



## Structure-Function Relationships of Multiple Rat Members of the 3 $\beta$ -Hydroxysteroid Dehydrogenase Family

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The conversion of 3 $\beta$ -hydroxy-5-ene steroids by the membrane-bound enzyme 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase, hereafter called 3 $\beta$ HSD, is an essential step in the biosynthesis of all classes of hormonal steroids. We have recently characterized 3 types of cDNAs encoding rat 3 $\beta$ HSD (1, 2). The predicted rat type I and type II 3 $\beta$ HSD-expressed proteins share 94% homology (1), while they share only 80% similarity with the rat type III 3 $\beta$ HSD, which is also a 372-amino acid protein (2) as observed for the human (3-5), macaque (6), and bovine (7) 3 $\beta$ HSD predicted proteins. Using the highly sensitive RNase protection method, we have shown that the type I and type II 3 $\beta$ HSD mRNAs are present in several rat tissues, including the ovary, testis, adrenal, and adipose tissue (1, 2), whereas the type III was only found in liver (2). In addition, we have demonstrated by computer analysis that the type I 3 $\beta$ HSD and the type III 3 $\beta$ HSD-encoded proteins possess 2 predicted *transmembrane-spanning domains* (MSD) (1, 2, 8), whereas type II 3 $\beta$ HSD is devoid of one of the 2 MSDs (1, 8). Moreover, transient expression of rat type I and type II 3 $\beta$ HSD cDNAs in non-steroidogenic cells revealed that these two 42-kd proteins catalyze both the oxidation and isomerization of  $\Delta^5$ -3 $\beta$ -hydroxysteroid precursors into  $\Delta^4$ -3-ketosteroids, as well as the interconversion of 3 $\beta$ -hydroxy and 3-keto-5 $\alpha$ -androstane steroids (1, 8). This chapter investigates the structure-function relationships of the 3 rat 3 $\beta$ HSD isoenzymes so far isolated.

## Materials and Methods

### Construction of Wild-Type and Chimeric Rat 3 $\beta$ HSD cDNAs by Site-Directed Mutagenesis

We used the full-length cDNA inserts corresponding to the rat type I (ro3 $\beta$ -HSD56) and type II (ro3 $\beta$ -HSD112) 3 $\beta$ HSD clones (1). We have also constructed the full-length rat liver 3 $\beta$ HSD clone by ligating the *EcoRI/SauI* DNA fragment corresponding to nucleotides -84 to +205 of clone rl 3 $\beta$ -HSD 20, the *SauI/TaqI* DNA fragment corresponding to nucleotides +206 to 720 of clone rl 3 $\beta$ -HSD 33 (2), and the *TaqI/EcoRI* DNA fragment corresponding to nucleotides +721 to +1871 of clone rl 3 $\beta$ -HSD 35 (2). Those full-length rat type I, type II, and type III 3 $\beta$ HSD clones were then cloned into the unique *EcoRI* site of the pCMV vector, downstream from the *cytomegalovirus* (CMV) promoter, to produce the recombinant plasmids pCMV type I 3 $\beta$ HSD, pCMV type II 3 $\beta$ HSD, and pCMV type III 3 $\beta$ HSD, respectively. Those plasmids were sequenced in both orientations, amplified, and subsequently purified by 2 cesium chloride-ethidium bromide density gradient ultracentrifugations. We then constructed by site-directed mutagenesis 2 chimeric cDNAs in which the 4 codons for Ala<sup>83</sup>, Ile<sup>85</sup>, Val<sup>87</sup>, and His<sup>89</sup> potentially involved in an MSD predicted between residues 75 and 91 in the type I 3 $\beta$ HSD protein using the SOAP program (9) of the PC/GENE software (release 6.01; Intelli Genetics, Inc./Genofit SA: Mountain View, CA) were substituted by the codons Ser<sup>83</sup>, Met<sup>85</sup>, Phe<sup>87</sup>, and Arg<sup>89</sup> present in the type II 3 $\beta$ HSD protein and vice versa, thus leading to cDNA inserts encoding a type I 3 $\beta$ HSD protein without the potential MSD (I - MSD) or a type II 3 $\beta$ HSD protein containing type I MSD (II + MSD) as represented schematically in Figure 14.1. Briefly, the full-length cDNA inserts ro3 $\beta$ -HSD56 (type I) and ro3 $\beta$ -HSD112 (type II) were excised from the respective pCMV plasmids by partial *EcoRI* digestion, and the DNA fragments released were size-fractionated on a 1.2% low-melting-temperature agarose gel. The purified full-length cDNA inserts were then digested with *KpnI*, and the generated *EcoRI-KpnI* and *KpnI-EcoRI* fragments were purified. The small *EcoRI-KpnI* DNA fragment from type I (ro3 $\beta$ -HSD56) was ligated with the long *KpnI-EcoRI* DNA fragment from type II (ro3 $\beta$ -HSD112) and then subcloned into pCMV vector to produce the pCMV-I - MSD plasmid. Similarly, the small *EcoRI-KpnI* DNA fragment from type II (ro3 $\beta$ -HSD112) was ligated with the long *KpnI-EcoRI* DNA fragment from type I (ro3 $\beta$ -HSD56) and then subcloned into the pCMV vector to produce the pCMV-II + MSD plasmid. The only differences between the deduced protein sequences encoded by the corresponding 2 small *EcoRI-KpnI* fragments are amino acid positions 83, 85, 87, and 89.

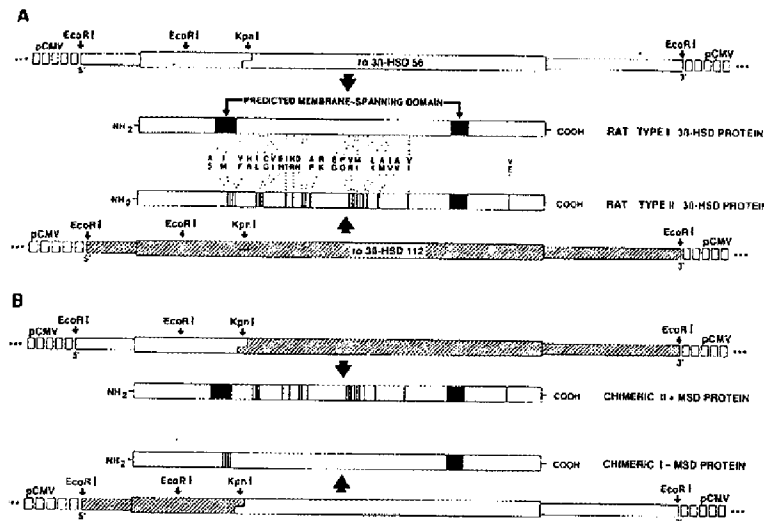


FIGURE 14.1. Structures of wild-type rat types I and II  $\beta$ HSD proteins and chimeric  $\beta$ HSD proteins. *A*: Shown are a schematic representation and comparison of the structures of wild-type rat type I and type II  $\beta$ HSD proteins encoded by pCMV type I  $\beta$ HSD containing the ro $\beta$ -HSD56 cDNA insert and pCMV type II  $\beta$ HSD containing the ro $\beta$ -HSD112 cDNA insert, respectively. The 23 nonidentical amino acid residues between the two rat  $\beta$ HSD types are indicated by vertical lines in the schema illustrating the deduced type II  $\beta$ HSD protein. The predicted MSDs are represented by black boxes. Only the *EcoRI* and *KpnI* restriction sites that are of interest for the construction of chimeric cDNAs are illustrated. *B*: Shown is a schematic representation of the chimeric  $\beta$ HSD proteins encoded by site-directed mutated rat  $\beta$ HSD cDNAs constructed as described in "Materials and Methods" and "Results" sections in the pCMV expression vector. The chimeric protein II + MSD was generated by introducing a putative MSD, while the chimeric protein I - MSD, on the other hand, was generated removing the same putative MSD. Reprinted with permission from Simard, de Launoit, and Labrie (8), © by The American Society for Biochemistry and Molecular Biology.

#### Transient Expression of Multiple Rat $\beta$ HSD cDNAs

Expression of the plasmids was carried out in the HeLa human cervical carcinoma cells by the transfection method previously described (1, 4, 5, 8). Briefly, the plasmids were introduced into HeLa cells by the calcium phosphate precipitation procedure. Mock transfections were carried out with the pCMV alone, while transfection efficiency was monitored by cotransfecting the tested plasmids with the control pXGH5 plasmid that expresses growth hormone. In order to determine  $\beta$ HSD

activity, cells were incubated for the indicated time periods at 37°C in the presence of <sup>3</sup>H-labeled steroid substrates in 50 mM Tris buffer (pH 7.5) containing 1 mM of the appropriate cofactor, namely NAD<sup>+</sup> for *dehydroepiandrosterone* (DHEA, Steraloids), *5α-androstane-3β,17β-diol* (3β-diol, Steraloids) and *pregnenolone* (PREG, Steraloids) or NADH for *5α-dihydrotestosterone* (DHT, Steraloids) and *5α-androstane-dione* (A-dione, Steraloids). The enzymatic reaction was stopped by chilling the incubation mixture in an ice-water slurry and adding 4 volumes of ether/acetone (9/1, v/v). Cell protein content was measured by the method of Bradford (1, 8) using bovine serum albumin as standard, while the relative amounts of translated type I, type II, and type III, as well as the 2 chimeric 3βHSD 42-kd proteins, were evaluated by immunoblot analysis and quantification with an image analyzer of the integrated optical intensity of the corresponding 42-kd bands, followed by the correction of transfection efficiency with GH expression (8). Values of K<sub>m</sub> as well as relative V<sub>max</sub> and relative specificity (V<sub>max</sub>/K<sub>m</sub> ratio) values were calculated by the Lineweaver-Burk method, as previously described (8).

#### Thin-Layer Chromatography and High-Performance Liquid Chromatography Analyses

The organic phase was then evaporated and separated either on TLC plates using a 4:1 mixture of benzene and acetone or by HPLC. For TLC, substrates and formed steroids were identified by comigration on each TLC plate of the nonlabeled steroid(s). The corresponding area was cut and transferred to scintillation vials containing 0.1-mL ethanol to which 10-mL scintillation fluid was added for measurement of radioactivity (1, 4, 5, 8).

Steroids were also identified by HPLC analysis using a System Gold (Beckman) unit consisting of a model 126 pump, a 507 automatic injector, a Radial-Pak NovaPak C<sub>18</sub> column (8 mm × 10 cm) and a model Beckman 168 photodiode array detector. The mobile phase for A-dione, DHT, and 3β-diol was H<sub>2</sub>O/methanol/tetrahydrofuran/acetonitrile (50/35/10/5, v/v/v/v) at 1.5 mL/min flow rate over a 30-min period. Radioactivity was monitored in the eluent using a Beckman 171 HPLC Radioactivity Monitoring System using Formula 963 (NEN) as a scintillation mixture at a flow rate of 4.5 mL/min.

## Results and Discussion

As mentioned above, the deduced amino acid sequences of rat type I and type II 3βHSDs display 93.8% similarity with only 23 nonidentical residues (1). Following transient expression, we have recently observed, however, that rat type I 3βHSD possesses much higher activity than the type II 3βHSD protein for all substrates tested (1). As predicted by

computer analysis (9), there is a potential MSD common to the deduced rat type I, type II, and type III, as well as to human, macaque, and bovine 3 $\beta$ HSD protein sequences, corresponding to a membrane-associated alpha helical segment present in all proteins between residues 287 and 303. Furthermore, such analysis of rat 3 $\beta$ HSDs indicates that the change of residues 83, 85, 87, and 89 observed in the wild-type rat type II 3 $\beta$ HSD protein prevents the formation of a second potential MSD present in the rat type I enzyme between residues 75 and 91 as well as in human and macaque 3 $\beta$ HSD proteins. In order to characterize the functional significance of MSD 75–91 in type I 3 $\beta$ HSD, we have compared the catalytic properties of wild-type rat types I and II 3 $\beta$ HSD proteins 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) by site-directed mutagenesis.

As illustrated in Figure 14.2, pCMV type I 3 $\beta$ HSD encodes a protein having a 3 $\beta$ HSD/ $\Delta^5$ - $\Delta^4$  isomerase relative specificity, as determined by the relative  $V_{max}/K_m$  ratio (relative  $V_{max}$  values were calculated assuming

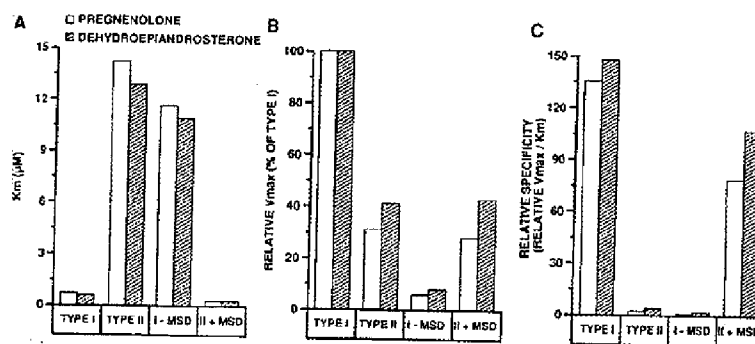


FIGURE 14.2. Catalytic properties of the expressed rat type I and type II isoenzymes of the 3 $\beta$ HSD family, as well as of the chimeric pCMV I – MSD- and pCMV II + MSD-encoded proteins. The procedure for transient expression of wild-type and chimeric cDNAs in HeLa cells and the 3 $\beta$ HSD assay are as described in “Materials and Methods.” Kinetic parameters were determined using Lineweaver-Burk plot ( $1/v$  vs  $1/[S]$ ) analysis of the catalytic activity of expressed proteins. The enzymatic reaction was performed during 30 min for expressed type I 3 $\beta$ HSD and II + MSD proteins, while the reaction was stopped after 60 min with homogenates from cells transfected with pCMV type II 3 $\beta$ HSD and pCMV I – MSD cDNAs. Relative  $V_{max}$  values were calculated assuming the  $V_{max}$  for the wild-type type I 3 $\beta$ HSD enzyme equal to 100. The specific activity values for the type I 3 $\beta$ HSD enzyme using PREG and DHEA as substrates were 5.1 and 4.1 nmol/min/mg protein, respectively. Reprinted with permission from Simard, de Launoit, and Labrie (8). © by The American Society for Biochemistry and Molecular Biology.

that the rate for the pCMV type I 3 $\beta$ HSD encoded protein is equal to 100), that is 64 times higher than that of the pCMV type II 3 $\beta$ HSD-encoded protein with corresponding  $K_m$  values of 0.74  $\mu$ M (type I) and 14.3  $\mu$ M (type II), and 100 and 30.8 relative  $V_{max}$  values, respectively (Fig. 14.2). The much higher relative specificity of rat type I 3 $\beta$ HSD compared to type II 3 $\beta$ HSD was confirmed using DHEA as labeled substrate (Fig. 14.2). The present data thus indicate that the lower activity and lower relative specificity of type II 3 $\beta$ HSD result primarily from an approximately 95% decrease in affinity for both substrates, while the  $V_{max}$  value differs by only 60%–70%.

As illustrated in Figure 14.2, the chimeric I – MSD protein, which lacks MSD at position 75–91, shows a markedly reduced affinity for PREG and DHEA, with  $K_m$  values of 11.7 and 11  $\mu$ M, respectively, compared to 0.74 and 0.68  $\mu$ M, while its relative specificity was dramatically decreased to 0.35% and 0.47% compared to wild-type type I when PREG and DHEA were used as substrates, respectively, thus demonstrating that removal of the putative MSD in the type I 3 $\beta$ HSD markedly affects the specific activity as well as the affinity of the enzyme (Fig. 14.2). In an opposite fashion, the chimeric II + MSD protein gained an affinity for PREG and DHEA comparable to that of the wild-type type I 3 $\beta$ HSD protein, with  $K_m$  values of 0.36 and 0.40  $\mu$ M, respectively. The present data show that the introduction of a putative MSD in the type II 3 $\beta$ HSD protein increased the relative specific activity of the type II protein to 58% (for PREG) and 73% (for DHEA) compared to the wild-type I 3 $\beta$ HSD protein. The present data provide strong evidence supporting the crucial role of the predicted MSD between residues 75 and 91 for the high level of enzymatic specificity of rat type I 3 $\beta$ HSD.

Surprisingly, we have observed that the expressed type I 3 $\beta$ HSD protein can also convert DHT into its 17 $\beta$ -oxidative form (i.e., A-dione), thus demonstrating that this enzyme possesses a secondary 17 $\beta$ HSD activity. In fact, as illustrated in Figure 14.3B, the endogenous 17 $\beta$ HSD activity in HeLa cells is very low (Fig. 14.3B), whereas homogenates from cells transfected with pCMV type I 3 $\beta$ HSD convert about 65% of DHT into A-dione after a 10-h incubation period in the presence of 1 mM  $NAD^+$  (Fig. 14.3C). In contrast, type II 3 $\beta$ HSD isoenzyme, as well as expressed chimeric I – MSD or II + MSD protein did not have such 17 $\beta$ HSD activity (data not shown). The fact that the chimeric II + MSD 3 $\beta$ HSD protein is devoid of 17 $\beta$ HSD activity strongly suggests that lack of such 17 $\beta$ HSD enzymatic activity of expressed type II 3 $\beta$ HSD protein is not only due to the absence between residues 75 and 91, but should rather be due to other specific differences between the type I and type II isoenzymes. Although dual enzymatic activity has been previously demonstrated with purified steroid enzymes (10, 11), this represents the first demonstration of such dual catalytic activity after transient expression of a specific cDNA.

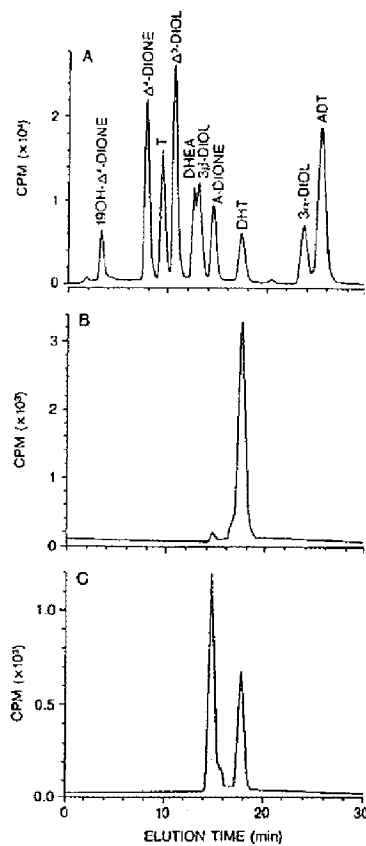


FIGURE 14.3. Secondary  $17\beta$ HSD activity of the rat type I  $3\beta$ HSD enzyme. Twenty  $\mu$ g of protein from homogenates of cells transfected with the pCMV alone or pCMV type I  $3\beta$ HSD plasmid were incubated in the presence of 1 mM  $\text{NAD}^+$  and 300 nM tritiated DHT for 12 h. Steroids were extracted, and the organic phase was then evaporated and steroids were identified by HPLC, as described in "Materials and Methods." DHT and A-dione were separated with a mobile phase of  $\text{H}_2\text{O}$ -methanol-tetrahydrofuran/acetonitrile (50/35/10/5, v/v/v/v) at a flow rate 1.5 mL/min over a 30-min period.

We then made the unexpected observation that the expressed liver-specific type III isoform of the  $3\beta$ HSD family is unable to catalyze the oxidation and isomerization of  $\Delta^5$ - $3\beta$ -hydroxysteroid precursors, such as PREG, DHEA, or  $\Delta^5$ -androstenediol, into their  $\Delta^4$ -3-keto forms nor to convert  $3\beta$ -hydroxysteroids, such as  $3\beta$ -diol, into their corresponding 3-

TABLE 14.1. Lineweaver-Burk plot analyses of the catalytic properties of the expressed rat type I and liver-specific type III isoforms of the 3 $\beta$ HSD family using increasing concentrations of DHT as substrate and the indicated cofactor.

Expressed protein	$K_m$ ( $\mu$ M)	Relative $V_{max}$	Relative specificity (relative $V_{max}/K_m$ )
Rat type I			
NADH	4.9	100.0	20.4
NADPH	2.9	47.8	16.5
Rat type III			
NADH	5.5	27.3	5.0
NADPH	0.7	15.4	22.0

Incubations were performed for 20 min at 37°C as described in "Materials and Methods."

keto-5 $\alpha$ -androstane form (data not shown). However, time course studies with homogenates from cells transfected with pCMV type III 3 $\beta$ HSD plasmid in the presence of A-dione or DHT supplemented with 1 mM NADH clearly showed a potent reductase activity, this reductase activity being shared with type I 3 $\beta$ HSD. As indicated in Table 14.1, Lineweaver-Burk plot analysis shows that the  $K_m$  values for DHT using homogenate from cells transfected with pCMV type I 3 $\beta$ HSD or pCMV type III 3 $\beta$ HSD are in the same range with respective values of 4.9  $\mu$ M and 5.5  $\mu$ M using NADH as cofactor. The type I 3 $\beta$ HSD protein possesses, however, a relative  $V_{max}$  and a relative specificity 3.7- and 4.1-fold higher than those of expressed type III isoform, respectively (Table 14.1). The  $K_m$  value for the type I 3 $\beta$ HSD protein in the presence of DHT supplemented with 1 mM NADPH is 2.9  $\mu$ M, a value that is in the same range as the value obtained when NADH is used as cofactor. However, the affinity of the expressed type III isoform for DHT is much higher with NADPH, with a  $K_m$  value of 0.69  $\mu$ M compared to 5.51  $\mu$ M with NADH. Using NADPH as cofactor, the relative  $V_{max}$  value is 3.1-fold higher in type I than in type III, whereas the relative specificity shows comparable values (16.5 vs 22.0). The present data indicate that NADPH is the preferred cofactor for the reductase activity of the expressed type III isoform (Table 14.1). Detailed investigation of the structure-function characteristics of the catalytic sites of the multiple 3 $\beta$ HSD isoenzymes by site-directed mutagenesis should provide crucial information concerning the molecular basis for their differential enzymatic activity.

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