Molecular Characterization of a First Human 3(α→β)-Hydroxysteroid Epimerase*

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In this report, we describe the isolation and characterization of a cDNA encoding an enzyme that exhibits catalytic characteristics of a 3(α→β)-hydroxysteroid epimerase (3(α→β)-HSE). The enzyme overexpressed in human 293 embryonic kidney cells transforms androstene into epi-androsterone in two steps: the oxidation of androsterone to 5α-androstan-3,17-dione, followed by the reduction of the latter to epi-androsterone. The reverse reaction, 3(β→α)-hydroxysteroid epimerization, is approximately 10-fold weaker. These results are confirmed by Vmax/Km determination, which shows that the enzyme catalyzes the oxidation of androsterone to 5α-androstan-3,17-dione and the reduction of 5α-androstan-3,17-dione to epi-androsterone more efficiently than the reverse reactions. The selective catalysis of the reaction following the 3(α→β) direction is also observed in intact transfected cells in culture, which better reflect physiological conditions. In vitro assays reveal that the recombinant enzyme prefers NAD+ and NADH as cofactors and could recognize both C-19 and C-21 3α-hydroxysteroids as substrates. DNA sequence analysis predicts a protein of 317 amino acids. Tissue distribution analysis using RT-PCR reveals that the mRNA of the enzyme is expressed in various tissues, including liver, brain, prostate, adrenal, and uterus, with the most abundant expression in the liver. Because active hydroxysteroids generally exert their effect in a stereospecific manner, 3(α→β)-HSE could thus potentially play an important role in regulating the biological activities of various steroids.

Epimerization reactions have been shown to play important roles in both bacterial and mammalian systems (1, 2). However, the most studied epimerases are nucleotide-sugar epimerases of bacterial or fungal origin (2–4). Only a few human epimerases have been characterized at the molecular level. One of the best known human epimerases is the human UDP-glucose 4-epimerase (EC 5.1.3.2), which catalyzes the conversion of UDP-glucose into UDP-galactose and has been found to be associated with the disease called galactosemia (5–8).

The first report of a hydroxysteroid epimerase activity in animal systems was made several decades ago: a 16-epimerase acting on estril and 16-epi-estriol in the human placenta was documented in 1968 (9). The activity that transforms 5α-androstan-3α,17β-diol (5α-diol) into its 3β-epimer in the rat ovary was also reported, although it was speculated that the conversion was due to the combined actions of 3α-HSD and 3β-HSD (10). An ecdysone 3-epimerase from the midgut of Manduca sexta (L.) was partially purified and was shown to act on position 3 to yield the 3-epimer of ecldysone (11). In addition, epimeric conversion of corticosteroids has also been documented for both human and hamster liver preparations (12). Despite the fact that the past few decades have seen rapid progress in the area of steroidogenesis at the molecular level, few reports can be found in recent literature on the cloning and characterization of steroid epimerases.

The biological activity of steroid hormones and their metabolites is often associated with the stereo conformation of the molecule. A well-documented example of this is the neuroactive steroid 5α-pregnane-3α,20S-diol (allo-pregnalone), one of the most abundant naturally occurring neuroactive steroids. It has been reported that this neurosteroid can play a role in the modulation of the reproductive function by suppressing the release of hypothalamic gonadotropin-releasing hormone in female rats (13). However, a recent report has demonstrated that its 3β-epimer, 5α-pregnane-3β,20S-diol (iso-pregnalone), is ineffective in regulating the hypothalamic activities (14). Recent findings have also suggested that this molecule might be, at least in part, responsible for the premenstrual syndrome (15). Another example of inactivation of steroids via carbon-3 (C-3) epimerization is that of the secosteroid hormone 1α,25-dihydroxyvitamin D₃, which, when converted to 1α,25-epi-dihydroxyvitamin D₃, becomes biologically much less active (16, 17). These examples illustrate well the importance of C-3 epimerization in regulating the biological activities of steroid hormones.

In the present study, we report the cloning and characterization of a human cDNA encoding a 3(α→β)-hydroxysteroid epimerase (3(α→β)-HSE), which catalyzes the transformation of C-19 and C-21 steroids. To our knowledge, this is the first molecular characterization of a mammalian 3(α→β)-HSE reported to date.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF223225.

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EXPERIMENTAL PROCEDURES

Isolation of Human 3(α→β)-HSE—A cDNA fragment of 174 bp, corresponding to the conserved region situated near the N-terminal of the enzymes belonging to the RoDH family, was obtained by reverse transcription from human liver poly(A)1 (CLONTECH Inc., Palo Alto, CA) followed by 30 cycles of amplification using PCR and specific oligoprimer pairs (5′-GCC-GAA-TTC-GTG-GTG-AGC-CAT-CTA-CAT-3′ and 5′-CGC-GGA-TCC-CAC-TGT-CTC-CAG-CCT-GGA-3′). The restriction sites EcoRI and BamHI were added to the oligoprimers for subsequent subcloning. The cDNA fragment thus obtained was used for the subsequent screening of a human liver lgt11 cDNA library (CLONTECH). The positive recombinant plaques were purified, and phage DNA was isolated by centrifugation for 90 min at 105,000 g, followed by phenol extraction. DNA inserts from positive phages were digested with EcoRI and ligated into pBluescript II SK1 for sequencing with the T7 sequencing kit (Amersham Pharmacia Biotech). The longest inserts containing the ATG codon and the poly(A)1 tail were cloned into a pCMV-neo expression vector for activity studies. Plasmid DNA was prepared using a Mega kit (Qiagen, Chatsworth, CA).

Transient and Stable Expression of 3(α→β)-HSE in HEK-293 Cells—Transient transfection of the expression vector pCMV-3(α→β)-HSE into HEK-293 cells was performed using the calcium phosphate precipitation procedures (18). For stable transfection, cells were cultured at half-confluence in 6-well dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum 24 h before transfection. 5 μg of pCMVneo-3(α→β)-HSE vector was transfected into HEK-293 cells using the Lipofectin transfection method (Life Technologies, Inc., Burlington, Ontario, Canada). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 0.7 mg/ml of G-418 to inhibit the growth of non-transfected cells. The medium was changed every 2 days until resistant colonies were observed. The positive clones were selected according to their ability to transform androsterone (ADT) into 5α-androstane-3β-ol-17-one (epi-ADT).

Site-directed Mutagenesis—The change from E to D and from G to R at positions 63 and 105 was performed using oligonucleotide primer pairs: 5′-GTG-GGG-GAC-AGA-AGA-CTC-TGG-GGA-CTG-G-3′, 5′-CCA-GTC-CCC-AGA-GTC-TTC-TGT-CCC-CCA-C-3′, and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Successive mutations were introduced for additional changes in the sequence with the following oligonucleotide pairs: 5′-GGA-GAG-CAC-GGG-GGA-AGA-ATT-GTC-AAT-G-3′, 5′-CAT-TGA-CAA-TTC-TTC-CCC-CGT-GCT-CTC-C-3′, and 5′-CTT-TCT-TTG-TAG-AGG-CTA-CTG-TGT-C-3′, 5′-GAC-ACA-GTA-GCC-TCT-ACA-AAG-AAA-G-3′. The integrity of the construct was verified by sequencing the inserted DNA fragment using T7 sequencing kit (Amersham Pharmacia Biotech). Plasmid DNA was prepared using the Qiagen Mega Kit. Oligonucleotide primers were synthesized with a DNA synthesizer ABI-394 (Perkin-Elmer).

Epimerase Activity Assays—The determination of the enzyme activity in intact cells was done following the method previously described (19). Briefly, 0.1 μM of the radiolabeled steroid was added to a freshly changed culture medium in a 24-well culture plate. When cell homogenates or 100,000 g fractions were used, the reaction was performed in

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**Fig. 1.** Nucleotide and putative amino acid sequences of the human 3(α→β)-HSE (AF223225). Numbers above the sequences correspond to the nucleotide sequence relative to the translation start site; numbers below the sequences correspond to the amino acid sequence. The AATAAA polyadenylation signal is underlined.
a 50 mM sodium phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, 0.1 mM radioactive steroids, and 1 mM of the required cofactor. Incubations were performed at 37 °C for 2 h. After the incubation, the steroids were extracted twice with 1 ml of ether. The organic phases were pooled and evaporated to dryness. The steroids were solubilized in 50 ml of dichloromethane, applied to a Silica Gel 60 thin layer chromatography (TLC) plate, then separated by migration in the toluene-acetone (4:1) solvent system. Metabolites were identified by visualization in 5% by volume of anisaldehyde and heating at 110 °C. The samples were further confirmed by high performance liquid chromatography (HPLC, data not shown). The results thus strongly suggest that this single enzyme possesses stereo-selectivity for the oxidative and reductive reactions. To further confirm that the reductive reaction preferentially produces the 3β-OH isomer, we incubated the enzyme with [14C]3α-dione in the presence of NADH, as illustrated in Fig. 3B. The major end product was indeed 3α-HSD activity (21). 71.4% identity with the rat type 6 RoDH (21); 67.2% and 67.5% identity, respectively, with the two newly reported human sterol/retinol dehydrogenases (22, 23); 66–67% identity with the three rat RoDHs (24–26); and 50% sequence identity with human 11-cis-RoDH or 9-cis-RoDH (27, 28).

Determination of Enzymatic Activity of the Recombinant Enzyme—Because the clone we obtained shows 94.7% identity with one of the previously reported enzymes, we wanted to determine whether our clone also encodes a protein exhibiting the same activity. To our surprise, when we incubated the microsomal fraction of HEK-293 cells stably overexpressing the enzyme with [3H]ADT in the presence of NAD+, we obtained two metabolites. One was 5α-dione, the expected oxidative metabolite of ADT, whereas the second was identified as 3α,5α-epidione (Fig. 3A). The identity of this second metabolite was further confirmed by high performance liquid chromatography (data not shown). The results thus strongly suggest that this single enzyme possesses stereo-selectivity for the oxidative and reductive reactions. To further confirm that the reductive reaction preferentially produces the 3β-OH isomer, we incubated the enzyme with [14C]3α-dione in the presence of NADH, as illustrated in Fig. 3B. The major end product was indeed 3α-HSD activity (21). 71.4% identity with the rat type 6 RoDH (21); 67.2% and 67.5% identity, respectively, with the two newly reported human sterol/retinol dehydrogenases (22, 23); 66–67% identity with the three rat RoDHs (24–26); and 50% sequence identity with human 11-cis-RoDH or 9-cis-RoDH (27, 28).

Determination of the Preferred Reaction: 3α-HSD versus 3β-HSD (21), ADT (Fig. 3A). The identity of this second metabolite was further confirmed by high performance liquid chromatography (data not shown). The results thus strongly suggest that this single enzyme possesses stereo-selectivity for the oxidative and reductive reactions. To further confirm that the reductive reaction preferentially produces the 3β-OH isomer, we incubated the enzyme with [14C]3α-dione in the presence of NADH, as illustrated in Fig. 3B. The major end product was indeed 3β-diol (two-thirds of the products), as expected, whereas approximately one-third of the products was found to be ADT.

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Fig. 3. Characterization of the reactions catalyzed by human 3(α→β)-HSE. A: lanes 1 and 2, autoradiography of standard 14C-labeled ADT and epi-ADT; lanes 3 and 4, metabolites extracted after the incubation of 0.1 μM [14C]ADT with the microsomal fraction of HEK-293 cells overexpressing 3(α→β)-HSE and mock-transfected, respectively, in 1 ml of 50 mM phosphate buffer (pH 7.4) and 1 mM NAD+; B: lane 1, autoradiography of standard 14C-labeled 5α-dione; lanes 2 and 3, metabolites extracted after a reaction performed under the same conditions as described for lanes 3 and 4 of A, except that 0.1 μM [14C]5α-dione and 1 mM NADH were used as substrate and cofactor, respectively. The reactions were performed at 37 °C for 2 h and were stopped by adding 1 ml of ether. Extraction, separation on TLC, and quantification of steroids were performed as described under “Experimental Procedures.” Histograms showing the amount of products obtained in the corresponding reactions appear below the autoradiographs.

Fig. 4. Determination of the reaction direction preferably catalyzed by human 3(α→β)-HSE. Lanes 1–3, autoradiography of standard [14C]5α-dione, [14C]ADT, and [14C]epi-ADT, respectively. Metabolites extracted after incubation of 0.1 μM [14C]ADT (lanes 4 and 5) and 0.1 μM [14C]epi-ADT (lanes 6 and 7) with the microsomal fraction of HEK-293 cells overexpressing 3(α→β)-HSE and mock-transfected cells, respectively. The reactions were performed at 37 °C for 2 h in 50 mM phosphate buffer (pH 7.4) containing 1 mM both NAD+ and NADH. The reactions were stopped by adding 1 ml of ethyl ether. The procedures were as described under “Experimental Procedures.” Histograms showing the percentage of the products in the corresponding reactions appear below the autoradiograph.

of 13 and 1.17 for ADT and epi-ADT as substrates, respectively), whereas in the reductive reaction the 3β-isomers are the major end products (V_{max}/K_{m} values of 12.5 and 3.5 for the reduction of 5α-dione to epi-ADT and ADT, respectively).

Epimerase Activity in Intact Transfected Cells in Culture—Previously, we have shown that intact transfected cells in culture are more suitable for determining the preferred reaction of oxidoreductases and for characterizing their physiological activities (19, 29, 30). Thus, to further determine whether the enzyme could catalyze epimeration without the addition of exogenous cofactors and under conditions more similar to physiological conditions, we incubated HEK-293 cells stably overexpressing 3(α→β)-HSE in a culture medium with 0.1 mM ADT and epi-ADT for various time periods. As illustrated in Fig. 5, the enzyme efficiently catalyzes the transformation of ADT into epi-ADT, whereas the transformation of epi-ADT into ADT is done at a much lower rate. The activity catalyzed by non-transfected HEK-293 under the same conditions was not significant.

Substitution of Amino Acid Residues in 3(α→β)-HSE with the Corresponding Ones in the RoDH-like Enzyme—Because the reported human RoDH-like enzyme having oxidative 3α-HSD activity shows only a few differences in amino acid residues with the 3(α→β)-HSE, we examined whether and how these changes in amino acid residues could affect the 3(α→β)-HSE activity. We thus performed site-directed mutagenesis to change the Glu at position 63 into Asp (E63D) and the Gly at position 105 into Arg (G105R) separately and in combination with the insertion and deletion of the nucleotide G to introduce the changes in 17 amino acid residues from 157 to 174 in the amino acid sequence. Expression vectors bearing these mutant sequences were analyzed for their activities. As illustrated in Table II, although the E63D substitution does not significantly affect the 3(α→β)-HSE activity, the G105R substitution and the changes in the 17 amino acid residues both completely inactivate the 3(α→β)-HSE activity of the recombinant enzyme.

### Table I

| Substrate | Cofactor | Product | K_m | V_max | V_{max}/K_m |
|-----------|----------|---------|-----|-------|-------------|
| ADT | NAD+ | 5α-Dione | 0.4 | 5.2 | 13 |
| epi-ADT | NAD+ | 5α-Dione | 1.7 | 2.0 | 1.17 |
| 5α-Dione | NADH | ADT | 0.4 | 1.4 | 3.5 |
| 5α-Dione | NADH | epi-ADT | 0.2 | 2.5 | 12.5 |
| Allo-pregnanolone | NAD+ | Iso-pregnanolone | 3.0 | 8.6 | 2.9 |

$K_{m}$ values represent the averages of two separate experiments with eight substrate concentrations. Duplicate samples were made for each concentration in each experiment. Each reaction was run for 1 h with the microsomal fraction of 20,000 HEK-293 cells stably expressing the enzyme.
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The activity was determined using intact cells in culture transiently transfected with the expression vector described above. The conversion of ADT to epip-ADT was examined. Data was obtained from duplicate assays.

| Positions of changes in nucleotide sequence (relative to ATG) | Mutations and positions in amino acids (relative to Met) | Activity (nmol/10^6 cells/h) |
|---------------------------------------------------------------|----------------------------------------------------------|-----------------------------|
| hHSE (wild type)                                              | wild type                                                | 0.021                       |
| G160 to T169                                                   | Glu^63 to Asp^63                                         | 0.019                       |
| G313 to A313                                                  | Gly^105 to Arg^105                                       | ND                          |
| Insertion of G468 and deletion of G520                       | Mutations of Gly^157 to Gly^174                          | ND                          |
| G160 to T169, G189 to A184, and insertion of G468 and deletion of G520 | Glu^63 to Asp^63, Gly^105 to Arg^105, and mutations of Gly^157 to Gly^174 | ND                          |

* ND, not detectable.

**Tissue Distribution of the 3(α→β)-HSE mRNA**—The tissue distribution of the epimerase mRNA was assessed by semiquantitative RT-PCR using poly(A)^+ RNA from CLONTECH. As illustrated in Fig. 6, we detected the expression of epimerase transcript in various tissues, namely, adrenal, brain, liver, lung, mammary gland, placenta, prostate, testis, and uterus. Relative levels of the transcript were determined by varying the quantity of poly(A)^+ RNAs for the reverse transcriptase reaction and the number of PCR amplification cycles to determine the linear range of PCR amplification for each tissue. It was determined that the highest level of expression is in the liver. In the other tissues, the order of the epimerase transcript levels is as follows: spleen > prostate > adrenal > brain > uterus > mammary gland > placenta (data not shown). No amplification product was detected from the testis tissue, even when the PCR product was probed by an epimerase-specific oligonucleotide with a longer exposure time.

**DISCUSSION**

In this report, we describe cloning and expression of a cDNA encoding an enzyme possessing 3(α→β)-HSE activity. Our results clearly indicate that the epimerization reaction is the result of catalysis by a single enzyme possessing both oxidative and reductive activities. As illustrated in Fig. 7, the overall reaction is composed of two steps, the oxidation of a 3α-hydroxy group to a 3-keto group, followed by the reduction of this 3-keto group into a 3β-hydroxy group. The 3-ketosteroid conformation is thus an intermediate in the 3(α→β)-hydroxysteroid epimeration. To our knowledge, this is also the first observation of an enzyme possessing stereo-selectivity for the oxidative and reductive reactions. Except for the 17 amino acids located from positions 158–174, and 2 amino acid substitutions at positions 63 and 105 (E63D and G105R) (Fig. 2), the sequence of our clone is identical to that of the RoDH-like human oxidative 3α-hydroxysteroid dehydrogenase, previously reported by Biswas and Russell (21). It is interesting to observe that the deletion and insertion of a G, respectively, at positions 467 and 520 relative to ATG in the sequence reported by Biswas and Russell (21), will transform these 17 different amino acid residues into the ones reported here.

To further understand the effect of these differences on the activity of the enzyme and to compare the epimerase activity reported here with the RoDH-like oxidative 3α-HSD activity reported by Biswas and Russell (21), we performed site-directed mutagenesis and examined the activities of each as described in Table II. The E63D substitution, which does not affect 3(α→β) activity, is probably due to polymorphism. On the other hand, because the enzyme described by Biswas and Russell (21) is an active one, the G105R substitution and the differing 17 amino acid residues (resulting from the deletion and the insertion of a G at positions 467 and 520, respectively) can probably be explained by sequencing errors. Further evidence that the sequence described by Biswas and Russell (21) contains sequencing errors is a sequence submitted by Kedishvili to GenBank under the accession number AF016509. This sequence is almost identical to ours except for two nucleotides located at positions 18 and 133 downstream from the ATG initiation codon. These changes do not alter the amino acid sequence. In addition, this sequence is probably obtained by PCR amplification, because it does not contain the 5’- and 3’-untranslated region. Therefore, the possibility that the difference of two nucleotides is due to amplification error is not to be excluded. Under the experimental conditions described by Biswas and Russell (21), when the period of incubation is very short, only one reaction can be detected and the direction of this
reaction is determined by the added cofactor. Although this is a regular procedure used to analyze enzyme activities in vitro, it is not suitable for this particular enzyme, which catalyzes both the oxidative and the reductive reactions in a stereo-selective manner. This explains why these authors failed to associate both activities into one epimeration reaction. Although an in vitro assay using purified enzyme or preparations of subcellular fractions is a common procedure for performing kinetic or ligand binding studies, we believe that transfected intact cells are more suitable to characterize enzyme activities in living cells, especially for oxidoreductases. This way, the preferred reaction catalyzed by the enzyme, either oxidation or reduction, can be clearly identified (29), and unstable or labile enzymes can be studied with certainty (19). In the present study, using intact transfected cells in culture without the addition of exogenous cofactors, a system which better reflects the actual physiological conditions, we demonstrated clearly that 3(α→β)-HSE possesses an epimerase activity capable of converting a 3α-hydroxysteroid into its 3β-hydroxy epimer (Fig. 5). This is further confirmed by in vitro assays in the presence of both NAD$^+$ and NADH as cofactors (Fig. 4).

The physiological significance of the conversion of 3α-hydroxysteroid by the epimerase has been demonstrated by recent findings regarding the differential biological activities of some stereoisomers of steroids. In fact, 3α-diol and 3β-diol have long been considered as breakdown products of androgens. However, recent studies provided evidence that 3α-diol, but not its 3β-epimer, might be an active hormone required for parturition in the rat (31). Furthermore, allo-pregnanolone, readily formed from progesterone in the brain, is one of the most potent neuroactive steroids. It can suppress the release of hypothalamic gonadotropin-releasing hormone via the γ-aminobutyric acid A receptor, whereas its 3β-epimer does not exert the same effect (14). Allo-pregnanolone is strongly suspected of being involved in the premenstrual syndrome (15). The conversion of 3α-diol and allo-pregnanolone into their respective 3β-isomers by 3(α→β)-HSE could inactivate these molecules. The detection of expression of the epimerase transcript in the brain provides evidence for the possible involvement of the epimerase in the regulation of allo-pregnanolone levels and, therefore, the possible implication of the epimerase in regulating hormonal changes in the brain.

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