Structure of the Human 3α-Hydroxysteroid Dehydrogenase Type 3 in Complex with Testosterone and NADP at 1.25-Å Resolution*

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The first crystallographic structure of human type 3 3α-hydroxysteroid dehydrogenase (3α-HSD3, AKR1C2), an enzyme playing a critical role in steroid hormone metabolism, has been determined in complex with testosterone and NADP at 1.25-Å resolution. The enzyme’s 17β-HSD activity was studied in comparison with its 3α-HSD activity. The enzyme catalyzes the inactivation of dihydrotestosterone into 5α-androstane-3α,17β-diol (3α-diol) as well as the transformation of androstenedione into testosterone. Using our homogeneous and highly active enzyme preparation, we have obtained 150-fold higher 3α-HSD specificity as compared with the former reports in the literature. Although the rat and the human 3α-HSDs share 81% sequence homology, our structure reveals significantly different geometries of the active sites. Substitution of the Ser222 by a histidine in the human enzyme may compel the steroid to adopt a different binding to that previously described for the rat (Bennett, M. J., Albert, R. H., Jez, J. M., Ma, H., Penning, T. M., and Lewis, M. (1997) Structure 5, 799–812). Furthermore, we showed that the affinity for the cofactor is higher in the human 3α-HSD3 than the rat enzyme due to the presence of additional hydrogen bonds on the adenine moiety and that the cofactor is present under its reduced form in the active site in our preparation.

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Human 3α-hydroxysteroid dehydrogenases (3α-HSDs,1 EC 1.1.1.213) work in concert with the 5α/5β-reductases to convert steroid hormones into the 3α5α and 3α5β-tetrahydrosteroids. These isozymes catalyze the inactivation of androgens, estrogens, progestins, and glucocorticoids. However, the inactivation of the most potent androgen 5α-dihydrotestosterone (5α-DHT) to 5α-androstan-3α,17β-diol (3α-diol) is its best known function (1). These isoenzymes thus play a major role in the regulation of the intracellular concentration of 3α-DHT in peripheral tissues, especially in the androgen-sensitive prostate that is susceptible to benign prostatic hyperplasia and prostate cancer. Testosterone, after entering prostatic cells, is transformed to 5α-DHT by 3-oxo-5α-androst-4-dehydrogenase (2, 3). 5α-DHT is a more potent androgen than testosterone in stimulating prostate cancer growth (4, 5) and preferentially binds to the androgen receptor (dissociation constant (Kd) for the androgen receptor of 10–11 M) (6). Elevation of 5α-DHT content in prostate has been associated with benign prostatic hyperplasia in humans (6, 7) and with human prostate carcinoma (8, 9). The action of 5α-DHT may be terminated by 3α-HSD, which catalyzes the inactivation of 5α-DHT to 3α-androstanediol (a weak androgen; Kd for the androgen receptor of 10–6 M) (1). It has also been proposed that, by catalyzing the reverse reaction, 3α-HSD may function as a molecular switch and, in this manner, may regulate the amount of 5α-DHT available for androgen receptor binding and activation. Three isoforms of 3α-HSD have been described in human tissues (10–12) as playing critical roles in sex hormone metabolism and action, such as regulating the occupancy of the androgen receptor. At least two related 3α-HSD isoforms (type 2 and type 3) have been detected in the human prostate (13, 14). These reports have shown that both enzymes eliminate 5α-DHT but that only the type 3 forms the active hormone 5α-DHT. Thus, 3α-HSD3 may increase the pool of active androgens in the prostate. Through its action on allopregnanolone, 3α-HSD also regulates the GABA A receptor (Kd = 10–9 M), a membrane-bound chloride ion-gated channel, and may have profound effects on the receptor function (15, 16). The types 1 and 3 3α-HSDs (AKR1C4 and AKR1C2, respectively) are highly homologous and catalyze the above reactions. It is reported that the type 1 enzyme is the most efficient in DHT reduction, with its substrate specificity (expressed in kcat/Km) being 10- to 40-fold higher than the other members of the family. The type 2 enzyme (AKR1C3) shows a significantly higher activity in catalyzing the transformation of androstenedione (4-dione) to testosterone than the other two types of 3α-HSD. In this re-

1 The abbreviations used are: HSD, hydroxysteroid dehydrogenase; DHT, dihydrotestosterone; 3α-diol, 5α-androstan-3α,17β-diol; 4-dione, androstenedione; AKR, aldo-keto-reductase; MCM, Monte Carlo minimization.
3α-HSD3, a Member of the AKR Family

Monte Carlo Minimization (MCM)—To predict the optimal position of testosterone in the active site of 3α-HSD3, we have used a computational strategy similar to that proposed by Zhorov and Lin (33) in the modeling study of estradiol binding to type 1 17β-HSD. In addition to the energy components described in Ref. 33, the energy expression also included electron density calculation by the method of Laskowski and Kapplus (34) and energy of deformation of bond angles in testosterone. The enzyme was represented by a two-shell model based on our crystallographic structure. The first (inner) shell of the model included amino acid residues having at least one atom within 6 Å of testosterone or the cofactor in the x-ray structure. All residues in the inner shell were treated as flexible, their torsion angles being allowed to vary during energy minimizations. Cα atoms of the flexible-shell residues were constrained by pins, flat bottom penalty functions that allowed penalty-free deviation of Cα atoms up to 1 Å from their crystallographic positions. The second (outer) shell of the model included amino acid residues not belonging to the flexible shell and having at least one atom within 10 Å of testosterone or the cofactor. The residues in the outer shell were kept rigid during energy minimizations.

A vector (pivot) was drawn between testosterone atoms O17 and O3 in the crystallographic structure of the ternary complex to specify the direction from the entrance to the steroid-binding pocket toward the cofactor. Testosterone was pulled along the pivot with a step of 1 Å, and an MCM energy profile against the testosterone position was computed as follows: A given position of the steroid was imposed by constraining O17 and the plane normal to the pivot and crossing it at the level characterized by a displacement parameter s, hereafter referred to as the testosterone position. The displacement s = 0 Å (position 0) corresponds to our x-ray structure, positive values of s indicating displacement of the steroid toward the cofactor. It should be noted that the atom-plane constraint removes only one of the six positional and rotational degrees of freedom of the ligand. Twelve MCM trajectories were calculated for 12 positions of testosterone. In each MCM trajectory, torsion angles of amino acids and testosterone and generalized coordinates characterizing position and orientation the steroid were sampled. In the subsequent energy minimizations, all generalized coordinates of the system, including those of the cofactor, were allowed to vary. Each MCM trajectory predicted the lowest energy structure for the given displacement of the steroid. These structures are visualized with the help of program RASMOL. Several energetic and geometric parameters of these structures are plotted against position of the steroid. The MCM energy profile was computed with the help of the ZMM program described in Ref. 33 and references therein.

Radiochemical Determination of Kinetic Parameters—Unlabeled dihydrotestosterone (DHT), 4-dione, testosterone, NADP(H), K2HPO4/KH2PO4 buffer, Tris-HCl buffer, bovine serum albumin, EDTA, glycerol, and phenylmethylsulfonyl fluoride were obtained from Sigma. 14C-DHT, 14C-4-dione, and 14C-testosterone were purchased from PerkinElmer Life Sciences. Diethyl ether, dichloromethane, toluene, and acetonitrile were obtained from Fisher. The Bio-Rad Protein Assay Kit was purchased from Bio-Rad. Silica-coated alumina thin layer chromatography plates and 14C-labeled steroids were purchased from BDH. All other reagents were purchased from Sigma. The Storm imaging system was from Molecular Dynamics, Inc. (Sunnyvale, CA). The software used for kinetic data analysis was ENZFITTER (Biosoft, Cambridge, UK) version 1.05.

Steady-state Kinetics—All reactions were performed at 37.0 ± 0.5 °C, and the reaction mixtures contained 0.1 M potassium phosphate buffer, pH 7.5, 0.05 mg/ml bovine serum albumin, 100 μM NADP(H), and varying amounts of 14C-labeled steroids. The final content of ethanol for each reaction was standardized to 2%. Reactions were initiated by the addition of the enzyme sample, aliquots were taken, and the reaction was then stopped by the addition of 3 volumes of diethyl ether on ice. The stopped reaction mixture was chilled using a dry ice/ethanol bath, and the aqueous phase was discarded. Diethyl ether was then evaporated, and steroids were reconstituted in 60 μl of dichloromethanol for TLC separation. The migration solvent system consisted of a 4:1 ratio of toluene to aceton. TLCs were then exposed and quantified using a STORM device. Initial velocities were measured with less than 10% substrate consumption and were expressed as nmol/min/mg). Experimental data were compiled, duplicated, and then treated by the ENZFITTER program.

RESULTS AND DISCUSSION

Structure of the Human 3α-HSD3 Ternary Complex—The human type 3 3α-HSD, in addition to its 3α-HSD activity, also possesses some 17β-HSD activity, but the latter activity is...
much weaker than in the case of the type 2 3α-HSD. We thus attempted to obtain the enzyme ternary complex with 4-dione and NADP. The crystal structure of the human 3α-HSD type 3 in the ternary complex was determined by the molecular replacement method using the program EPMR (35). The quality of the refined ternary complex can be assessed from the statistics given in Table I. All data between 10 and 1.25 Å were used in the refinement, yielding a crystallographic $R$-factor of 18% for 180,677 reflections and a free $R$-factor of 20% for 8672 reflections. There are two molecules in the asymmetric unit related by a 2-fold noncrystallographic symmetry axis, although the protein is a monomer in solution. The refined model includes two complete ternary complexes, each of which contains an NADP molecule, a testosterone molecule, and an ac- cinate molecule (Fig. 1). In addition, the model includes 858 water molecules.

The protein adopts a well known triose-phosphate isomerase barrel motif, namely an ($\alpha$/$\beta$)$_8$-barrel with two additional helices. A ternary complex exhibiting good stereochemistry was refined to 1.25 Å. Refinement of this model against the 1.25-Å data has enabled atomic positions in the model to be defined by electron density that discriminates between carbon, nitrogen, and oxygen. Out of the 323 amino acids contained in the protein, 323 were modeled into the electron density for molecule A and 318 for molecule B. In molecule B, the loop (positions 132–136) is disordered, whereas this latter is stabilized by a symmetric molecule in molecule A. Individual isotropic temperature ($B$) factors were also refined and resulted in the same $B$ values in molecules A and B for the protein and the acetate. Nevertheless, the $B$ factor for testosterone in molecule B is higher than in molecule A. The Ramachandran plot for the ternary complex positions 94.4% of the backbone dihedral angles in the core regions, as defined by Kleywegt and Jones (36). The only residue that falls into the disallowed region is the residue Ser221, which is also in a disallowed region in the rat structure, more precisely because the nitrogen of Ser221 makes a hydrogen bond with the cofactor pyrophosphate group. With the exception of the N-terminal region (Asp1–Cys8, implicated in the crystal packing), Ser$^{220}$–Tyr$^{223}$ in the C terminus (dis- ordered in the rat structure), and the loops interacting with the steroid (see below), the structure obtained for the human 3α- HSD3 ternary complex is highly similar to the structure reported for the rat 3α-HSD3 ternary complex (24), as shown by the root mean square deviation of 0.678 Å for 242 Cα positions. However, important shifts are observed for the five loops involved in the geometry of the active site, namely loops Tyr$^{247}$– Ser$^{252}$, Ala$^{259}$–Asn$^{267}$, His$^{117}$–Leu$^{144}$, Gly$^{220}$–Pro$^{233}$, and Leu$^{298}$– Phe$^{319}$. Furthermore, in the human 3α-HSD3, the crystal protein sequence begins with an Asp$^1$–Asp$^2$–Ser$^3$ sequence instead of the expected Met$^1$–Asp$^2$–Ser$^3$ sequence. This is due to the expression with the addition of the glutathione S-transferase. Since thrombin did not cut all of the linker sequence, Asp$^4$ was found to remain in the purified protein.

Although the human and the rat models show the same overall structure, the two enzymes demonstrate important differences in the cofactor and steroid binding regions.

**NADP Binding Site**—The electron density map clearly shows that NADP is bound tightly to 3α-HSD3. NADP binds at the C-terminal end of the barrel, threading through a short tunnel.

**Fig. 1.** Schematic stereoview representation of the 3α-HSD3 ternary complex. $\alpha$-Helices, $\beta$-strands, and coils are represented by helical **ribbons**, *arrows*, and **ropes**, respectively. The testosterone, acetate, and NADP molecules are shown as **ball and stick representations** with purple, yellow, and white bonds, respectively. This image was produced using MolScript (43) and Raster3D (44).
mainly formed by van der Waals contacts between the β1-α1 loop (residues 26 and 27), the loop B (residues 222–226), and Lys270, with the adenine ring at the periphery and the nicotinamide ring toward the core of the barrel (Figs. 1 and 2). Similarly to the rat structure (37), the orientation of NADP in the cofactor binding site is determined by several hydrogen bonds and salt bridges. At the core of the barrel, the nicotinamide ring stacks against the side chain of Tyr24 and makes three hydrogen bonds between the carboxy-amide group and the side chains of Ser166, Asn167, and Gln190. The ribose group is also stabilized via hydrogen bonds with the main chain of the Tyr24 residue and the side chain of the Asp30 residue. The pyrophosphate portion is involved in hydrogen bonding with the side chain of Ser237 and the main chains of Leu219, Ser221, and Lys270. At the periphery of the barrel, the adenosine 2′,5′-diphosphate portion of NADP is stabilized by hydrogen bonds and a salt bridge. Hydrogen bonds occur with side chains of Gln279, Ser271, and Arg276 and the main chain of Tyr272. At the same time, Arg276 forms a salt bridge with the 2′-phosphate (Fig. 2).

Although several attempts to crystallize the apoenzyme form of 3α-HSD3 were performed, none of them were successful. In fact, whenever crystals of the enzyme were grown in the absence of any ligand, the electron density obtained from the diffraction of these crystals showed the presence of the NADP in the cofactor binding site. This observation suggests that the cofactor bound to the enzyme comes from the cell itself during the overproduction and that its affinity for the enzyme is very high. However, because the structure of the apoenzyme has been solved for rats, it is possible that its cofactor affinity is lower than that of humans. Two substitutions in the cofactor binding site of the human enzyme can be at the origin of this difference: Asn280 (human) instead of Leu280 (rat) and Lys270 (human) instead of Arg270 (rat). These two residues are local-

ized in the periphery of the NADP binding site. Whereas Leu280 (rat) does not form any interaction with the cofactor, the human homologue Asn280 is able to form two hydrogen bonds with the adenine part of NADP. Furthermore, Lys270 (human) forms a salt bridge with the 2′-phosphate of NADP, an interaction that is absent in the rat enzyme because the corresponding amino acid Arg270 is positioned farther apart (Fig. 2). Although all of the other interactions existing between NADP and the tunnel are similar in the rat and human enzyme, these three extra interactions support the higher affinity of NADP in the human enzyme.

It was previously established that the cofactor binding or release is rate-limiting in 3α-HSDs (38), which could be a function of making or breaking the many interactions between the enzyme and the cofactor. Our results show that there are three additional hydrogen bonds in the human structure on the part of the enzyme and the cofactor. These three hydrogen bonds seem to be important for the stabilization of the cofactor and could contribute to the lower $k_{cat}$ values obtained for the human enzyme 3α-HSD3 (AKR1C2) ($k_{cat}$ for the oxidation of androstenedione = 0.42 min$^{-1}$, and $k_{cat}$ for the reduction of 4-dione = 1.39 min$^{-1}$ (14)) than those obtained for the rat ($k_{cat}$ for the oxidation of androstenedione = 66 min$^{-1}$, and $k_{cat}$ for the reduction of 4-dione = 19 min$^{-1}$). This hypothesis is corroborated by the fact that for the other members of the human AKR family, namely 20α-HSD (AKR1C1), 3α-HSD2 (AKR1C3), and 3α-HSD1 (AKR1C4), residues 270 and 280 are also substituted by Lys and Asn, respectively, and the $k_{cat}$ values are lower than the rat ($k_{cat}$ for the oxidation of androstenedione = 0.060 min$^{-1}$ and 1.39 min$^{-1}$ for human AKR1C1 and AKR1C4, respectively, and $k_{cat}$ for the reduction of 4-dione =
Steroid Binding Pocket—The excellent quality of the electron density map calculated at nearly atomic resolution enabled the unambiguous localization of the steroid. Surprisingly, although 4-dione was introduced before crystallization, the electron density observed in the active site of 3α-HSD3 corresponds to testosterone (Fig. 3). Therefore, a reduction occurred before or during the crystallization process. Further kinetic investigation revealed that the overnight incubation of our enzyme preparation in the presence of 14C-labeled 4-dione but without the addition of cofactor produced 14C-testosterone, while the control experiment, namely the overnight incubation of 14C-4-dione in the absence of the enzyme, did not produce any 14C-testosterone (Fig. 4). Furthermore, and as explained previously, whenever crystals of the purified enzyme were grown in the absence of steroid and cofactor, a clear electron density for NADP was always observed in the cofactor’s binding site (data not shown). This evidence supports on one hand that 3α-HSD3 is expressed and purified with NADP(H), this latter being tightly and stably bound to 3α-HSD3, and on the other hand that this cofactor remains in its reduced state under the experimental conditions. To date, it still remains unclear whether NADP(H) is present in its reduced state from the beginning of the expression process in Escherichia coli, this latter being stabilized in the binding tunnel (see below), or if the cofactor was reduced later (disordered in the rat structure). No detectable increase of the absorbance at 340 nm could be observed from a solution containing NADP, buffer, dithiothreitol in the presence or absence of the enzyme, this suggesting that NADP is not reduced due to small molecule chemicals present in the experimental environment (data not shown).

Interestingly, testosterone is positioned with its O3 pointing toward the nicotinamide ring, a position that is not in the expected binding mode resulting from the simple reduction of 4-dione. Indeed, because the observed density corresponds to testosterone, this means that the C17 ketone has been reduced. This observation suggests that 4-dione should enter into the active site with its C17 ketone pointing toward the nicotinamide ring of the cofactor, permitting the hydride transfer and therefore the formation of testosterone. The position of testosterone within the binding cavity obtained from the crystallographic structure, with its O3 and not O17 pointing toward the NADP, suggests that the testosterone formed from the reduction of 4-dione leaves the catalytic site and reenters in the binding site with its O3 forward.

As observed in the rat ternary complex, the testosterone molecule is bound in a cylindrical cavity formed by five loops at the C-terminal end of the barrel, namely loop A (residues 117–143), loop B (residues 217–238), the C-terminal tail (residues 299–323), and the shorter connections β1-α1 (residues 23–32) and β2-α2 (residues 51–57). The A and B rings of the testosterone are in stacking with Trp277 (Fig. 5) and interact with Tyr24, Trp86, and Ile229 while the β-face interacts mainly with Val54. If the A and B rings interact extensively with the human 3α-HSD3, the C and D rings make only a few van der Waals contacts, principally with Val128 and Ile129. In such binding, the C18 and C19 angular methyl groups are directed toward the side of the cavity containing residue Val54, whereas in the rat structure the C18 and C19 angular methyl groups were directed toward the side of the cavity containing Trp277.
Comparison of the binding of testosterone in the human and the rat structures reveals that the positions and orientations of testosterone in the binding pocket are different in 3α-HSD3 from the two sources (Fig. 5). Whereas the testosterone in the human enzyme is bound at the entrance of the active site, the steroid is deeply engulfed in the catalytic site of the rat structure. Although different residues implicated in the binding of the testosterone molecule in the rat structure are substituted in the human sequence (Tyr^24, Val^54, Ile^{129}, Pro^{226}, and Ile^{310} for the human enzyme instead of Thr^24, Leu^54, Phe^{129}, Thr^{226}, and Tyr^{310} for rat enzyme), they cannot by themselves explain this difference in the steroid binding mode.

Close inspection of the binding site of testosterone in both enzymes shows that a large shift is observed for the important Trp^{227} residue. Indeed, although Trp^{227} is conserved in both species, this amino acid adopts a very different position in the two models; in the human enzyme, this amino acid is oriented at 134 degrees (C^α to C^β) from the position observed in the rat enzyme (Fig. 5). The change in the position of the Trp^{227} side chain is caused by the substitution of Ser^{222} (rat) by His^{222} (human). His^{222} belongs to the loop B that is implicated in the binding of the cofactor and the steroid. In the human enzyme, the side chain of His^{222} penetrates deeply to the enzyme’s active site and compels the Trp^{227} side chain to generate a 134°
rotation of the latter. In fact, this orientation of Trp^227 disallows the binding of the testosterone molecule in the same position, because otherwise the C18-methyl group would be at least 1 Å from Trp^227. As a result, testosterone cannot occupy the same position in both mammalian enzymes.

In such binding, the distance between the C3 atom of the steroid and the C4 of the nicotinamide ring is about 6 Å, prohibiting the hydride transfer. The crystallographic structure reveals the presence of an acetate molecule that sits between the nicotinamide and the testosterone, being 3.3 Å apart from the nicotinamide ring on one side and 4.3 Å apart from the C3 testosterone on the other side (Fig. 1). The acetate molecule occupies exactly the same position on the C2-C3-C4-O4 from the D-ring of testosterone as positioned in the rat structure. This acetate molecule is very stable in the active site, with B-values of 29.2 Å² for molecule A and 26.8 Å² for molecule B, which are equivalent to the B-values of testosterone (24.8 Å² for molecule A and 34.1 Å² for molecule B). The ketone group of the acetate molecule mimics the O3 ketone of the steroid by making hydrogen bonds with His^117 and Tyr^55. A catalytic mechanism has been proposed for 3α-HSDs that involves three residues near the nicotinamide ring. In this mechanism, Tyr^55 acts as the general acid, Lys^84 decreases the pKₐ of the tyrosine by hydrogen bonding to it, and Asp^50 is salt-bridged to the lysine (40, 41). His^117 has also been implicated in catalysis by hydrogen bonding to it, and Asp^50 is salt-bridged to the lysine, making hydrogen bonds with His^117 and Tyr^55. A catalytic site function: the enzyme prep-}

When we compare the results we obtained for human 3α-
HSD (3α-reduction of DHT K_m = 1.1 μM, k_cat = 1.5 min⁻¹, k_cat/K_m = 1.3 μM⁻¹ min⁻¹) with formerly published results (e.g. 3α-reduction of DHT K_m = 26 μM, k_cat = 0.23 min⁻¹, k_cat/K_m = 0.0088 μM⁻¹ min⁻¹ from Penning et al. (14)), our enzyme preparation shows a higher k_cat and a lower K_m, thus, the specificity is nearly 150-fold higher than formerly reported results, in agreement with the high resolution crystals obtained from the same preparation.

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