}C]testosterone (53.6 mCi/mmol), [4-^{14}C]Δ^4-androstene-3,17-dione (53.6 mCi/mmol) and [4-^{14}C]17β-oestradiol (50 mCi/mol) were purchased from New England Nuclear. [4-^{14}C]3α-Androstanediol was synthesized enzymically from [^{14}C]5α-DHT using recombinant rat liver 3α-HSD (specific activity 1.5 μmol of androsterone oxidized/min per mg [26]), and [4-^{14}C]20α-hydroxypregn-4-ene-3,20-dione (20α-hydroxyprogesterone) was synthesized enzymically from [^{14}C]progesterone using recombinant rat ovarian 20α-HSD (specific activity 2.09 μmol of 20α-hydroxyprogesterone oxidized/min per mg respectively [27]. Homogeneous recombinant human 3α-HSD isoforms were purified from *Escherichia coli* BL21 (D3) host cells as previously described [28], to the following specific activities: 0.21 μmol of androsterone oxidized/min per mg (AKR1C4) and 2.8, 2.5 and 2.1 μmol of 1-acenapthenol oxidized/min per mg for AKR1C3, AKR1C2 and AKR1C1 respectively. Standard assay conditions contained either 75 μM androsterone or 1 mM 1-acenapthenol in 100 mM potassium phosphate (pH 7.0) containing 2.3 mM NAD^+ at 25 °C. All human polyadenylated RNA [poly(A)$^+$ RNA] samples were purchased from Clontech. All steroids were obtained from Steraloids (Wilton, NH, U.S.A.) and NAD(P)(H) was purchased from Boehringer–Mannheim. All other reagents were of ACS (American Chemical Society) grade or better and were obtained from Sigma–Aldrich.

Synthesis of [^{14}C]3α-androstanediol and [^{14}C]20α-hydroxyprogesterone

Aliquots of either [^{14}C]5α-DHT or [^{14}C]progesterone (50 mCi/mmol) were air-dried and re-dissolved in an acetonitrile solution containing unlabelled steroid to give a final specific radioactivity of 10000 c.p.m./nmol. The steroid substrate (final concentration 35 μM) was then added to 4 × 1.0 ml systems containing 100 mM potassium phosphate (pH 6.0), 200 μM NADPH and 4 % (v/v) acetonitrile. The reaction was initiated with excess recombinant rat liver 3α-HSD or recombinant rat ovary 20α-HSD. The reaction was monitored at 340 nm until the predicted absorbance change in NADPH indicated that the entire substrate had been depleted. The reaction mixtures were extracted with 2 × 2.0 ml of ethyl acetate, and [^{14}C]3α-androstanediol and [^{14}C]20α-hydroxyprogesterone were isolated by TLC on multi-channel plates after development in chloroform/ethyl acetate (4:1, v/v). The steroid was eluted from the silica with ethyl acetate, and the specific radioactivity of the substrate was used to determine the amount of recovered steroid. The steroids were then diluted to yield stock solutions of 1.75 mM 3α-androstanediol or 20α-hydroxyprogesterone containing 10000 c.p.m./nmol. The radiochemical purity of [^{14}C]androstanediol and [^{14}C]20α-hydroxyprogesterone was verified by TLC and autoradiography before being used.

Spectrophotometric assays

Ketosteroid reduction was monitored spectrophotometrically in 100 mM potassium phosphate (pH 7.0) containing a constant NADPH concentration (200 μM) and various amounts of the following steroids: 5α-DHT (3.75–75 μM); androsterone (3.75–75 μM); 5α-androstane-3,17-dione (2.5–50 μM), progesterone (2.5–50 μM) or 3β-hydroxy-5β-pregnan-20-one (2.5–50 μM) dissolved in 4 % acetonitrile. Hydroxysteroid oxidation was monitored spectrophotometrically in the same buffer system containing a constant amount of $NADP^+$ (2 mM) and various amounts of the following steroids: 3α-androstanediol (3.75–75 μM); androsterone (3.75–75 μM); 5α-DHT (3.75–75 μM), 20α-hydroxyprogesterone (2.5–50 μM) or 20α-hydroxy-5β-pregnan-3-one (2.5–50 μM). Kinetic parameters for testosterone oxidation were measured radiometrically as described below. Reactions were initiated by the addition of enzyme and were corrected for non-enzymic rates. All reactions were followed at 340 nm at 25 °C by monitoring the change in absorbance of the nicotinamide nucleotide cofactor ($\epsilon = 6270\ M^{-1} \cdot cm^{-1}$). Kinetic constants were calculated using the ENZFITTER (Biosoft, Cambridge, U.K.) non-linear regression analysis program to fit untransformed data to a hyperbolic function [29], as originally described by Wilkinson [30], yielding estimated values of k_{cat}, K_m and their associated standard errors.

Radiochemical detection of reaction products

To identify reaction products, ketosteroid reduction was conducted in 0.1 ml systems containing 100 mM potassium phosphate (pH 6.0), 2.3 mM NADPH and 35 μM ^{14}C-radiolabelled steroid (40000 c.p.m.) in 4 % acetonitrile. Similarly, assays of hydroxysteroid oxidation were conducted in 0.1 ml systems containing 100 mM potassium phosphate (pH 7.0), 2.3 mM $NADP^+$ and 35 μM ^{14}C-radiolabelled steroid (40000 c.p.m.) in 4 % acetonitrile. Reactions were initiated by the addition of 5 μg of recombinant enzyme and incubated at 37 °C for 90 min. Reactions were quenched by the addition of 400 μl of ethyl acetate. The resulting extracts were evaporated to dryness and re-dissolved in 40 μl of methanol and applied to LK6D Silica TLC plates. Chromatograms were developed in chloroform/ethyl acetate (4:1, v/v). The positions of the substrate and products in each case were confirmed by reference to standards that were applied to the outside lanes and visualized by spraying with a methanol/H_2SO_4 (1:1, v/v) solution and heating. The TLC

plates were then exposed to X-ray film for autoradiography. The amounts of substrate and product were quantified by scraping the corresponding sections of the TLC plate into a toluene-based scintillation fluid and converting the corrected c.p.m. into nmol of product using the specific radioactivity of the starting steroid. Scintillation counting was performed on a TriCarb 2100 with a counting efficiency of 95% for ^{14}C-radioactivity.

Radiochemical determination of kinetic parameters

To measure kinetic parameters for either 5α-DHT reduction or testosterone oxidation the reactions were performed as described above except the buffer used was 100 mM potassium phosphate (pH 7.0) and the [^{14}C]steroid concentration was varied from 3 to 50 μM. The reactions were quenched at various times to ensure that they were linear with respect to time. The nmoles of [^{14}C]-product formed were used to calculate the initial velocity. The, k_{cat}, K_m and k_{cat}/K_m values were calculated in the manner described for the spectrophotometric assays.

Tissue distribution of human 3α-HSD isoforms by RT-PCR

All poly(A)$^+$ RNA (1 mg/ml) was diluted with diethylpyrocarbonate-treated water to a concentration of 0.1 μg/μl. First-strand cDNA synthesis was performed in systems containing: 2 μl of (0.1 μg/μl) poly(A)$^+$ RNA, 9 μl of H_2O and 1 μl of (0.5 μg/μl) oligo-(dT) primer. The mixture was denatured at 70 °C for 10 min and allowed to anneal at 42 °C for 2 min. The mixture was then supplemented with 4 μl of 5× first-strand buffer (final conc. 1×), 2 μl of 0.1 M dithiothreitol (final conc. 0.01 M) and 1 μl of 10 mM dNTP (final conc. 0.5 mM) plus 1 μl of SuperScript II reverse transcriptase (final conc. 10 units/ml). The mixture was then incubated for 30 min at 42 °C and the reaction terminated by heating at 50 °C for 30 min. PCR-amplification of 3α-HSD isoforms or β-actin was performed from the library of first-strand cDNAs obtained for each tissue.

To amplify the 3α-HSD isoforms the PCR mixture (100 μl) contained: 1 μl of first-strand cDNA library, 1 μl of 3′-primer (final conc. 1 μM), 1 μl of 5′-primer (final conc. 1 μM), 10 μl of 10× *Taq* DNA polymerase buffer (final conc. 1×), 4 μl of 25 mM $MgCl_2$ (final conc. 1 mM), 1 μl of 10 mM dNTPs (final conc. 0.1 mM), 1 μl of *Taq* DNA polymerase (final conc. 0.05 unit/μl) and 81 μl of H_2O. The isoform specific primers used were as follows: AKR1C1 forward primer (5′-primer), 5′-dGTAAAGCTTTAGAGGCCAC-3′ (corresponding to bp 119–137) and a reverse primer (3′-primer), 5′-dCACCCATGC-TTCTTCTCGG-3′ (corresponding to bp 690–708) yielding a PCR product of 500 bp; AKR1C2 forward primer (5′-primer), 5′-dGTAAAGCTCTAGAGGCCGT-3′ (corresponding to bp 119–137) and a reverse primer (3′-primer), 5′-dCACCCATG-GTTCTTCTCGA-3′ (corresponding to bp 690–708) yielding a PCR product of 500 bp; AKR1C3 forward primer (5′-primer), 5′-dGTAAAGCTTTGG-AGGTCAC-3′ (corresponding to bp 119–137) and reverse primer (3′-primer), 5′-dCACCCATCGTT-TGTCTCGT′-3′ (corresponding to bp 690–708) yielding a PCR product of 500 bp; and AKR1C4 forward primer (5′-primer), 5′-dACAGAGCTGTAGAGGTCAC-3′ (corresponding to bp 119–137) and reverse primer (3′-primer), 5′-dCACCCATAGT-TTATGTCGT-3′ (corresponding to bp 690–708) yielding a PCR product of 500 bp.

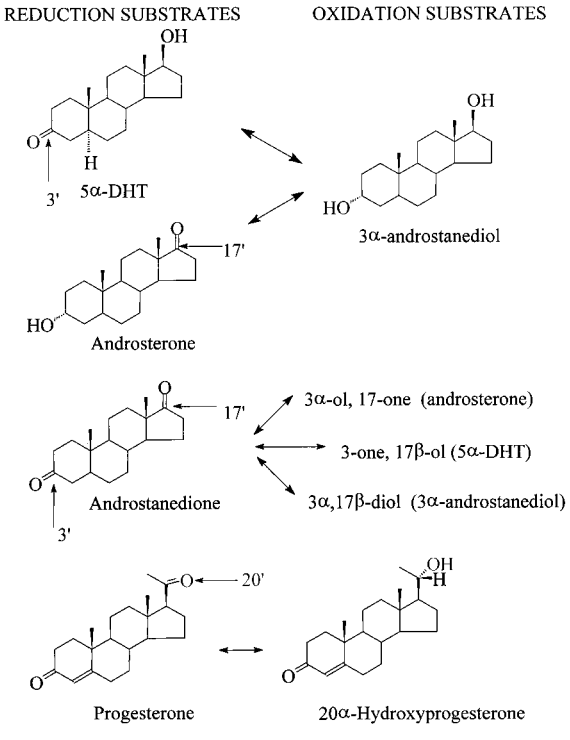
The PCR program included denaturation at 94 °C for 2 min, and then for each cycle a 45 s denaturation step at 94 °C, a 45 s annealing step at 60 °C and a 2 min extension step at 72 °C. Aliquots (10 μl) were removed at cycles 15, 18, 21, 25 and 30 and electrophoresed on a 1% (w/v) agarose gel. PCR amplification of β-actin was performed from the first-strand cDNA libraries in an identical manner except the final concentration of the forward and reverse primers was 0.4 μM. The β-actin primers (CLONTECH) correspond to 5′-primer: 5′-dATCTGGCACCACACC-TTCTACAATGAGCTGCG-3′ and 3′-primer: 5′-dCGTCATA-CTCCTGCTTGCTGATCCACATCTGC-3′ (GenBank accession number M10277) and yielding a PCR product of 838 bp.

To quantify the abundance of transcripts for each 3α-HSD isoform relative to β-actin within a particular tissue Southern-blot analysis was performed. The probe used to detect 3α-HSD isoforms corresponded to a 855-bp *Eco*RI fragment of AKR1C1 (and shared a minimum sequence identity of 84% with each isoform) and the probe for β-actin was a 1.8 kb fragment of β-actin (Clontech). Following a high-temperature wash at 55 °C the membranes were placed on a PhosphorImage screen for 1 h and spots quantified by PhosphorImaging. For each tissue the $\log_{10}$ density of the PhosphorImage was plotted against the number of PCR cycles and the linear portion of the plot for the amplification of each 3α-HSD isoform and β-actin was identified. For each tissue examined 18 cycles of PCR fell within the linear range for the amplification of 3α-HSD and β-actin and relative densities were reported for this cycle. The linear range of the assay covered 4 log units suggesting that this assay has the sensitivity to detect differences in transcript level that could differ by 10000-fold.

RESULTS

Cloning, expression, purification and kinetic characterization of homogeneous recombinant human 3α-HSD isoforms

The cDNAs for four previously reported human 3α-HSDs were obtained by isoform selective RT-PCR of human hepatoma (HepG2) cell poly(A)$^+$ RNA. The resultant cDNAs were sub-



Scheme 2 Keto- and hydroxy-steroid substrates used to discriminate between the positional- and stereo-specificity of human 3α-HSD isoforms

Table 2 Kinetic parameters for 3-ketosteroid reduction and 3α-hydroxysteroid oxidation catalysed by homogeneous recombinant human 3α-HSD isoforms

Kinetic parameters for 5α-DHT reduction were measured radiochemically (R) and spectrophotometrically (S). For the type 3 3α-HSD and the 20α(3α)-HSD the most reliable parameters were obtained radiochemically and only these data are given. ND, not determined.

Enzyme	K_m (μM)	V_{max} (nmoles/min per mg)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
5α-DHT reduction				
AKR1C1 (R)	80.6 ± 28.8	18.0 ± 4.8	0.66	8
AKR1C2 (R)	26.0 ± 6.6	6.24 ± 0.82	0.23	9
AKR1C3 (R)	26.2 ± 3.1	4.18 ± 0.26	0.25	6
AKR1C3 (S)	19.8 ± 7.2	7.1 ± 1.09	0.26	13.2
AKR1C4 (R)	8.3 ± 1.5	51.9 ± 3.1	1.92	231
AKR1C4 (S)	3.4 ± 1.0	53.9 ± 2.8	1.99	586
5α-Androstane-3,17-dione reduction				
AKR1C1	6.77 ± 1.78	12.8 ± 1.1	0.47	70
AKR1C2	6.26 ± 1.59	37.8 ± 1.5	1.39	222
AKR1C3	5.00 ± 0.86	7.63 ± 0.39	0.28	56
AKR1C4	1.44 ± 0.10	48.4 ± 0.6	1.79	1243
3α-Androstanediol oxidation				
AKR1C1	ND	ND	ND	ND
AKR1C2	22.0 ± 7.5	6.58 ± 1.24	0.24	11
AKR1C3	27.2 ± 4.2	3.99 ± 0.36	0.15	6
AKR1C4	10.5 ± 2.4	55.5 ± 5.2	2.1	200
Androsterone oxidation				
AKR1C1	41.7 ± 3.7	1.59 ± 0.07	0.060	1.4
AKR1C2	9.73 ± 1.88	11.3 ± 0.6	0.42	43
AKR1C3	ND	ND	ND	ND
AKR1C4	5.04 ± 1.22	37.7 ± 1.8	1.39	276

Table 3 Kinetic parameters for 17-ketosteroid reduction and 17α-hydroxysteroid oxidation catalysed by homogeneous recombinant human 3α-HSD isoforms

Enzyme	K_m (μM)	V_{max} (nmol/min per mg)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)
Androsterone reduction				
AKR1CI	21.0 ± 9.1	4.79 ± 0.76	0.18	9
AKR1C3	8.96 ± 1.2	10.2 ± 0.4	0.37	42
5α-Dihydrotestosterone oxidation				
AKR1C1	31.7 ± 14.6	0.72 ± 0.18	0.026	0.8
AKR1C3	11.9 ± 4.4	1.25 ± 0.18	0.046	3.8
Testosterone oxidation				
AKR1C1 (R)	39.8 ± 6.6	1.20 ± 0.11	0.044	1.1
AKR1C3 (R)	24.3 ± 7.5	1.34 ± 0.18	0.049	2.0

cloned into pET16b vectors which were used to transform *E. coli* BL21(D3) cells. Following induction by isopropyl β-D-galactoside, the overexpressed proteins were purified to homogeneity in milligram quantities. Because of the high sequence identity that exists between these isoforms, their identity was confirmed by functional assays in which kinetic parameters (k_{cat}, K_m and k_{cat}/K_m) for standard substrates were compared with those previously reported for the native isoforms purified from human liver. This work has been reported by us elsewhere [28]. As a result, AKR1C1–AKR1C4 were obtained in sufficient quantities for further characterization of their steroid substrate specificity.

Previous reports from this and other laboratories have indicated that human 3α-HSD isoforms may not exhibit positional- and stereo-specificity for steroid substrates. For example

Table 4 Kinetic parameters for 20-ketosteroid reduction and 20α-hydroxysteroid oxidation catalysed by homogeneous recombinant human 3α-HSD isoforms

Enzyme	K_m (μM)	V_{max} (nmol/min per mg)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)
Progesterone reduction				
AKR1C1	2.65 ± 0.66	7.85 ± 0.35	0.29	109
3β-Hydroxy-5β-pregnan-2-one reduction				
AKR1C1	16.2 ± 4.7	29.7 ± 3.3	1.10	68
20α-Hydroxyprogesterone oxidation				
AKR1C1	14.7 ± 3.5	31.9 ± 2.8	1.20	82
20α-Hydroxy-5β-pregnan-3-one oxidation				
AKR1Cl	19.4 ± 4.1	87.5 ± 7.6	3.23	167

AKR1C1, although predominantly identified as a 20α-HSD, has marginal 3α-HSD activity [17]. In addition, AKR1C3 has also been identified as a type 5 human 17β-HSD [23,24]. In the present study, four steroid substrates were chosen to measure positional specific ketone reduction; 5α-DHT (C3-ketone reduction only); androsterone (C17-ketone reduction only); 5α-androstane-3,17-dione (C3 and C17 ketone reduction), and progesterone (C20-ketone reduction only) (Scheme 2). These steroids were tested as substrates for each human isoform in the presence of NADPH. Five steroid substrates were also chosen to measure stereoselective hydroxysteroid oxidation; androsterone (3α-hydroxysteroid oxidation only); 5α-DHT and testosterone (17β-hydroxysteroid oxidation only); 3α-androstanediol (3α- and 17β-hydroxysteroid oxidation); and 20α-hydroxyprogesterone (20α-hydroxysteroid oxidation only). These steroids were tested as substrates for each human isoform in the presence of $NADP^+$. In each instance, K_m, V_{max} and k_{cat}/K_m were determined either spectrophotometrically by measuring the change in absorbance of the nicotinamide nucleotide cofactor or radiochemically by measuring the appearance of steroid product. Radiochemical assays were used for 5α-DHT reduction and testosterone oxidation to obtain the necessary increased sensitivity (Tables 2–4).

With 5α-DHT as substrate for C3-ketone reduction, AKR1C4 was the most efficient with a k_{cat}/K_m that exceeded the other isoforms by 15–90-fold. With 5α-androstane-3,17-dione, a substrate for C3- and C17-ketone reduction, all four 3α-HSD isoforms displayed an enhanced catalytic efficiency relative to 5α-DHT, and this enhancement was of the order of 2–25-fold. The greatest enhancement was seen with AKR1C1–AKR1C3. Since this increase could be due to C3- and C17-ketone reduction, it was interesting to note that only AKR1C1 and AKR1C3 gave measurable rates of androsterone reduction (C17-ketone reduction) indicating their ability to reduce the C17 ketone group (Table 3). These observations suggest that AKR1C1 and AKR1C3 also function as both 3- and 17- ketosteroid reductases. With progesterone as substrate (C20-ketone reduction), AKR1C1 also gave a measurable catalytic efficiency suggesting that it was a 3-, 17- and 20-ketosteroid reductase (Table 4).

With androsterone, a substrate for 3α-hydroxysteroid oxidation, AKR1C4 exhibited a 5–200-fold greater catalytic efficiency over the other isoforms. Importantly, AKR1C3 failed to yield a measurable rate of androsterone oxidation in the spectrophotometric assay, which is consistent with our earlier findings with this enzyme [19]. Using 5α-DHT and testosterone as substrates (for 17β-hydroxysteroid oxidation) it was found that only AKR1C1 and AKR1C3 had measurable activity and that the values obtained with testosterone were similar to those

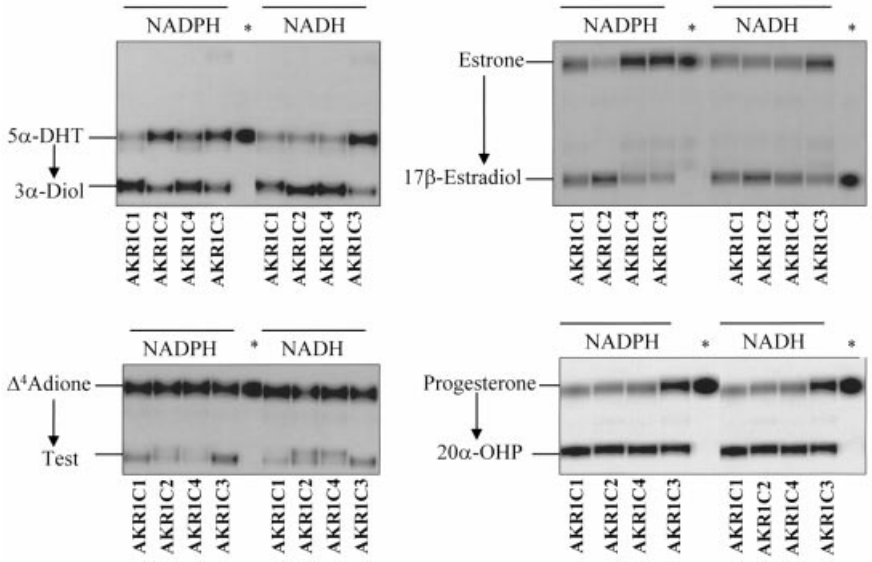


Figure 1 Radiochemical assay of C3-, C17- and C20-ketone reduction catalysed by human 3α-HSD isoforms

Homogeneous recombinant 3α-HSD (AKR1C1–AKR1C4; 5 μg) was incubated with 35 μM [^{14}C]DHT, [^{14}C]Δ^4-androstene-3,17-dione (Δ^{14}Adione), [^{14}C]oestrone or [^{14}C]progesterone plus 2.3 mM NAD(P)H for 90 min at 37 °C. At the end of the reaction the radiolabelled steroids were extracted, subjected to chromatography and the chromatograms were exposed to X-ray film for autoradiography. *Marks the position of radioactive standards. No products were observed in either the absence of cofactor or enzyme. 3α-Diol, 3α-androstanediol; Test, testosterone; 20α-OHP, 20α-hydroxyprogesterone.

seen with 5α-DHT. With 20α-hydroxyprogesterone as substrate, only AKR1C1 gave a measurable catalytic efficiency. These observations indicate that the purified AKR1C3 isoform will function as a bi-directional 3α- and 17β-HSD, and that the purified AKR1C1 may function as bi-directional, 3α-,17β- and 20α-HSD.

Because AKR1C1 displays broad specificity for steroid substrates, the ability of this enzyme to catalyse the NADPH-dependent reduction of 3β-hydroxy-5β-pregnan-20-one (A/B *cis*-ring fusion) and the NADP$^+$-dependent oxidation of 20α-hydroxy-5β-pregnan-3-one (also A/B *cis*-ring fusion) was also examined. Only modest differences were found to exist in the catalytic efficiencies for these substrates and they were similar to those observed with either progesterone or 20α-hydroxyprogesterone.

Radiochemical assay of C3-, C17- and C20-ketone reduction catalysed by human 3-HSD isoforms

In the previous section, spectrophotometric based assays indicated that human 3α-HSDs were not positional-specific. However, these assays did not reveal the identity of the reaction product(s). In these studies the ability of recombinant 3α-HSD isoforms to catalyse the NAD(P)H-dependent reduction of [^{14}C]DHT, [^{14}C]Δ^4-androstene-3,17-dione, [^{14}C]oestrone and [^{14}C]progesterone to yield 3α-androstanediol, testosterone, 17β-oestradiol and 20α-hydroxyprogesterone, respectively, was examined under identical reaction conditions using radiochromatography (Figure 1). It was found that AKR1C1–AKR1C4 functioned as dual nicotinamide nucleotide-specific 3-keto-, 17-keto-and 20-keto-steroid reductases. When the percentage conversion of steroid substrate into product was considered it was apparent that each isoform had superior 3- and 20-ketosteroid reductase activity compared with 17-ketosteroid reductase activity. While all the enzyme isoforms produced 3α-androstanediol, 17β-oestradiol and 20α-hydroxyprogesterone from 5α-DHT, oestrone and progesterone, respectively, only AKR1C3 produced significant amounts of testosterone from Δ^4-androstene-3,17-dione.

Radiochemical assay of 3α-, 17β- and 20α-hydroxysteroid oxidation catalysed by human 3α-HSD isoforms

In these studies the ability of recombinant 3α-HSD isoforms to catalyse the NAD(P)$^+$-dependent oxidation of [^{14}C]3α-androstanediol, [^{14}C]testosterone, [^{14}C]17β-oestradiol and [^{14}C]20α-hydroxyprogesterone to yield 5α-DHT, Δ^4-androstene-3,17-dione, oestrone and progesterone, respectively, under identical reaction conditions was determined by radio-chromatography (Figure 2). With 3α-androstanediol as substrate, only AKR1C2 produced significant amounts of 5α-DHT. AKR1C1 as well as AKR1C3 preferentially produced androsterone and 5α-androstane-3,17-dione from 3α-androstanediol, indicative of their associated 17β-HSD activities. The results obtained with AKR1C3 confirm our previous observations, which showed that this enzyme oxidized 3α-androstanediol to products other than 5α-DHT [19]. With testosterone as substrate, the formation of Δ^4-androstene-3,17-dione by AKR1C3 and AKR1C4 was preferred when NAD$^+$ was used as cofactor. All four isoforms were capable of oxidizing 17β-oestradiol and 20α-hydroxyprogesterone to oestrone and progesterone respectively.

In summary, the radio-chromatography indicates that the 3α-HSD isoforms display broad positional specificity for steroid substrates. Salient features are that discrete isoforms make active steroid hormones: only AKR1C3 makes testosterone; only AKR1C2 makes 5α-DHT; while all isoforms make 17β-oestradiol and progesterone. The physiological role of these isoforms in steroid hormone metabolism will be influenced by their distribution within human tissues.

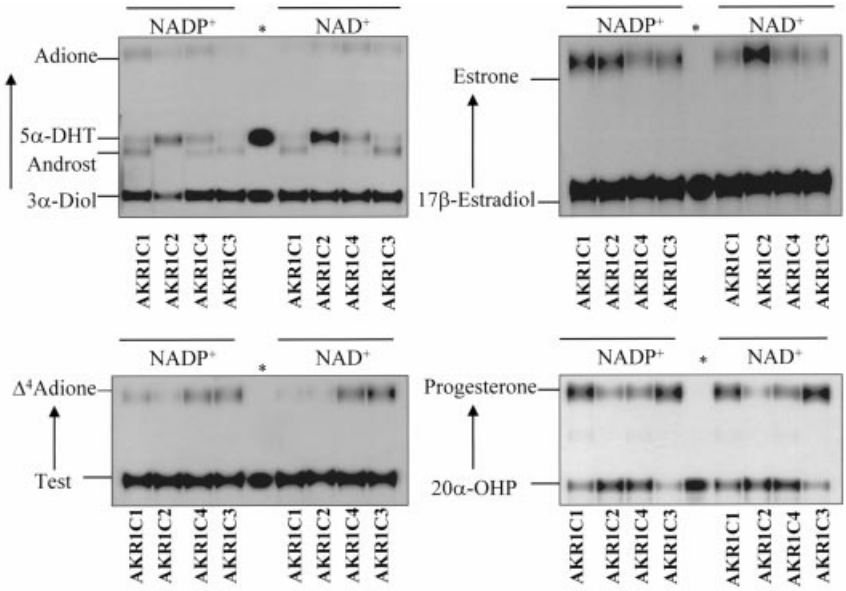


Figure 2 Radiochemical assay of 3α-, 17β- and 20α-hydroxysteroid oxidation catalysed by human 3α-HSD isoforms

Homogeneous recombinant 3α-HSD (AKR1C1–AKR1C4; 5 μg) was incubated with 35 μM [^{14}C]3α-androstanediol (3α-Diol), [^{14}C]testosterone (Test), [^{14}C]17β-oestradiol or [^{14}C]20α-hydroxyprogesterone (20α-OHP), plus 2.3 mM NAD(P)$^+$ for 90 min at 37 °C. At the end of the reaction the radiolabelled steroids were extracted, subjected to chromatography and the chromatograms were exposed to X-ray film for autoradiography. *Marks the position of radioactive standards. No products were observed in the absence of either cofactor or enzyme. Androst, androsterone; Adione, 5α-androstane-3,17-dione.

Tissue distribution of human 3α-HSD isoforms

Several reports have appeared describing the tissue distribution of human 3α-HSD isoforms [18,19,23]. Some of these reports have relied on Northern-blot analysis using either a probe for the open reading frame of one particular isoform [19] or a probe for the 3′-UTR of the isoform under study to achieve specificity of hybridization [19,23]. It has been our experience that both approaches are unsatisfactory, since they do not discriminate between isoforms. Because of this difficulty the distribution of AKR1C3 and AKR1C4 was previously studied by RT-PCR, but the specificity of the primer pairs was not established [18]. For these reasons, we developed a quantitative RT-PCR assay for each isoform which permitted us to report the abundance of each transcript relative to β-actin within a given tissue. In developing this assay, we first designed isoform-specific primers which would permit the amplification of a 500 bp fragment for each isoform. Specificity of the primers was verified by demonstrating that each primer pair only amplified a product of the desired size from the correct cDNA template. For example, the primer pair for AKR1C1 produced a 500 bp fragment only from the AKR1C1 cDNA, but failed to produce a product of the correct size when the cDNA for any other 3α-HSD isoform was substituted as the template (Figure 3).

In using these primer pairs to measure isoform-specific tissue distribution, we established the linear range for PCR amplification of the isoform and β-actin by monitoring the appearance of cDNA over incremental PCR cycles. This approach revealed that 18 cycles of PCR amplification from first-strand cDNA synthesis were sufficient to remain in the linear range to detect 3α-HSD isoforms and β-actin within a given tissue. The distribution of each isoform was then recorded in eight human tissues (liver, lung, prostate, uterus, mammary gland, brain, small intestine and testis) at 18 cycles relative to β-actin (Figure 4). Quantification was achieved by Southern-blot analysis using randomly primed cDNA fragments for AKR1C1 and β-actin followed by PhosphorImaging. In this method the linear range of the assay spanned 4 log units.

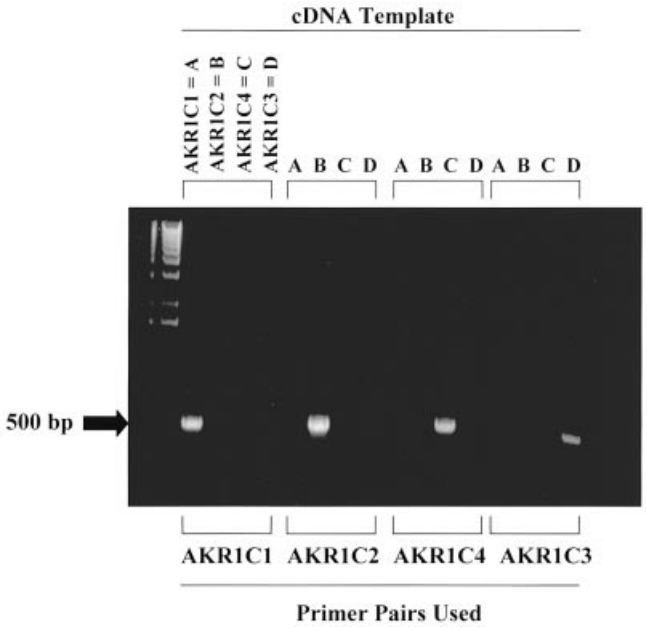


Figure 3 Detection of human 3α-HSD isoforms by isoform specific RT-PCR

AKR1C1–AKR1C4 isoform specific primers were designed and validated to amplify a 500-bp fragment from only the targeted cDNA. The cDNAs used as templates in each reaction are listed above the lanes. The isoform-specific primer pair used for amplification is listed beneath the lanes. All reactions were run for 30 cycles in the thermal cycler.

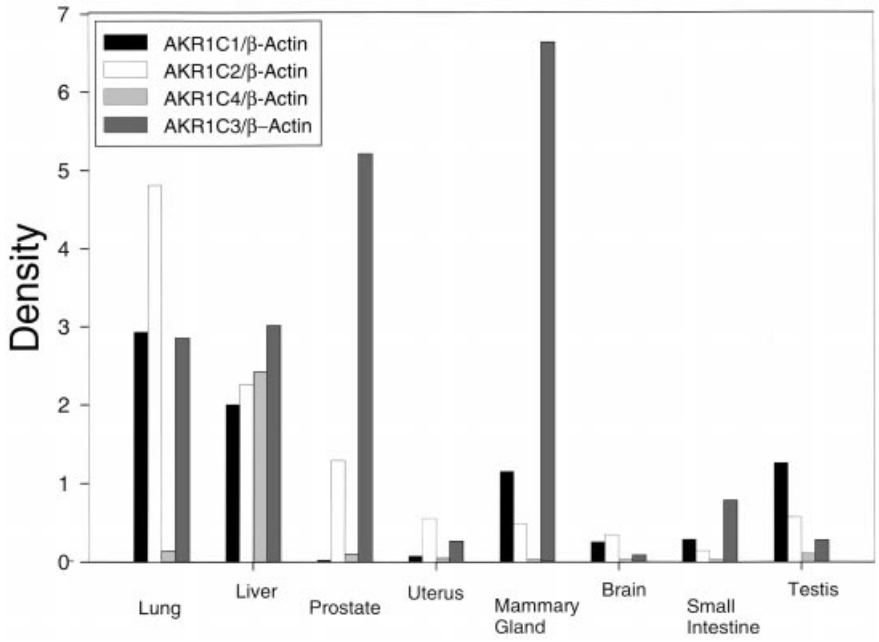


Figure 4 Distribution of 3α-HSD isoforms in human tissues relative to β-actin

First-strand cDNA synthesis was performed on poly(A)$^+$ RNA isolated from eight human tissues. RT-PCR was then performed using either the primer pairs specific for each isoform (validated in Figure 5) or a primer pair for the amplification of β-actin. After 18 cycles, the PCR products were separated by agarose gel electrophoresis, transferred on to nitrocellulose and Southern-blot analysis was performed using cDNA probes that would detect either all 3α-HSD isoforms or β-actin. The amount of 3α-HSD present was then scored relative to β-actin using PhosphorImaging analysis. Eighteen cycles of PCR falls within the linear-range of amplification of each transcript within each tissue. The linear range spans for 4 log units.

These studies revealed that the liver was the only tissue that expressed similar levels of all four human 3α-HSD isoforms. In addition AKR1C4 was found to be fairly liver-specific; only minor amounts were found in lung and brain. The lung displayed high expression of all isoforms, except AKR1C4, with respect to β-actin. The dominant forms in prostate were found to be AKR1C2 and AKR1C3, both of which have been previously cloned from this tissue [19,20]. In the testis, AKR1C1 was the major isoform. In the mammary gland, AKR1C3 was by far the most dominant isoform. In the uterus the expression of the 3α-HSD isoforms was modest relative to β-actin, where AKR1C2 and AKR1C3 isoforms predominated. In the brain, isoform expression was low but AKR1C1 and AKR1C2 were expressed to a larger extent than the other isoforms.

DISCUSSION

We have described the kinetic properties and substrate specificity of each homogeneous recombinant human 3α-HSD isoform. We have identified their reaction products and also report their tissue distribution by RT-PCR. Combined, our data provide new insights into the biological importance of these AKRs with respect to steroid hormone metabolism in target and non-target tissues.

Kinetic properties and substrate specificity

Spectrophotometric assays indicated that with typical 3-ketosteroid and 3α-hydroxysteroid substrates, AKR1C4 was the most catalytically efficient. For example, this isoform reduced 5α-DHT with a k_{cat}/K_m that was 15–30-fold greater than that observed with other 3α-HSD isoforms. Similarly, AKR1C4 oxidized androsterone with a k_{cat}/K_m that was 5–200-fold greater than that observed with AKR1C1 or AKR1C2. It was also clear that both AKR1C1 and AKR1C3 displayed 17β-HSD activity since they both reduced androsterone. Although 17β-HSD activity had been previously assigned to the latter enzyme this was not true of the former isoform. Because AKR1C1 oxidized 5α-DHT, testosterone and 20α-hydroxyprogesterone this indicated that one of the human 3α-HSD isoforms could function as a 3α-, 17β- and 20α-HSD. The plasticity of AKR1C1 was further revealed by its ability to reduce 3β-hydroxy-5β-pregnan-20-one with a catalytic efficiency similar to progesterone, indicating no preference for A/B-*cis*-ring-fused steroids over Δ^4-3-ketosteroids. To attain this broad substrate specificity, k_{cat} has been sacrificed since the k_{cat} values observed were 100–1000-fold smaller than those measured with the homogeneous recombinant rat 3α-HSD (AKR1C9), which by contrast is a robust catalyst with a k_{cat} of 50–200 min^{-1} for steroid substrates.

Radiometric assays revealed that each 3α-HSD isoform was indeed functionally plastic. Each enzyme acted as a 3-, 17- and 20-ketosteroid reductase in the presence of NAD(P)H. Since this method led to product identification, it was apparent that all isoforms could reduce 5α-DHT to 3α-androstanediol, oestrone to 17β-oestradiol, and progesterone to 20α-hydroxyprogesterone. Of the human 3α-HSD isoforms, AKR1C3 was the best in converting Δ^4-androstene-3,17-dione into testosterone. This is the first time that all human 3α-HSD isoforms have been implicated in androgen, oestrogen and progesterone metabolism.

Each enzyme was also able to function as a 3α-, 17β- and 20α-hydroxysteroid oxidase with NAD(P)$^+$ as cofactor. Although, each isoform was able to oxidize 17β-oestradiol to oestrone, and 20α-hydroxyprogesterone to progesterone, important differences were observed with 3α-hydroxysteroids and testosterone. AKR1C1, AKR1C2 and AKR1C4 will oxidize androsterone (see above) but the only isoform capable of converting 3α-

androstanediol into 5α-DHT is AKR1C2. Similarly, the dominant enzymes capable of oxidizing testosterone to yield Δ^4-androstene-3,17-dione appear to be AKR1C3 and AKR1C4. The ability of each of these AKRs to interconvert active C_{18}-, C_{19}- and C_{21}-steroid hormones into their cognate inactive metabolites may have consequences that relate to steroid hormone action.

The strength of this *in vitro* characterization is that it permits the inherent specificity of these enzymes to be assigned. The metabolic role of each isoform will be governed by its tissue localization. For these reasons an isoform-specific RT-PCR assay was established.

Tissue distribution and consequences for steroid metabolism/action

The quantitative RT-PCR analysis used permits the abundance of each isoform to be scored relative to β-actin within a given tissue. This coupled with the substrate specificity described allows conclusions to be drawn about the possible tissue-specific functions of each 3α-HSD isoform.

RT-PCR indicated that each human 3α-HSD isoform was expressed equally in human liver. However, AKR1C4 was almost exclusively detected in human liver indicating that it is predominantly a liver-specific isoform. Given its superior k_{cat}/K_m for 3-ketosteroids relative to the other 3α-HSD isoforms, it is safe to conclude that this is the major form that works in concert with the 5α/5β-reductases to yield inactive 5α/5β-tetrahydrosteroids for conjugation and eventual elimination from the liver. Thus a major role for AKR1C4 is protection against circulating steroid hormone excess. Importantly, the 5β-reductase that precedes 3α-HSD in hepatic steroid hormone metabolism is also an AKR, which shares high sequence identity with AKR1C4, suggesting that this pathway of steroid hormone metabolism may have arisen by gene duplication. In contrast, AKR1C2 is potently inhibited by bile acids, and is identical to human bile acid binding protein [16] and its k_{cat}/K_m values for steroid substrates are relatively poor. Thus unlike the situation in rat liver where a single AKR isoform (AKR1C9) functions as both a robust catalyst for steroid turnover and a bile acid binding protein, these functions are performed by different proteins (AKR1C4 and AKR1C2 respectively) in human liver.

In the human lung, each 3α-HSD isoform except AKR1C4 was highly expressed. The broad substrate specificity of these isoforms for 3-, 17- and 20-ketosteroids, coupled with their ability to catalyse the oxidoreduction of a variety of xenobiotics, including polycyclic aromatic hydrocarbon *trans*-dihydrodiols [28,31], suggests that these enzymes may be involved in the metabolism of these agents and contribute to their clearance.

In the human prostate, the two isoforms most abundantly expressed are AKR1C2 and AKR1C3. Our studies show that both enzymes eliminate 5α-DHT, but only AKR1C2 forms the active hormone 5α-DHT. Thus AKR1C2 may increase the pool of active androgens in the prostate. The ability of AKR1C3 to also function as a 17β-HSD in this tissue requires comment. This 17β-HSD activity leads to the oxidation of 3α-androstanediol to yield androsterone, and eventually 5α-androstane,3-17-dione is formed [19]. In the reduction direction this activity will convert Δ^4-androstene-3,17-dione into testosterone. This latter reaction is clearly a property of the recombinant enzyme and is observed with the isolated enzyme and when the cDNA is stably expressed in human embryonal kidney cells [24]. However, our radiometric assays do not support the presence of a robust 17β-HSD activity ascribed to the stably expressed enzyme. In fact, percentage conversions indicate that the enzyme prefers to function as a 3α- and 20α-HSD. The higher 17β-HSD activity observed with the stably expressed enzyme was previously explained on the basis of the lability of this enzyme [24]. However, the 3α-HSD activity catalysed by the same transiently expressed protein was not labile. It is not possible to explain these differential effects on activity unless steroid hormone turnover is catalysed at different active sites on the same enzyme. No precedent exists for this in the AKR superfamily.

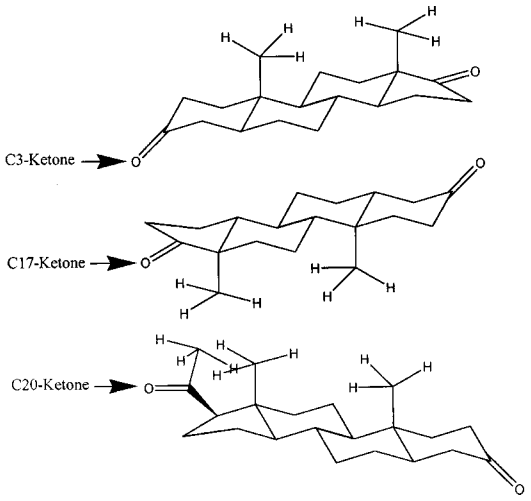
It has been argued that AKR1C3 contributes to the intracrine production of testosterone within the prostate [23,24]. However, since the Leydig cells of the testis are the major source of circulating testosterone it is uncertain what the contribution of AKR1C3 is in maintaining testosterone levels in the prostate. The relevance of this enzyme to prostatic testosterone production may be more important in individuals that have undergone castration. It is concluded that in intact male adults, AKR1C3 exists in the prostate to eliminate 5α-DHT as originally proposed, and that AKR1C2 is responsible for converting 3α-androstanediol into 5α-DHT. AKR1C2 is not the only enzyme capable of synthesizing 5α-DHT, since this property is shared with a *cis*-retinol dehydrogenase, which is a short-chain dehydrogenase/reductase [32]. Importantly, PC3 cells stably expressing AKR1C2 can drive a p(androgen response element)$_2$-simian virus 40-chloramphenicol acetyltransferase reporter gene construct when the cells are transiently transfected with the androgen receptor and challenged with 3α-androstanediol [33].

An unexpected result was the high expression of AKR1C3 in the human mammary gland. If this enzyme functions predominantly as a reductase, it would convert Δ^4-androstene-3,17-dione into testosterone, increasing the available pool of steroids that can be aromatized to 17β-oestradiol. Further, it will reduce oestrone to 17β-oestradiol and progesterone to 20α-hydroxyprogesterone, which collectively could contribute to a pro-oesterogenic state in the breast. Whether this enzyme contributes to oestrogen dependent growth will depend on the level of its expression compared with type 1 17β-HSD in normal and diseased breast tissue.

In the uterus AKR1C3 and AKR1C2 dominate, and the latter is more highly expressed. It is postulated that AKR1C2 may be the major contributor to the production of uterine 3α-androstanediol. Additionally, the 20α-HSD activity associated with this isoform may contribute to the conversion of progesterone into 20α-hydroxyprogesterone, which in rodents is necessary for parturition. The production of 3α-androstanediol and 20α-hydroxyprogesterone by the same enzyme in the uterus may have important consequences for the termination of pregnancy.

In the brain, AKR1C1–AKR1C3 are all present. However, it is AKR1C1 and AKR1C2 that are more highly expressed. In Northern-blot analysis using 3′-end probes to detect the presence of AKR1C3 and AKR1C2 both mRNAs were previously found to be expressed across many brain regions [25]. However, it is unlikely that this method actually discriminated between these two isoforms. Based on spectrophotometric and radiometric data, all isoforms are capable of producing the neuroactive tetrahydrosteroids that may modulate the $GABA_A$ receptor. Of these, AKR1C1 and AKR1C2 are the most abundant in whole brain. The expression of AKR1C1 in human brain has not been previously reported.

The strength of our *in vitro* characterization of the steroid specificity of each 3α-HSD isoform coupled with tissue distribution studies, is that it lays the groundwork for transfection studies. Each isoform now needs to be transiently or stably expressed in steroid-hormone-responsive and non-responsive cells and challenged with the appropriate substrate to determine its directionality in androgen, oestrogen and progesterone



Scheme 3 Binding modes required to achieve 3α-, 17β- and 20α-HSD activity at the same AKR active site

metabolism. Up until now the ability of each of these isoforms to make active androgens, oestrogens or progestins, or eliminate these hormones in a whole-cell context has not been studied in a systematic fashion. The production of active hormones could also be assessed in stable transfectants by transiently co-transfecting steroid hormone receptors and reporter gene constructs driven by hormone response elements. *Trans*-activation of a receptor by a steroid substrate would provide a read-out of the production of active hormone. The distribution of each isoform with regard to hormonal status in the prostate, uterus, mammary gland and brain also needs to be examined.

Functional plasticity of human 3α-HSD isoforms

It is remarkable that the human 3α-HSD isoforms have evolved to function as 3α-, 17β- and 20α-HSDs. Structure–function studies on AKRs indicate that the cofactor binding site is invariant with respect to $NADP^+$. This cofactor binds in an extended *anti*-conformation across the α/β-barrel [34]. The nicotinamide ring is orientated so that the stereochemistry of hydride transfer is maintained, and the 4-*pro-R* hydrogen is always transferred from the *re*-face. Because the cofactor is locked in position so that the C4 position is conserved relative to the catalytic tetrad, functional plasticity can only be achieved if the steroid substrate is bound in several orientations. Based on the crystal structure of the 3α-HSD–$NADP^+$–testosterone ternary complex [35], 3α-HSD activity is achieved when the A-ring of the steroid is directed towards the catalytic tetrad and the β-face of the steroid is facing a flexible loop B; 20α-HSD activity can only be achieved when the steroid binds backwards so that the D-ring binds in the A-ring position; and 17β-HSD activity can only be achieved when the D-ring binds in the A-ring position and if the steroid is bound upside down so that the angular methyl groups point away from loop B (Scheme 3). To achieve 3α-, 17β- and 20α-HSD all three binding modes must be allowed by AKR1C1–AKR1C4 (Scheme 3). Interestingly, rat liver 3α-HSD was converted into a positional and stereospecific 20α-HSD by a loop-chimaera approach, in which loops A, B and C of rat ovarian 20α-HSD were introduced into 3α-HSD [36]. These considerations indicate that steroid-induced changes in the flexible loops (loops A, B and C) are probably responsible for the functional plasticity of the human 3α-HSD isoforms. Determination of the crystal structures of ternary complexes of the human isoforms will provide a more detailed structural basis for their plasticity.

Note: since this paper was originally submitted, the cloning, expression and tissue distribution of AKR1C1 have been independently reported [37]. In that report the distribution of AKR1C1 was compared with other human isoforms across tissues using a similar RT-PCR assay to that described in the present study. Importantly, these authors failed to detect AKR1C2 and AKR1C3 in human prostate yet both these isoforms have been cloned from cDNA libraries prepared from this tissue [19,20].

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