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Structure–Function Relationships and Molecular Genetics of the 3β-Hydroxysteroid Dehydrogenase Gene Family

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The isoenzymes of the 3β-hydroxysteroid dehydrogenase/5-ene-4-ene-isomerase (3β-HSD) gene family catalyse the transformation of all 5-ene-3β-hydroxysteroids into the corresponding 4-ene-3-keto-steroids and are responsible for the interconversion of 3β-hydroxy- and 3-keto-5α-androstane steroids. The two human 3β-HSD genes and the three related pseudogenes are located on the chromosome 1p13.1 region, close to the centromeric marker D1Z5. The 3β-HSD isoenzymes prefer NAD^+ to $NADP^+$ as cofactor with the exception of the rat liver type III and mouse kidney type IV, which both prefer NADPH as cofactor for their specific 3-ketosteroid reductase activity due to the presence of Tyr^{36} in the rat type III and of Phe^{36} in mouse type IV enzymes instead of Asp^{36} found in other 3β-HSD isoenzymes. The rat types I and IV, bovine and guinea pig 3β-HSD proteins possess an intrinsic 17β-HSD activity specific to 5α-androstane 17β-ol steroids, thus suggesting that such "secondary" activity is specifically responsible for controlling the bioavailability of the active androgen DHT. To elucidate the molecular basis of classical form of 3β-HSD deficiency, the structures of the types I and II 3β-HSD genes in 12 male pseudohermaphrodite 3β-HSD deficient patients as well as in four female patients were analyzed. The 14 different point mutations characterized were all detected in the type II 3β-HSD gene, which is the gene predominantly expressed in the adrenals and gonads, while no mutation was detected in the type I 3β-HSD gene predominantly expressed in the placenta and peripheral tissues. The mutant type II 3β-HSD enzymes carrying mutations detected in patients affected by the salt-losing form exhibit no detectable activity in intact transfected cells, at the exception of L108W and P186L proteins, which have some residual activity (~1%). Mutations found in nonsalt-loser patients have some residual activity ranging from ~1 to ~10% compared to the wild-type enzyme. Characterization of mutant proteins provides unique information on the structure–function relationships of the 3β-HSD superfamily.

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Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase/5-ene-4-ene-isomerase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3-KSR, 3-keto steroid reductase; 3α-HSD, 3α-hydroxysteroid dehydrogenase; PREG, pregnenolone (5-pregnene-3β-ol-20-one); 17-OH-PREG, 17-OH pregnenolone; DHEA, dehydroepiandrosterone (andros-5-ene-3β-17β-diol); 5-ene-DIOL (androst-5-ene-3β,17β-diol); PROG, progesterone (4-pregnene-3,20, dione); 17-OH-PROG, 17-OH progesterone; 4-ene-DIONE, 4-ene-androstenedione (androst-4-ene-17β-ol-3-one); DHT, 5α-dihydrotestosterone (5α-androstane-17β-ol-3-one); 3β-diol, 5α-androstane-3β17β-diol; DHP, 5α-dihydroprogesterone; ADT, androsterone (5α-androstane-3α-17βdiol); A-DIONE, androstanedione (5α-androstane-3-17-dione); CAH, congenital adrenal hyperplasia; PCR, polymerase chain reaction; membrane spanning domain, MSD; nonclassic 3β-HSD, NC3β-HSD; cytomegalovirus, CMV.

INTRODUCTION

The membrane-bound 3β-hydroxysteroid dehydrogenase/5-ene-4-ene-isomerase (3β-HSD) located in the endoplasmic reticulum and in mitochondrial membranes [1, 2] catalyzes the conversion of 5-ene-3β-hydroxysteroids, namely, pregnenolone (PREG), 17-OH pregnenolone (17-OH-PREG), dehydroepiandrosterone (DHEA) and androst-5-ene-3β,17β-diol (5-ene-DIOL) into the corresponding 4-ene-3-ketosteroids progesterone (PROG), 17-OH progesterone (17-OH-PROG), 4-ene-androstenedione (4-ene-DIONE) and testosterone, respectively. This enzymatic activity is required for the formation of all classes of steroid hormones, namely, progesterone, mineralocorticoids, glucocorticoids, as well as androgens and estrogens [3–5]. In addition, to be involved in the irreversible dehydrogenation and isomerization of 5-ene-3β-hydroxysteroid precursors, the enzymes of the 3β-HSD family are also closely involved in the formation and/or degradation of the 5α-androstanes, thus catalyzing the transformation of 3β-hydroxy and 3-keto-5α-androstane steroids [6–11].

Recently, the structure of cDNAs encoding isoenzymes of the 3β-HSD family have been characterized in the human and several other vertebrate species [3–5, 8, 12–22] (Fig. 1). Transient expression of recombinant human types I and II [6, 7, 22], rat types I, II and IV [8–10, 16, 23], mouse types I and III [18], guinea pig type I [19] and rainbow trout type I [20] isoenzymes reveals that 3β-HSD and 5-ene-4-ene isomerase activities reside within a single protein. These studies have demonstrated that NAD^+ is the cofactor required for 3β-HSD activity, which acts as an allosteric activator of the isomerase activity.

3β-HSD activity is not only found in classical steroidogenic tissues, namely the placenta, adrenal cortex, ovary, and testis, but also at significant levels in several peripheral tissues, including the liver, skin, adipose tissue, lung, endometrium, myometrium, prostate, epididymis, seminal vesicle, mammary gland, intestine, kidney, salivary gland, heart, and brain [5, 24–26]. The localization and ontogeny of 3β-HSD in the human adrenal, testis, ovary and placenta as well as in the same rodent tissues have been studied [27–32] and recently reviewed [33]. The widespread distribution of 3β-HSD expression indicates that this enzyme is likely to play an important role in the intracrine formation of sex steroids in peripheral target tissues [34].

Little is known about the structure–function relationships of the 3β-HSD family of enzymes. However, affinity radiolabelling of purified bovine adrenal 3β-HSD with 5′-[*p*-(fluorosulfonyl)benzoyl][adenine-8^{14}C]adenosine identified the presence of two cysteine residues at or near the putative NAD-binding site found in peptides I^{18} to K^{26} and E^{274} to R^{282} [35]. More recently, affinity radiolabelling of human type I 3β-HSD with 2α-bromo[2′-^{14}C]acetoxyprogesterone identified two tryptic peptides, Lys^{175} and Arg^{250}, comprising amino acids Asn^{176} to Arg^{186} and Gly^{251} to Lys^{274}, respectively, that should contain residues involved in the putative substrate-binding domain [36]. These two last regions are highly conserved in human type II 3β-HSD and other members of this family of enzymes and might be involved in the substrate-binding domain.

HUMAN 3β-HSD ISOENZYMES AND THEIR GENES

Structure and expression of type I and type II 3β-HSD isoenzymes

Following purification of 3β-HSD from human placenta [37, 38], a first placental cDNA type has been isolated and characterized [12] (Fig. 2). This sequence has also been confirmed in parallel by Lorence *et al.* [7]. The second 3β-HSD cDNA type, chronologically designated as type II 3β-HSD, was isolated from a human adrenal λgt22 cDNA library [22]. The type I (HSD3B1) gene encodes an isoenzyme of 372 amino acids, that is predominantly expressed in the placenta and peripheral tissues, such as the skin and mammary gland, whereas the type II (HSD3B2) gene encodes an isoenzyme of 371 amino acids sharing 93.5% homology with the type I, which is almost exclusively expressed in the adrenals and gonads [22].

The type I enzyme possesses a 3β-HSD/5-ene-4-ene isomerase activity higher than type II with respective K_m values of 0.24 and 1.2 μM for PREG, 0.18 and 1.6 μM for DHEA. The specific activity (V_{max} of both types is equivalent [22]. Incubation of cell homogenates in the presence of NADH and [^{3}H]DHT shows that the 3β-hydroxysteroid oxidoreductase activity, as measured by the formation of 5α-androstane-3β,17β-diol, is also higher for type I than that of the type II 3β-HSD protein with K_m values of 0.26 and 2.7 μM, respectively.

The higher K_m value of type II 3β-HSD mainly expressed in steroidogenic tissues could be related to the higher levels of endogenous substrates present in these classical steroidogenic tissues. The approximately 10-fold higher affinity of type I 3β-HSD, which is preferentially expressed in peripheral intracrine tissues could greatly facilitate steroid formation from relatively low concentrations of substrates usually present in these tissues.

Structure and chromosomal localization of type I and type II 3β-HSD genes

The complete structure of both type I (HSD3B1) and type II (HSD3B2) 3β-HSD genes, which consists of four exons included within a genomic DNA fragment of about 7.8 kb, was determined [6, 39–41]. Comparison of the nucleotide sequences of the two genes indicates that they share 77.4, 91.8, 94.5, 91.0,

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                      10         20         30         40         50         60
HUMAN I        TGWSCLVTGA GGFLGQRIIR LLVKEKELKE IRVLDKAFGP ELREEFSKLQ NKTKLTVLEG
HUMAN II       .......... ..L.....V. ...E...... ..A.....R. .......... .R........
MACAQUE        .......... ........V. ...E...... ........R. .......... ..........
BOVINE         A........G .........C ...E..D.Q. ......V.R. .V........ S.I...L...
MOUSE I        A......... ...V.....K M..Q....Q. V.A...V.R. .TK....... T...V.....
MOUSE II       ---------- ---------- ---------- ---------- ---------- ----------
MOUSE III      P......... .........Q ...Q..D.E. ......V.K. .T..Q.FN.G TSI.V.....
MOUSE IV       P......... ........V. M..Q.E..Q. ..A.FRT..R KQE..L.... T...V...K.
RAT I          P......... ...V...... M..Q....Q. V.A...V.R. .TK....... T.A.V.M...
RAT II         P......... ...V...... M..Q....Q. V.A...V.R. .TK....... T.A.V.M...
RAT III        P......... ........VQ M..Q....Q. V...YRT.S. KHK..L.... T.A.V...R.
RAT IV         P......... ........VQ ...Q..D... V.....V.R. .T....FN.G TSI.V.....
RAINBOW TROUT SLQ.DV.V.... C....E.LV. ..LE.DK.T. ..M..INVR. Q.IQCLEEIR GD.LVS.F..

                      70         80         90        100        110        120
HUMAN I        DILDEPFLKR ACQDVSVIIH TACIIDVFGV THRESIMNVN VKGTQLLLEA CVQASVPVFI
HUMAN II       .......... .......V.. .......... .......... .......... ..........
MACAQUE        .......... .......V.. .......... .......... .......... ..........
BOVINE         .....QC..G ...GT..V.. ..SV...RNA VP..T..... .......... ..........
MOUSE I        ....AQC.R. ...GI..V.. ..AV...T.. IP.QT.LD.. L....N.... .......A..
MOUSE II       ---------- ---------- ---------- ---------- -------... .I.....A..
MOUSE III      ....TQY.R. ...GI..V.. ..A....T.. IP.QT.LD.. L....N.... .I.....A..
MOUSE IV       ....AQC... ...GM.AV.. ..AA..PL.A AS.QT.LD.. L.......D. ..E.N..T..
RAT I          ....AQY.R. ...GI..V.. ..AV...SH. LP.QT.LD.. L....NI... ..E....A..
RAT II         ....AQY.R. ...GI..V.. ..SVM.FSR. LP.QT.LD.. L....N.... GIH....A..
RAT III        ..V.AQ..R. ...GM..... ..AAL.IA.F LP.QT.LD.. ........D. ..E....A..
RAT IV         ....TQC.R. ...GI..V.. ..AL...T.. NP.QT.LD.. L....N.... .......A..
RAINBOW TROUT  ..S.SEL.R. ..KGA.LVF. ..SL...T.K VLYSELHR.. .........T ...EN.VS..

                     130        140        150        160        170        180
HUMAN I        YTSSIEVAGP NSYKEIIQNG HEEEPLENTW PAPYPHSKKL AEKAVLAANG WNLKNGGTLY
HUMAN II       .......... .......... .......... .T...Y.... .......... ......D...
MACAQUE        ...TL..... .......... .......... .....Y.... .......... .T........
BOVINE         H..T...... ...R....D. R...HH.SA. SS...Y.... ......G... .A........
MOUSE I        FC..VD.... ....K.VL.. ...QNH.S.. SD...Y...M .......... SM.......N
MOUSE II       FS..VD.... ......VL.. ....CH.S.. SD...Y...M .......... SM.......Q
MOUSE III      FS..VD.... ....D.VL.. ..D.HR.S.. SD...Y...M .......... SM.......Q
MOUSE IV       .S..VL.... .......L.A ....HH.S.. SN...Y...M .......... SI.......H
RAT I          .C.TVD.... ....K.VL.. ....HH.S.. SDA..Y..RM .......... SI.......H
RAT II         .C.TVD.... ....KT.L.. R...HH.S.. SN...Y...M .......... SI.......H
RAT III        .S..TG.... .....T.L.D R...HR.S.. SN...Y..RM .......... SI......FH
RAT IV         .C.TVD.... ....K..L.. ....HH.S.. SN...Y...M .......... SI.......H
RAINBOW TROUT  .......... .ANGDP.I.. D.NT.YTCSL KF..SKT..E ..QVT.Q.Q. EV.Q...R.A

                     190        200        210        220        230        240
HUMAN I        TCALRPMYIY GEGSRFLSAS INEALNNNGI LSSVGKFSTV NPVYVGNVAW AHILALRALQ
HUMAN II       ......T... ...GP..... .......... .......... .......... .........R
MACAQUE        .......... ...GP..... .......... .......... .......... .........R
BOVINE         .......... ....P....Y MHG....... .TNHC...R. .......... .........R
MOUSE I        .......... ..R.P.IFNA .IR..K.K.. .CVT....IA .....E.... .....A.G.R
MOUSE II       .......C.. ..R.PLI.NI .IM..KHK.. .R.F...N.A .......... .....A.G.R
MOUSE III      .......C.. ..R.Q...NT .IK..K.KF. .RGG.....A .......... .....A.G.R
MOUSE IV       .....LSF.. ..ECQVT.TT VKT..K..S. IKKNAT..IA .......A.. .....A.S..
RAT I          .......... ..R.P...VM .LA..K.K.. .NVT....IA .......... .....A.G.R
RAT II         .......... ..RGQ...RI .IM..K.K.V .NVT....I. .......... .....A.G.R
RAT III        .....LPF.. ..E.QII.TM V.R..K.NS. IKRHAT..IA .......A.. .....A.G.R
RAT IV         .......... ..R.P...VM .LA..K.K.. .NVT....IA .......... .....A.G.R
RAINBOW TROUT  .......... ...C...LGH MGDGIR.GDM .YRTSREAQ. .......A.L ..LQ.A...R
                                                        ^
                                                        P
                     250        260        270        280        290        300
HUMAN I        DPKKAPSIRG QFYYISDDTP HQSYDNLNYT LSKEFGLRLD SRWSFPLSLM YWIGFLLEIV
HUMAN II       .......V.. .......... .........I .......... ....L..T.. ........V.
MACAQUE        .......VQ. .......... .........I .......C.. ....L..A.. ........V.
BOVINE         ....V.N.Q. .......... .....D.... ....W.FC.. ..M.L.I..Q ..LA......
MOUSE I        ....ST..Q. .......... .....D.... ....W...PN AS..L..P.L ..LA....T.
MOUSE II       ....S.N.Q. E......... ...F.DIS.. ....W.FCP. .S..L.VP.L ..LA....T.
MOUSE III      N...S.N.Q. E......... .....D.... ....W.FC.N ...YL.VPIL ..LA....T.
MOUSE IV       ....S...Q. .....T.... .....D.KC. ....W..... TS..L..P.L ..LA....T.
RAT I          ....SQNVQ. .......... .....D..C. ....W..... .S..L..P.L ..LA....T.
RAT II         ....SQN.Q. .......... .....D..C. ....W..... .S..L..P.L ..LA....T.
RAT III        ..E.SQ..Q. .......... .....D.... ....W.FC.. .S..L..P.L ..LA....T.
RAT IV         ....SQNVQ. .......... .....D.... ....W..H.. .S..L..P.L ..LA......
RAINBOW TROUT  ..QRRAA.G. N......... PV..SDF.HA VLSPL.FSIQ EKPIL.IPVL .LLC..M.ML

                     310        320        330        340        350        360
HUMAN I        SFLLRPIYTY RPPFNRHIVT LSNSVFTFSY KKAQRDLAYK PLYSWEEAKQ KTVEWVGSLV
HUMAN II       ....S...S. Q......T.. .......... .......... .......... ..........
MACAQUE        ....S.V.S. Q......T.. .......... .......... .......... ..........
BOVINE         ....S...K. N.C....L.. .......... .......G.E ...T...... ..K..I....
MOUSE I        ......V.R. ..L....LI. ....T..... .......G.E ..VN...... ..S..I.TI.
MOUSE II       ....S...R. I......L.. ..G.T..... .......G.E ..V....... ..S..I.T..
MOUSE III      ....S...R. I......L.. .TA.T..... .......G.E ..V....... ..S..I.T..
MOUSE IV       ......V.N. ......LLI. VL........ .......G.E ..V....... ..S..I.T..
RAT I          ......F.N. ......C.L. ....K..... .......G.V ..V....... ..S..I.T..
RAT II         ......F.N. ......C.L. ....K..... .......G.E ..V....... ..S..I.T..
RAT III        ......F.N. .......FM. IL....I... .......G.E ..V....... ..S..I.T..
RAT IV         ..F.H.V.N. ..S....L.. ....K..... .......G.E ..V....... ..S..I.T..
RAINBOW TROUT  QI..C.FKRF T..I..QLL. ML.TP.S... RR....MG.A .R......RK R.MD..A.QL

                     370
HUMAN I        DRHKETLKSK TQ
HUMAN II       .......... ..
MACAQUE        .......... ..
BOVINE         KQ......T. IH
MOUSE I        EQ.R.I.DT. C.
MOUSE II       EQ.R...DT. S.
MOUSE III      EQ.R...DT. S.
MOUSE IV       MQ.R.IGNK. S.
RAT I          EQ.R...DT. S.
RAT II         EQ.R...DT. S.
RAT III        EQ.R...DT. S.
RAT IV         EQ.R...DT. S.
RAINBOW TROUT  PKER.RI.V. --
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Fig. 1. Comparison of the deduced amino acid sequences of human types I and II, macaque ovary, bovine ovary, rainbow trout ovary, rat types I, II, III and IV as well as mouse types I, II, III and IV members of the 3β-HSD enzyme family. Amino acid sequences are designated by the single-letter code. Residues common to human type I 3β-HSD are designed by a dot (.). The noncharacterized NH_2-terminal sequence in mouse type II 3β-HSD is indicated by a dashed line (----). Note that the members of the mammalian 3β-HSD family have been chronologically designated as a function of their elucidation in each species.

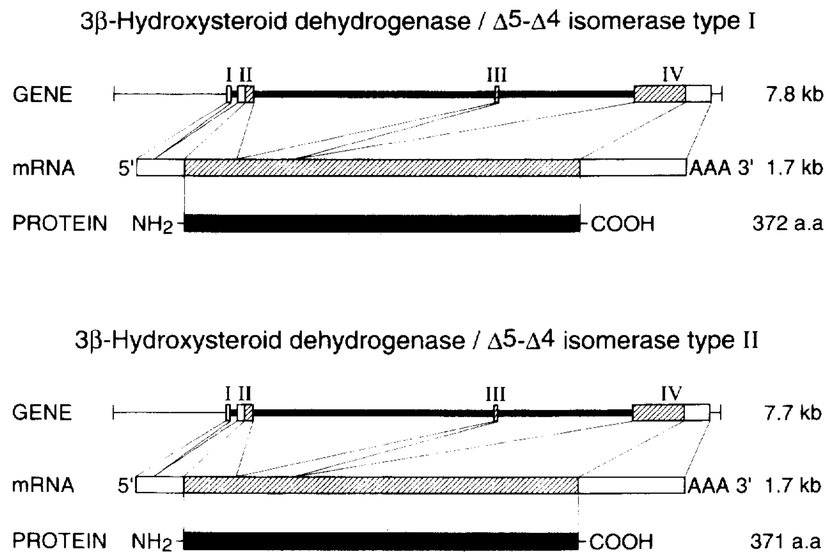

Fig. 2. Structure of human type I and type II 3β-HSD genes, mRNA species and the corresponding proteins. Exons are represented by boxes in which hatched lines demarcate the coding regions, while open boxes represent the noncoding regions. Introns are represented by black bold lines.

84.0, 80.4 and 74.2 homology in exons I, II, III and IV as well as in introns I, II and III, respectively. The 1250 nucleotides in the 5′ flanking region share 81.9% homology. We have also recently demonstrated that the structural organization of rat types I, II and IV 3β-HSD is closely similar to that of the human 3β-HSD genes [42].

The HSD3B1 and HSD3B2 genes were assigned by *in situ* hybridization to the chromosome 1p13.1 region [43, 44]. As demonstrated by genetic linkage analysis the D1S514 marker was closely linked to HSD3B1 ($\theta < 0.001$; Lod score = 14.13) and HSD3B2 ($\theta = 0.008$; Lod score = 35.36); the HSD3B loci are located at 1–2 cM of the centromeric marker D1Z5 [45]. To obtain information on the physical linkage of the HSD3B1 and HSD3B2 genes, *Not* I and *Sac* II digests of genomic DNA were resolved by pulse-field gel electrophoresis and hybridized with type I and type II 3β-HSD cDNAs used as probes. A single band of about 0.6 megabase was detected after digestion with *Not* I, whereas digestion with *Sac* II suggests that the two genes are located within an approximately 0.29 megabase DNA fragment [45]. This observation is in agreement with the unique localization of the HSD3B gene family to 1p13.1 by *in situ* hybridization. Furthermore, the detection of a single band after hybridization of *Not* I or *Sac* II digested DNA under low stringency conditions strongly suggests that the three pseudogenes recently characterized [46] and sharing a high sequence homology with the HSD3B1 and HSD3B2 genes, are also included within this 0.29 megabase *Sac* II DNA fragment. The close linkage and the high sequence identity of these genes and pseudogenes strongly suggest that the human 3β-HSD gene family exists as a tandem cluster of related genes which have evolved through duplication and divergence of a single ancestral gene. A similar conclusion was recently proposed concerning the four related mouse 3β-HSD genes, which are closely linked on a segment of chromosome 3 that is conserved on human chromosome 1 [47].

Molecular genetics of 3β-HSD deficiency

Congenital adrenal hyperplasia (CAH) is the most frequent cause of ambiguous genitalia and adrenal insufficiency in newborns [48]. It comprises a group of syndromes caused by specific enzymatic deficiencies in the adrenal cortex that affect the pathway of cortisol biosynthesis, leading to a compensatory hypersecretion of adrenocorticotropin by the anterior pituitary gland with consequent hyperplasia of the adrenal cortex [48, 49]. In contrast to the two most frequent causes of CAH, 21-hydroxylase and 11β-hydroxylase deficiencies, which are adrenal defects, the severe form of 3β-HSD deficiency impairs steroidogenesis in both the adrenals and the gonads, resulting in decreased secretion by these tissues of not only cortisol and aldosterone, but also of progesterone, androgens, and estrogens [48, 50, 51].

In newborns of both sexes, CAH due to classic 3β-HSD deficiency is characterized by varying degrees of salt wasting [51 and refs therein]. The expected severe inhibition of testosterone biosynthesis by the fetal testis resulting in a marked decrease in 3β-HSD activity, provides an explanation for the incomplete masculinization of the external genitalia seen in the male patients studied. On the other hand, as frequently seen, complete or partial inhibition of 3β-HSD activity in the adrenals and ovaries was not accompanied by a noticeable alteration of the differentiation of the external genitalia of female patients, as indicated by the absence of ambiguity of external genitalia in the female patients.

Since the first report by Bongiovanni [50], many patients of both sexes have been described and the heterogeneity of the clinical presentation evidenced. The salt-losing form of classic 3β-HSD deficiency is usually diagnosed during the first few months of life because of insufficient biosynthesis of aldosterone and consequent salt loss [50–57]. In contrast, the nonsalt-losing form of the 3β-HSD deficiency may be diagnosed either at a young age in the presence of indicating factors, such as a family history of death during early infancy [58], perineal hypospadias in male newborns [59, 60], or failure to gain weight [54]; or the diagnosis may be made at a later age [55, 61–63]. Because sexual differentiation is normal in female newborns affected by nonsalt-losing 3β-HSD deficiency, the proper diagnosis is delayed until adrenarche [62] or puberty [61].

An elevated ratio of 5-ene- to 4-ene-steroids is considered the best biological parameter for the diagnosis of 3β-HSD deficiency [64, 65]. It is well recognized, however, that levels of 17-OH PROG and 4-ene-DIONE plasma and other 4-ene steroids are frequently elevated in 3β-HSD-deficient patients [50, 53, 57, 61, 63–65]. Such observations suggest functional 3β-HSD that is expressed in peripheral tissues and is responsible for the extraadrenal and extragonadal conversion of 5-ene-hydroxysteroid precursors into the corresponding 4-ene-3-keto-steroids.

We have recently reviewed in greater detail the molecular basis of classical 3β-HSD [51]; we will thus limit our present review to the data providing a molecular explanation for the enzymatic heterogeneity ranging from the severe salt-losing form to the clinically inapparent salt-wasting form of the disease. The findings providing pertinent information concerning the structure–function relationships of the 3β-HSD enzymes will be also briefly described.

Molecular basis of the salt losing form of classic 3β-HSD deficiency. To identify the molecular lesions in the type I and/or type II 3β-HSD genes responsible for the salt-losing form of classic 3β-HSD deficiency using selective PCR amplification, we determined the nucleotide sequence of the whole coding region, exon–intron splicing boundaries as well as 5′ flanking region, including the putative promoter and the 3′ noncoding region of each of the two genes. The complete sequence of the PCR products obtained from the type I 3β-HSD gene from six patients originating from five unrelated families was identical to that of the corresponding known regions in the normal gene [66–68], thus providing no evidence to suggest a genetic alteration affecting type I mRNA processing or a structural change in the type I 3β-HSD isoenzyme in these patients. In agreement with this finding, no mutation was detected in the type I 3β-HSD gene in three unrelated patients with the nonsalt-losing form of classic 3β-HSD deficiency [67, 69, 70].

The 8 different point mutations characterized in the 10 patients studied were all detected in the type II 3β-HSD gene, which is predominantly expressed in the adrenals and gonads (Table 1) [56, 66–68, 71]. The increased levels of some 4-ene-steroids and their metabolites in patients with classic 3β-HSD deficiency are thus best explained by normal peripheral type I 3β-HSD activity exerting its catalytic activity on the elevated levels of substrates resulting from a deficiency of type II 3β-HSD in the adrenals and gonads.

It is expected that no functional type II 3β-HSD isoenzyme is expressed in the adrenals and gonads of the patients bearing the homozygous nonsense W171X mutation (families 1 and 2), the compound

Table 1. Mutations in the type II 3β-HSD gene in patients with the salt-losing form of classic 3β-HSD deficiency

Family	Origin	Karyo-type	Case Report	Molecular Lesion: Mutation(s)	Molecular Lesion: Type	Mutation report
1	Swiss	46XX	[57, 79]	Trp171Stop	Nonsense	[66]
2	Swiss	46XX	[80]	Trp171Stop	Nonsense	[66]
3	American	46XY	[53]	Trp171Stop: 186/insC/187	Nonsense Frameshift	[66]
4	American	46XY	[64]	Glu142Lys: Trp171Stop	Missense Nonsense	[67]
5	Dutch	46XY	[81, 82]	186/insC/187: Tyr253Asn	Frameshift Missense	[67]
6	Afghan/ Pakistani	46XY	[56]	273ΔAA	Frameshift	[56]
7	Afghan/ Pakistani	46XY	[56]	273ΔAA	Frameshift	[56]
8	Afghan/ Pakistani	46XY	[56]	273ΔAA	Frameshift	[56]
9	Algerian	46XY	[60]	Gly15Asp	Missense	[71]
10	Spanish Portuguese	46XY	[55, 83]	Leu108Trp: Pro186Leu	Missense Missense	[68]

heterozygote W171X:186/insC/187 (family 3), and of the patients bearing the homozygous 273Δ AA mutation (families 6, 7, and 8), which is in agreement with the severity of the disorder in these index cases [51, 56, 66]. Both the recombinant mutant E142K protein or the mutant Y253N enzyme exhibited no detectable activity as measured in intact transfected COS-1 cells in culture and in homogenate preparations from transfected cells [67]. Thus, in the compound heterozygote patient E142K:W171X (family 4) as well as in the compound heterozygote 186/insc/187:Y253N (family 5), it is also expected that no functional type II 3β-HSD isoenzyme is expressed in the gonads and adrenals of these index cases with severe form of classic 3β-HSD deficiency [67].

We have also recently reported the detection of an homozygous G to A mutation converting codon Gly^{15} into Asp^{15} in the type II 3β-HSD gene in a male pseudohermaphrodite born from consanguineous parents and suffering from severe salt-losing 3β-HSD deficiency [71]. In addition to studying the effect of the substitution of Gly^{15} for Asp^{15}, we evaluated the effect of substituting Gly^{15} for Ala^{15} to further investigate the potential involvement of residue 15 in the βαβ dinucleotide-binding fold. This Gly at position 15 is located within a highly conserved Gly fingerprint found in all members of the 3β-HSD superfamily. This Gly-X-X-Gly-X-X-Gly fingerprint (where X is any amino acid) is similar to the common Gly-X-Gly-X-X-Gly conserved sequence of most NAD(H) binding enzymes [72]. In intact transfected cells, after a 2 h incubation, the percentage of conversion of $[^3H]$PREG into $[^3H]$PROG was 35 and 50% for the G15A and native type II 3β-HSD enzymes, respectively, whereas no detectable activity was observed in cells expressing the G15D protein. These findings are in close agreement with the severity of the disorder in the homozygote G15D [60, 71]. On the other hand, in homogenates from transfected cells, the mutant protein G15D shows an activity similar to that of G15A (Table 2). As measured by the first-order rate constant $V^{max}_{PREG}/K^{m}_{PREG}$, the mutant G15D and G15A enzymes possess about 27.5 and 29.0%, respectively, of the activity catalyzed by the native type II 3β-HSD using PREG as substrate. Moreover, the expressed G15D and G15A proteins had decreased affinities for NAD^+ (Table 2). Thus, the specificity for NAD^+ ($V^{max}_{NAD^+} + /K^{m}_{NAD^+}$) indicates that the mutant G15D and G15A enzymes possess about 23.6 and 17.1%, respectively, of the activity catalyzed by the normal type II 3β-HSD using NAD^+ as cofactor in the presence of an excess (30 μM) of PREG as substrate. The estimated overall efficiency $((V^{max}_{PREG} + V^{max}_{NAD^+}/2)/(K^{m}_{PREG} \times K^{m}_{NAD^+}))$ of the mutant G15D and G15A enzymes would be 5.2 and 3.7%, respectively, relative to the overall efficiency of the normal type II 3β-HSD enzyme. The marked difference in activity of the G15D and G15A proteins expressed in intact cells compared with their similar levels of activity in homogenates suggests that the G15D mutation could alter the proper intracellular localization of this integral membrane protein or that its association with intact membranes *in vivo* may exert some strain that prevents adoption of its final maximally efficient conformation. The analysis of these two mutant proteins illustrates well the inherent limitations of such experimental approaches which should be used in conjunction to better evaluate the impact of a mutation on *in vivo* enzymatic activity.

We have also assessed the effect of the L108W and P186L mutations, found in the compound heterozygote from family 10 [68], on 3β-HSD activity by *in vitro* analysis of mutant enzymes expressed in COS-1 cells (Table 2). Unexpectedly, we observed the presence of a low but detectable activity for both mutant L108W and P186L 3β-HSD proteins in intact transfected cells in culture [68]. As mentioned above, all the other patients characterized with a severe form of 3β-HSD deficiency associated with salt-wasting bear point mutations that completely abolish the activity of the enzyme when evaluated in intact transfected cells in culture [56, 66, 67, 71]. Using homogenates from transfected cells, it was clear that the mutant L108W and P186L proteins possess a lower affinity for the steroid substrate and the cofactor (Table 2). The

Table 2. Kinetics parameters of native type II 3β-HSD and mutant G15D, G15A, N100S, L108W, and P186L proteins [68, 70, 71]

Protein	K_m PREG	Relative V_{max} PREG	Relative V_{max} PREG / K_m PREG	K_m NAD	Relative V_{max} NAD	Relative V_{max} NAD / K_m NAD
Type II 3β-HSD	0.72	100	139	22	100	4.54
G15D	3.2	120	37.5	113	121	1.07
G15A	3.4	134	39.4	148	116	0.78
Type II 3β-HSD	1.7	100	58.8	24	100	4.2
L108W	12.0	16.1	1.3	678	17.4	0.025
P186L	18.0	20.7	1.2	920	17.4	0.019
Type II 3β-HSD	3.5	100	28.6	20	100	5
N100S	25	19.3	0.8	650	30	0.05

Relative V_{max} values were calculated assuming the V_{max} for the human type II enzyme equal to 100.

in vitro overall efficiency, relative to the normal enzyme, was approximated as ~0.1 to 0.3%. The presence of such a low level of activity is thus apparently inadequate to permit the formation of mineralocorticoids in quantities sufficient to avoid severe salt wasting.

Molecular basis of the nonsalt losing form of classic 3β-HSD deficiency. The 7 different point mutations characterized in the 6 families studied were all detected in the type II 3β-HSD gene (Table 3) as previously observed for the salt-losing form of classic 3β-HSD deficiency [67, 69, 70, 73–75].

The proband of family 11 was the first index case suffering from the nonsalt-losing form of classic 3β-HSD deficiency studied [67]. He is homozygous for the missense point mutation A245P. The observation that there was no detectable 3β-HSD activity in cell homogenates in the absence of glycerol, whereas significant enzymatic activity was measured in intact transfected cells and cell homogenates prepared in the presence of glycerol, a known stabilizing agent, might suggest that this mutation weakens the association of the mutant A245P enzyme with proper intracellular membranes [67]. The expressed mutant A245P protein (in the presence of glycerol) had an affinity for PREG comparable to that of the wild-type type II 3β-HSD enzyme, with K_m values of 4.64 and 2.63 μM, respectively, while the V_{max} values, on the other hand, were 13.02 and 62.06 pmol/min/μg of total protein, respectively. As measured by the ratio V_{max}/K_m, the mutant A245P enzyme possessed about 11.9% of the activity catalyzed by the native type II 3β-HSD [67]. In fact, the absence of salt wasting in this patient could well be explained by the weak but measurable 3β-HSD activity, which permits formation of mineralocorticoids sufficient to avoid severe salt wasting, in analogy with some nonsalt-losing CAH cases secondary to 21-hydroxylase deficiency [49, 76].

The molecular lesions in the type II 3β-HSD gene were also characterized in two siblings from family 12 with the well-described nonsalt-losing form of classic 3β-HSD deficiency (Table 3) [62, 69, 77]. Using homogenates from transfected COS-1 cells, the G129R enzyme showed a K_m value for PREG of 10μM compared with 1.0 μM for the native type II 3β-HSD enzyme. When DHEA was used as substrate, the K_m value for G129R 3β-HSD was 14 μM, compared with 2.1 μM for the native type II 3β-HSD enzyme. In addition, the G129R mutation caused a marked decrease in the apparent relative V_{max}, leading to apparent relative V_{max}/K_m values of 2.0 and 4.7% of the native type II 3β-HSD using PREG or DHEA as substrate, respectively. It appears likely that this low level of activity is sufficient to prevent salt loss, but it is also possible that part of the enzymatic activity comes from the putative remaining percentage of correctly spliced 6651→A allele in these patients [69].

The remarkable clinical finding of the male pseudohermaphrodite in family 13 [55, 70] was the poor male differentiation contrasting with a lack of salt loss. The child had never presented clinical salt loss and had normal growth. However, plasma renin activity, which for the child's age was elevated in basal conditions (28.8μg/l/h; normal values: 22.0 ± 11.2), was further increased during the test (57.48 μg/l/h). Basal aldosterone level (524 pmol/l) was normal in basal conditions but did not rise at the end of the test (441 pmol/l). These findings suggested that the child had a compensated salt loss resulting from a borderline normal but limited capacity of aldosterone biosynthesis at the price of a high renin synthesis. However, the child had a manifest craving for salt, which explains her high salt intake. She had adrenal crises (at the occasion of a pyretic episode) only twice in 12 years, requiring acute treatment with parenteral hydrocortisone and DOCA. The patient carried a homozygous N100S

Table 3. Mutations in the type II 3β-HSD gene in patients with the nonsalt-losing form of classic 3β-HSD deficiency

Family	Origin	Karyotype	Case report	Molecular Lesion: Mutation(s)	Molecular Lesion: Type	Mutation report
11	Turkish	46XX	[58]	Ala245Pro	Missense	[67]
12	American	46XX	[66, 77]	Glyl29Arg: 6651→A	Missense Splicing	[69]
12	American	46XY	[62, 77]]	Glyl29Arg: 6651→A	Missense Splicing	[69]
13	Algerian	46XY	[55, 70]	Asn100Ser	Missense	[70]
14	American	46XY	[61, 78]	Tyr254Asp:?	Missense ?	[73]
15	Scottish	46XY	[74]	Leu173Arg	Missense	[74]
15	Scottish	46XY	[74]	Leu173Arg	Missense	[74]
16	Brazilian	46XY	[63]	Ala82Thr	Missense	[75]
16	Brazilian	46XY	[63]	Ala82Thr	Missense	[75]
16	Brazilian	46XX	[63]	Ala82Thr	Missense	[75]
17	Brazilian	46XX	[75]	Ala82Thr	Missense	[75]

missense mutation [70] (Table 3). Using homogenates from transfected COS-1 cells, the N100S 3β-HSD enzyme showed a K_m value for PREG of 25 μM compared with 3.5μM for the native type II 3β-HSD enzyme (Table 2). In addition to decreasing apparent affinity, the N100S mutation also decreased the V_{max}, leading to a relative V_{max}/K_m value of 2.7% that of normal type II 3β-HSD using PREG as substrate. The mutant N100S protein also had a decreased apparent affinity for NAD^+. Thus, the relative specificity for NAD^+ indicates that the mutant N100S enzyme possesses 1% of the activity catalyzed by the normal type II 3β-HSD. The overall efficiency of N100S protein is closely similar (~0.1%) to that of L108W and P186L proteins. These findings suggest that this very weak residual activity of the mutated N100S enzyme is sufficient to prevent salt loss at the price of high renin synthesis, although it was insufficient for normal male sex differentiation in the patient.

Study was also undertaken to characterize the genetic alteration(s) responsible for the nonsalt-losing 3β-HSD deficiency diagnosed at puberty in the female (46 XX) patient [61, 78] from family 14. We detected the missense mutation, Y254D, in one allele of the patient's type II 3β-HSD gene [73]. Recombinant mutant type II 3β-HSD enzyme carrying the Y254D substitution exhibited no detectable activity with PREG or DHEA used as substrate. The absence of restriction fragment length polymorphism by Southern blot analysis and the finding that all the amplified DNA fragments possess the expected length suggest the absence of deletions, duplications, or rearrangements in the other allele in this patient. A putative second mutation could be located farther than 1427 bp upstream of the initiation codon, thus potentially affecting the normal expression of this gene or within intronic regions and generating an alternative aberrant splicing site. These possibilities remain to be elucidated.

Thus, in general, in patients with a severe form and a concurrent defect in aldosterone biosynthesis leading to salt wasting, the disorder results from point mutations abolishing the activity of the 3β-HSD enzyme encoded by the type II 3β-HSD gene [56, 66, 67, 71], the gene type primarily expressed in the adrenal, ovary, and testis [22]. In addition, the present study demonstrates that the nonsalt-losing form of classic 3β-HSD deficiency also results from missense mutation(s) in the type II 3β-HSD gene, which causes an incomplete loss of enzymatic activity, thus leaving sufficient enzymatic activity to prevent salt wasting [67, 69, 70]. On the other hand, it is noteworthy that the kinetic properties of N100S 3β-HSD protein, found in a nonsalt-losing patient [70] are quite similar to those of L108W and P186L mutant proteins, which were detected in a severe salt-losing form of classic 3β-HSD deficiency [68]. The functional characterization of these mutant proteins cannot provide an explanation for the heterogeneity responsible for the severe salt-losing form (L108W:P186L) down to the clinically inapparent form of salt loss (N100S) of classic 3β-HSD deficiency. Nevertheless, the hormonal profile of N100S mutation suggests that he compensated salt loss due to a limited capacity of aldosterone biosynthesis at the price of a high renin synthesis.

It is of special interest to note that the amino acids Gly^{15}, $Alà^{82}$, Asn^{100}, Leu^{108}, Gly^{129}, Glu^{142}, Leu^{173}, Tyr^{253} and Tyr^{254}, which are the sites of the missense mutations described above, are conserved in all members of the vertebrate 3β-HSD isoenzymes characterized thus far in the human [7, 12, 22], macaque [15], bovine [13], rat [8, 14, 16], mouse [17, 18], guinea pig [19], and rainbow trout [20]. This finding strongly suggests the crucial role of these residues for the catalytic activity of these enzymes. However, although amino acid Pro^{186} is also well conserved in the vertebrate 3β-HSD family, it is not conserved in all members; rat type III [10, 14] and mouse type IV [18], which are specific 3-ketoreductases responsible for the conversion of 3-keto-saturated steroids using NADPH as cofactor, do not share this amino acid at this position. Moreover, because Ala^{245} is not always conserved in the 3β-HSD superfamily, it is conceivable that the A245P mutation generating Pro^{245} causes a turn in the polypeptide chain that generates a structural change in the protein that is responsible for the decreased activity of this mutant type II 3β-HSD enzyme.

Nonclassic 3β-HSD deficiency. Over the last decade, cases of 3β-HSD deficiency occurring in older females have been described, termed nonclassic (also attenuated or late onset) 3β-HSD deficiency [84–86]. These cases, which have clinical similarities to the range of defects seen with the adrenal steroid 21-hydroxylase enzyme, and on analogy with the relationship between the classic and nonclassic forms of 21-hydroxylase, were termed nonclassic 3β-HSD deficiency. This hormonal defect was also suggested possibly to be an allelic variant of the classic forms of 3β-HSD deficiency. Allelism between the phenotypically less severe and variable nonclassic form and a classic form of 21-hydroxylase deficiency was postulated on a clinical basis [87], and has since been amply confirmed in many genetic and molecular genetic studies [88–91]. Because the frequency of occurrence of the nonclassic 21-hydroxylase disorder is very high relative to classic CAH [87], it was considered likely that milder forms of 3β-HSD deficiency, assuming a comparable genetic basis, would also occur with much greater frequencies than the classic form [92]. A recent review of the literature [93] suggests that NC3β-HSD deficiency might be even more frequent than nonclassic 21-hydroxylase deficiency.

The complete coding regions, the exon–intron junctions, as well as the 3′ noncoding region, including the polyadenylation site of both types I and II 3β-HSD

genes, were sequenced in both orientations in sex patients (#3, 7, 11, 12, 20, 24) reported as having NC3β-HSD deficiency [94]. Moreover, the 5′ region was also sequenced in both orientations up to 527 and 525 bp upstream of exon I of the type I and type II 3β-HSD genes, respectively. No mutation was detected for any of the six patients in the regions covered by the direct sequencing PCR analysis of both the type I and II 3β-HSD genes. In parallel, in Dr Morel's laboratory, no mutation was detected in another cohort of 20 patients having NC3β-HSD deficiency [95].

The fact that no mutation was found in patients suffering from NC3β-HSD deficiency strongly suggests that this disorder does not result from a mutant type II 3β-HSD isoenzyme. This finding is in clear contrast to salt-losing and nonsalt-losing forms of classic 3β-HSD deficiency described above in which point mutations have been detected. We cannot refute the possibility that inherited mutation(s) could be located farther upstream in the putative promoter region of the type II 3β-HSD gene, leading to an aberrant level of expression of a normal type II 3β-HSD protein. However, the latter hypothesis is weakened by the fact that all patients come from unrelated pedigrees and diverse ethnic origins [94, 95]. On the other hand, because 3β-HSD gene expression and activity are under a complex multiple hormonal regulation [see for reviews 3, 4, 5, 96], it cannot be ruled out that at least some forms of NC3β-HSD deficiency result from a genetic or acquired origin acting indirectly on these modulatory parameters. There is also the possibility of implication of a steroidogenic enzyme different from known 3β-HSD isoenzymes. Does it possibly involve dysregulation of 17α-hydroxylase and 17,20-lyase activities, as recently suggested [97]? If so, is this dysregulation of genetic origin? All these hypotheses remain to be studied further to gain more understanding of this puzzling but frequent disease.

STRUCTURE AND FUNCTION OF MEMBERS OF THE RAT 3β-HSD GENE FAMILY

Structure of rat types I, II, III, and IV 3β-HSD

In addition to the characterization of the structure of the type of 3β-HSD expressed in the adrenals and gonads of the macaque [15], the bovine [13, 98], the guinea pig [19], and the rainbow trout [20], the sequence of four types of rat [8, 14, 16, 21, 99] and four types of mouse [17, 18, 100] 3β-HSD cDNAs have recently become available (Fig. 1). The predicted rat type I and type II 3β-HSD proteins expressed in the adrenals, gonads, kidneys, placenta and adipose tissue share 93.8% homology. The deduced amino acid sequence of rat type IV 3β-HSD shares 90.9, 87.9 and 78.8% identity with that of rat types I, II and III 3β-HSD proteins, respectively, thus having 34, 45 and 79 non-identical residues with types I, II and III proteins. The rat liver-specific type III protein is a specific 3-keto-reductase (3-KSR) and its enzymatic characteristics activity will be further described below. The type IV 3β-HSD mRNA population was detectable in the placenta, ovary and, at a lower degree, in the adrenal gland, while under the conditions used, no signal was detectable in rat testis. The rat type IV mRNA population corresponds to the sole detectable 3β-HSD species in the skin. We have demonstrated that the affinity and the specific activity of rat type IV are similar to those of the rat type I 3β-HSD isoenzyme [16].

Enzymatic characteristics of expressed rat types I and II 3β-HSD

In vitro incubation with homogenates from cells transfected with pCMV-type I-3β-HSD or pCMV-type II-3β-HSD in the presence of 1 mM NAD$^+$ and ^{3}H-labeled PREG or DHEA showed that type I had a respective 3β-HSD/5-ene-4-ene isomerase relative specificity, as determined by a relative V_{max}/K_m ratio, 64 and 47-times higher than type II (Table 4) [23]. As predicted by computer analysis, there is a potential membrane-spanning domain (MSD) common to the deduced rat type I and type II as well as other mammalian 3β-HSD protein sequences, between residues 287 to 303 [3]. However, analysis of rat types I and type II 3β-HSDs indicates that the change of residues 83, 85, 87 and 89 in the type II 3β-HSD protein prevents the formation of another potential MSD present in the rat type I, type III and type IV and several other mammalian species enzymes between residues 75 and 91. The kinetic properties of wild type

Table 4. Kinetic properties of wild type rat type I and type II 3β-HSD as well as of chimeric I-MSD and II + MSD proteins [23]

	K_m (μM)		Relative V_{max}		Relative specificity (Relative V_{max}/K_m)	
Expressed protein	PREG	DHEA	PREG	DHEA	PREG	DHEA
Rat type I 3β-HSD	0.74	0.68	100	100	135	147
Chimeric I-MSD	11.7	11.0	5.5	7.60	0.47	0.69
Rat type II 3β-HSD	14.3	12.9	30.8	40.8	2.1	3.16
Chimeric II + MSD	0.36	0.40	28.0	42.7	77.8	106.7

Relative V_{max} values were calculated assuming the V_{max} for the rat type I enzyme equal to 100. The specific activity values for type I isoenzyme using PREG or DHEA as substrates were 5.1, and 4.1 nmol/min/mg protein, respectively.

rat type I and II 3β-HSD proteins were compared with those of a chimeric type I protein lacking this MSD (I-MSD) and of a chimeric type II protein having gained this putative MSD (II + MSD) [23]. The data presented in Table 4 provide strong evidence supporting the crucial role of the predicted MSD between residues 75 to 91 for the enzymatic specificity of rat type I 3β-HSD. It is thus likely that the absence of this putative MSD in type II 3β-HSD explains its much lower activity.

17β-HSD activity of rat type I and type IV 3β-HSD

Somewhat unexpectedly, we observed that homogenate obtained from cells transfected with the pCMV-type I 3β-HSD in the presence of NAD^+, converts DHT into androstanedione (A-DIONE), thus demonstrating that the expressed enzyme possesses 17β-HSD activity [9]. After a 12 h incubation in the presence of 1 mM NAD^+, homogenate from cells transfected with pCMV-type I 3β-HSD converts about 65% of DHT into A-DIONE.

In contrast to the type I 3β-HSD protein, type II 3β-HSD, as well as chimeric I-MSD and II + MSD proteins, do not possess significant 17β-HSD activity [9]. Finding that the chimeric II + MSD 3β-HSD protein is devoid of 17β-HSD activity strongly suggests that the lack of such 17β-HSD enzymatic activity for the type II 3β-HSD isoenzymes is not due to the absence of a MSD between residues 75 to 91, but rather to another structural difference resulting from one or several of the 19 other amino acid changes observed between the type I and type II enzymes [9].

As measured in HeLa cell homogenate, the K_m value for the transformation of DHT into 3β-diol (4.02 ± 0.67 μM) by the expressed type I 3β-HSD is in the same range as that obtained with the human type II 3β-HSD (Table 2) [22]. When NAD^+ is used as cofactor, the androgenic 17β-HSD activity of rat type I 3β-HSD shows a K_m of 7.97 ± 2.18 μM. The V_{max} of 3β-HSD activity of expressed type I 3β-HSD is much higher than that of 17β-HSD activity (24.9 ± 1.18 vs 1.67 ± 0.13 nmol/min/mg) [9].

However, when the same enzymatic assays are performed in unbroken JEG-3 human choriocarcinoma cells transfected with the rat type I 3β-HSD, 17β-HSD activity is clearly predominant over 3β-HSD activity. Cells transfected with rat type I 3β-HSD metabolize DHT exclusively into A-DIONE while no 3β-diol formation can be detected over basal values obtained in cells transfected with the pCMV plasmid [9].

In order to investigate if the rat type IV 3β-HSD isoenzyme also possesses such a 17β-HSD activity, we next studied the time course of formation of steroids in human SW-13 adrenal cortex adenocarcinoma cells in culture transfected with pCMV-type I 3β-HSD [Fig. 3(A)] or pCMV-type IV 3β-HSD [Fig. 3(B)] plasmid using [^{3}H]DHT as substrate. SW-13 cells have low endogenous 3β-HSD activity. In SW-13 cells transfected with pCMV-type I 3β-HSD plasmid, the 17β-HSD activity was also predominant [11]. It can be seen in Fig. 3(B) that in SW-13 cells transfected with pCMV-type IV 3β-HSD, the 17β-HSD activity was more important than the 3β-HSD activity. Using SW-13 cell homogenate preparations in the presence of 1 mM NAD^+ and [^{3}H]DHT as substrate. The K_m values for the 17β-HSD activity of rat types I and IV were 1.0 ± 0.1 and 0.8 ± 0.1 μM, respectively, with closely similar relative V_{max} values [11].

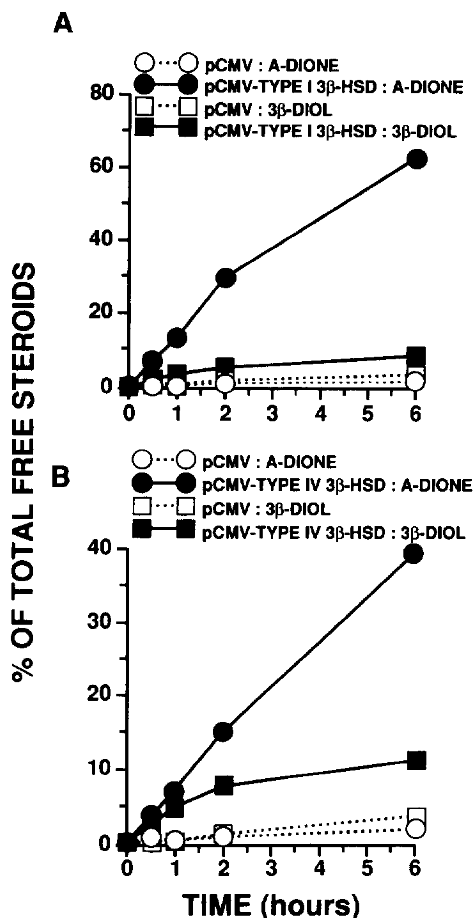

Fig. 3. Time course of the enzymatic conversion of DHT in intact SW-13 cells in culture transfected with control pCMV (A, B), pCMV-type I 3β-HSD (A) or pCMV type IV 3β-HSD (B) plasmid. Formation of A-dione is illustrated by circle symbols, whereas the formation of 3β-diol is illustrated by square symbols. Cells were exposed to 100 nM [^{3}H]DHT for the indicated time periods. Steroids were then extracted and determined by HPLC analysis as described [11]. The results are presented in % as the means of the free steroid metabolites present in culture medium as % of total free steroid. Because SEM overlaps with the symbol used, only the symbol is illustrated.

The predominance of such a high level of 17β-HSD activity of rat types I and IV 3β-HSDs offers an explanation for the formation of the inactive androgen A-DIONE as the major final product when DHT or

its 3-keto metabolite, 3β-diol, are used as radiolabeled substrate in mammalian cells lacking significant endogenous 3α-HSD activity such as JEG-3 cells and SW-13 cells. Moreover, the data obtained in HeLa cells [11], which have a high endogenous level of 3α-HSD activity responsible for the rapid conversion of DHT into 3α-diol, are also in close agreement with this conclusion as indicated by the high level of formation of the inactive androgen ADT as the major final product when either DHT or 3β-diol are used as labeled substrates [11]. In view of the data obtained, the deduced predominant pathway found in human JEG-3 cells expressing rat type I 3β-HSD is 3β-diol→DHT→A-DIONE. The predominance of this "secondary" 17β-HSD activity is also reflected in HeLa cells transfected with type I 3β-HSD by the deduced predominant pathway 3β-diol→DHT→3α-diol→ADT, in which formation of 3α-diol was due to endogenous 3α-HSD activity of HeLa cells, whereas the other reactions are catalyzed by the rat type I or type IV 3β-HSD isoenzyme (Fig. 4).

The apparent discrepancy between the oxidoreductase activity of the rat type I 3β-HSD, as revealed by interconversion of DHT and 3β-diol as well as A-DIONE and epi-ADT using homogenate preparations from mammalian cells expressing this recombinant isoenzyme [8, 10] and the lack of significant 3-keto reductase activity measured in intact cells in culture expressing the same enzyme, can be explained by low levels of the intracellular pool of NADH relative to the high bioavailability of NAD^+ in mammalian cells. In agreement with this explanation, in HeLa, JEG-3 and SW-13 cells in culture expressing rat types I and IV 3β-HSDs, 5-ene-hydroxysteroid precursors are efficiently converted into their corresponding 4-ene-3-keto steroids. In fact, 3β-HSD/5-ene-4-ene isomerase activity is well recognized to strictly require NAD^+ as an allosteric cofactor [101, 102]. The highly efficient conversion of DHT into 3β-diol in HeLa cells in culture expressing rat 3-KSR (type III) which is catalyzed by the latter enzyme also argues for the bioavailability of NADPH within these cells [11]. In

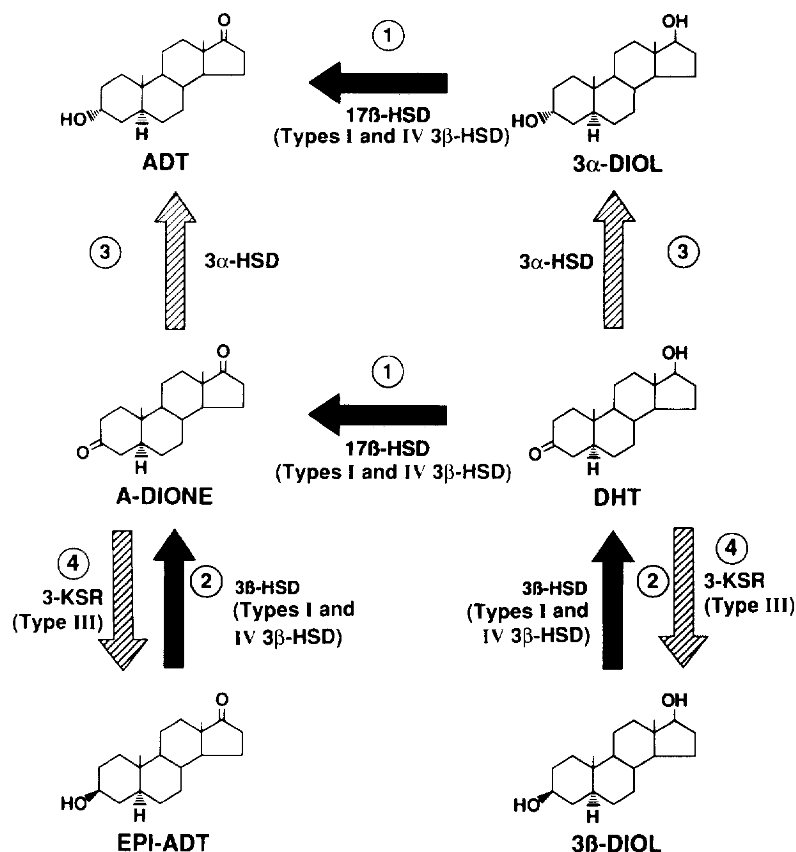

Fig. 4. Enzymatic 17β-HSD and 3β-HSD activities of rat types I and IV 3β-HSD expressed in intact cells [11]. Reaction 1 corresponds to the androgenic 17β-HSD activity measured in cells expressing the rat types I or IV 3β-HSD isoenzymes. Reaction 2 corresponds to the 3β-HSD activity present in cells expressing rat types I or IV 3β-HSD enzymes. Reaction 3 corresponds to 3α-HSD activity in some cells. Reaction 4 corresponds to the 3-keto-steroid reductase activity present in liver cells expressing the rat 3-KSR (type III) enzyme. The black arrows indicate predominant reactions expected to use primarily NAD^+ as cofactor, while the hatched arrows indicate the predominant reactions expected to use primarily NADPH as cofactor.

fact, the reaction catalyzed by the liver-specific member of the 3β-HSD family has recently been demonstrated to use NADPH as preferred cofactor [10]. It is also of interest to mention that 3α-HSD is known to use NADPH as cofactor, this also being in close agreement with the postulated predominant metabolic pathways in transfected cells in culture using NAD^+ or NADPH as cofactor (Fig. 4).

The observation that the affinity of the rat types I and IV 3β-HSD for conversion of DHT into A-DIONE is similar to that for their 3β-HSD/5-ene-4-ene isomerase activity in homogenates from transfected cells [9, 11, 16] also argues for the intrinsic androgenic 17β-HSD activity as playing an important role in the degradation of the potent androgen DHT. The low levels of bioavailable NADH in intact cells could then force the conversion of DHT into A-DIONE in spite of a 15-fold lower V_{max} value for this reaction compared to that observed for the conversion of DHT into 3β-diol in homogenates from HeLa cells expressing rat type I 3β-HSD [9]. However, such a high V_{max} value for the primary 3β-HSD activity can explain well the present findings of a rapid conversion of 3β-diol into DHT in the absence of detectable formation of epi-ADT in both HeLa and JEG-3 cells expressing the recombinant rat type I 3β-HSD.

The predominance of the "secondary" 17β-HSD activity of rat types I and IV 3β-HSD isoenzymes expressed in intact mammalian cells in culture thus strongly suggests the physiological importance of this activity in modulating the intracellular concentration of the active androgen DHT in androgen target tissues. It is of interest to mention that such "secondary" 17β-HSD activity was also observed with guinea pig type I 3β-HSD [19]. Moreover, it has recently been demonstrated that bovine purified 3β-HSD enzyme as well as adrenocortical mitochondria and microsomal preparations exhibit 17β-HSD activity [1]. Furthermore, these authors demonstrated that trilostane inhibited 17β-HSD activity and 3β-HSD activity to the same extent, thus strongly suggesting that the same binding site is involved for both activities [1].

It should be mentioned that the only other enzyme known to catalyze the oxidation of both 3β, and 17β-hydroxy groups of some hydroxysteroids is the NAD-dependent 3β,17β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* [103]. The recent elucidation of its cDNA sequence reveals that this enzyme is not a member of the 3β-HSD superfamily, but is rather homologous to the 17β-HSD superfamily [104]. In addition, dual activity at the active site of steroid-specific oxidoreductases has been also reported using preparations of purified enzymes, namely, 17β,20α-HSD from human placenta [105] and porcine testis [106], 3β,20α-HSD from bovine and sheep erythrocytes [107], 3α,20α-HSD from porcine adrenal [108], 3α,20β-HSD from *Streptomyces hydrogenans* [109] and estrophilic 3α,3β,17β,20α-HSD from rabbit liver [110].

It has been postulated that such "secondary" activity could be explained by the binding of the steroid in the inverted substrate orientation to the same active binding site as that responsible for the "primary" activity of the enzyme, this phenomenon also being named "wrong way binding" [111]. Nevertheless, the interpretation that a steroid can be anchored to an active site in alternative orientations is limited by the possibility of impure enzyme preparations. In the case of the 3β-HSD isoenzymes that possesses dual 3β,17β-HSD activity, it should be mentioned that the 3α-hydroxy group of 3α-diol as well as the 3-keto group of DHT permit (i) binding of the steroid in the "wrong way" and (ii) oxidation of the 17β-ol group, thus showing that this secondary 17β-HSD activity is not restricted to DHT. Such conclusion is in agreement with the finding that the kinetic properties of androgenic 17β-HSD activity of rat type I 3β-HSD leads to the conversion of 3α-diol into ADT as efficiently as measured for the conversion of DHT into A-DIONE [9, 11]. The availabilitity of such recombinant mammalian hydroxysteroid dehydrogenases showing dual 3β,17β-HSD activity offers the opportunity to better characterize the structure–function relationships of such enzymatic activity.

Enzymatic characteristics of the rat liver-specific 3-keto-steroid reductase (type III)

As mentioned above, the rat 3-KSR (type III) protein does not display oxidative activity for the classical substrates of 3β-HSD namely, PREG, 17OH-PREG, DHEA and 5-androstene 3β,17β-diol [10]. However, in the presence of NADH or NADPH, the type III enzyme, in common with other 3β-HSD isoenzymes, converts A-DIONE and DHT into the corresponding 3β-hydroxysteroids (Table 5). When NADPH is used as cofactor, the affinity of the type III protein for DHT becomes higher than that of the type I isoenzyme with K_m values of 0.12 and 1.18 μM, respectively. The type III is thus a 3-keto reductase using NADPH as preferred cofactor and is responsible for the conversion of 3-keto saturated steroids into less active steroids.

In addition to using the 5α-androstane steroids DHT and A-DIONE as substrates, the expressed 3-KSR also catalyzes the 3β-reduction of 5α-dihydroprogesterone (DHP) into 5α-pregnane-3β,20β-diol in HeLa and JEG-3 cells in culture [11]. The K_m and the V_{max} values of the expressed 3-KSR (type III) protein using DHP as substrate and NADPH as cofactor were calculated at 0.24μM and 0.83 nmol/min/mg protein. By comparison, the K_m value of the expressed type I 3β-HSD isoenzyme also using DHP as substrate and NADH as cofactor was measured as 0.55 μM, while the calculated V_{max}value was 0.18 nmol/min/mg protein [11].

Examination of the 3β-HSD isoenzymes shows a typical βαβ dinucleotide-binding fold with Asp^{36} located in the position predicted for the acidic residue

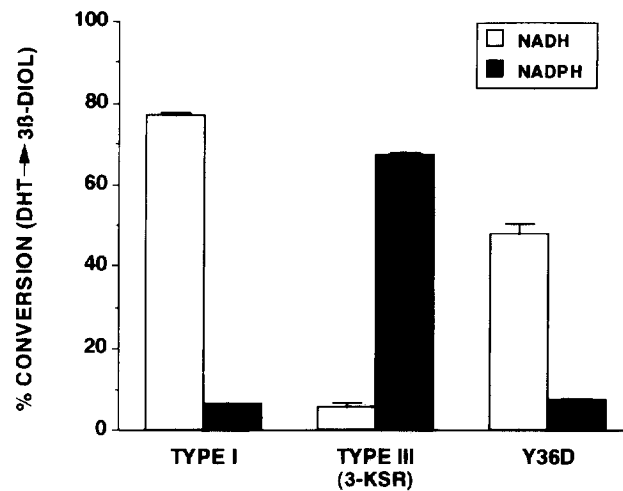

Fig. 5. Comparison of the relative activities of rat type I 3β-HSD, 3-KSR (type III) and the mutant Y36D proteins. The conversion of [³H]DHT into [³H]3β-diol in homogenates from 293 cells transfected with pCMV-type I 3β-HSD or pCMV3-KSR or pCMV Y36D plasmids. Incubation with 10 nM of [³H]DHT was carried out for 180 min in the presence of 1 mM of NADH or 1 mM NADPH using 5 to 20 μg of protein homogenates. The results are presented in % as the means ± SEM after normalization for the estimated amount of translated 42 kDa proteins measured by immunoblot analysis using polyclonal antibodies raised against human type I 3β-HSD.

Table 5. Kinetic properties of the expressed rat type I and 3-KSR (type III) enzymes using DHT as substrate [10]

Expressed protein	Cofactor	K_m (μM)	Relative V_{max}	Relative specificity (Relative V_{max}/K_m)
Rat type I	NADH	5.05	100	19.80
	NADPH	1.18	6.8	5.76
Rat 3-KSR (type III)	NADH	6.16	7.1	1.15
	NADPH	0.12	2.5	20.83

Relative V_{max} for type I isoenzyme incubated in the presence of NADH is equal to 100, the specific activity value being equal to 10.1 nmol/min/mg protein.

that participates in hydrogen bond formation with 2'hydroxyl moiety of all known NAD-dependent dehydrogenases. The Asp[36] residue is present in all vertebrate 3β-HSD characterized so far, with the exception of rat type III and mouse type IV, which both show a specific 3-KSR activity. To study the unique cofactor specificity of the rat 3-KSR (type III), we have introduced a point mutation in the βαβ dinucleotide-binding fold of this protein by substituting Tyr[36] for Asp[36] (Y36D) by site-directed mutagenesis [112]. Transient expression of the wild-type 3-KSR (type III) and mutant Y36D was carried out in 293 human primary embryonal kidney cells. When using [³H]DHT as substrate, the Y36D mutation yields to a marked improvement in overall catalytic activity with NADH as cofactor measured after a 3 h incubation period, whereas this mutation causes a dramatic decrease in the activity with NADPH (Fig. 5). Although the Y36D protein shows an affinity for NADH similar to the type I isoenzyme, this mutation only slightly improves the ability of the enzyme to catalyze the conversion of PREG and DHEA into their corresponding 4-ene-ketosteroids [112]. It is of interest to mention that the Y36D mutant protein also possesses the secondary 17β-HSD activity as measured by the rapid conversion of DHT into A-DIONE in transfected 293 cells [112]. These data thus demonstrate that the presence of the Tyr[36] instead of Asp[36] is responsible for the difference of cofactor specificity of the 3-KSR (type III) protein but is not sufficient to explain its low activity with 5-ene-3β-hydroxysteroid substrates.

The presence of multiple 3β-HSD isoenzymes offers the unique possibility of tissue- and/or cell-specific expression and regulation of this enzymatic activity that plays an essential role in the biosynthesis of all hormonal steroids in classical as well as in peripheral intracrine steroidogenic tissues.

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