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Crystallization and preliminary X-ray crystallographic analysis of the human type 3 3α-hydroxysteroid dehydrogenase at 1.8 Å resolution

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In androgen-sensitive target tissues, 3α-hydroxysteroid dehydrogenase regulates the androgen receptor (AR) activity by catalyzing the inactivation of 5α-dihydrotestosterone (the most natural potent androgen) to 5α-androstane-3α,17β-diol. In this report, the crystallization of a human prostatic type 3 3α-hydroxysteroid dehydrogenase, a member of the aldo–keto reductase superfamily, is described. Two different crystal forms of the complex between the human type 3 3α-HSD, $NADP^+$ and testosterone have been obtained using PEG as precipitant. Crystal form I, which diffracts to 1.6 Å, belongs to the monoclinic space group $P2_1$, with unit-cell parameters $a = 55.07$, $b = 87.15$, $c = 76.88$ Å, $\beta = 107.37^\circ$ and two subunits in the asymmetric unit. A complete data set has been collected at 1.8 Å. Crystal form II, which diffracts to 2.6 Å, belongs to the rhombohedral space group $R32$, with unit-cell parameters $a = b = 143.59$, $c = 205.86$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ and two subunits in the asymmetric unit.

1. Introduction

3α-Hydroxysteroid dehydrogenases (3α-HSDs; E.C. 1.1.1.50) work in concert with the 5α/5β-steroid reductases to convert steroid hormones into the 3α/5α- and 3α/5β-tetrahydrosteroids. 3α-HSDs catalyse the inactivation of androgens, progestins and glucocorticoids. However, the inactivation of the most potent androgen 5α-dihydrotestosterone (DHT; $K_d = 10^{-11}\,M$ for the androgen receptor) to 5α-androstane-3α,17β-diol (3α-diol; $K_d = 10^{-6}\,M$ for the androgen receptor) is its best known function (Bruchovsky & Wilson, 1968; Liao *et al.*, 1973). The enzyme thus plays a major role in the regulation of the intracellular concentration of the androgen DHT in peripheral tissues, especially in the androgen-sensitive prostate, which is susceptible to benign prostatic hyperplasia and prostate cancer. Three isoforms of 3α-HSD have been reported in human tissues (Dufort *et al.*, 1996; Khanna *et al.*, 1995; Deyashiki *et al.*, 1992). The types 1 and 3 3α-HSDs are highly homologous and catalyze mainly the above-mentioned reactions. On the other hand, the type 2 enzyme shows a significantly higher activity in catalysing the transformation of androstenedione (4-dione) to testosterone than the other two types of 3α-HSD. In this regard, type 2 3α-HSD is more similar to 17β-HSD and is therefore named type 5 17β-HSD (Dufort *et al.*, 1998). 3α-HSDs belong to the aldo–keto reductase superfamily (AKR) and have no significant sequence similarity to other HSDs belonging to the short-chain dehydrogenase–reductase superfamily (SDR) such as 17β-HSDs (Ghosh *et al.*, 1995; Labrie *et al.*, 1997, 2000; Peltoketo *et al.*, 1988), 11β-HSD (Agarwal *et al.*, 1989) and 3β-HSD (Luu-The *et al.*, 1989). Human type 3 3α-HSD cloned in our laboratory (Dufort *et al.*, 1996) shows a higher level of amino-acid sequence identity to human 20α-hydroxysteroid dehydrogenase (20α-HSD; Zhang *et al.*, 2000) (97.8%) than to the type 1 3α-HSD (81.7%) and type 5 17β-HSD (86%). In fact, there are only seven amino-acid differences between the type 3 3α-HSD and 20α-HSD.

Here, we report the crystallization and a preliminary crystallographic study of the human type 3 3α-HSD, the first example from the human 3α-HSD family. This human crystal form provides the highest resolution data for mammalian 3α-HSDs to date. Previously, the best reported was 2.5 Å for the rat structure (Bennett *et al.*, 1997). In the crystal, the enzyme molecule is in a ternary complex with testosterone and $NADP^+$. We will be able to describe in detail the interactions established with the steroid in the enzyme's active site and compare these with those already established in the rat model, thus shedding light on the substrate recognition and catalysis by these enzymes.

2. Materials and methods

2.1. Preparation and concentration of the sample

Human 3α-HSD3 with a glutathione S-transferase (GST) tag was overproduced in *Escherichia coli*. The enzyme was purified

Table 1
Crystallization and crystallographic data of crystal forms of human 3α-HSD type 3.

Values in parentheses are for the highest resolution shell; this was 1.90–1.80 Å for form I and 2.78–2.66 Å for form II.

	Form I	Form II
Crystallization		
Temperature (K)	293	293
Protein solution	10 m*M* potassium phosphate pH 7.0, 1 m*M* EDTA, 0.5 m*M* DTT, 0.06% β-octyl glucoside	10 m*M* potassium phosphate pH 7.0, 1 m*M* EDTA, 0.5 m*M* DTT, 0.06% β-octyl glucoside
Ligands	1 m*M* $NADP^+$ and testosterone	1 m*M* $NADP^+$ and testosterone
Well solution		
Precipitant	26%(*w*/*v*) PEG 4K	26%(*w*/*v*) PEG 4K
Protein concentration (mg ml^{-1})	15	15
Buffer	0.1 *M* sodium citrate pH 5.6	0.1 *M* sodium citrate pH 5.6
Additives	0.38 *M* ammonium acetate, 1.5% MPD, 0.4 m*M* DTT	0.16 *M* ammonium acetate, 1.5% MPD, 0.4 m*M* DTT
Time (d)	1–2	1–2
Largest crystal size (mm)	0.06 × 0.18 × 0.72	0.07 × 0.30 × 0.30
X-ray data collection		
Temperature (K)	100	100
Space group	$P2_1$	$R32$
Unit-cell parameters	a = 55.07, b = 87.15, c = 76.88 Å, $\alpha = \gamma = 90°$, $\beta = 107.365°$	$a = b$ = 143.59, c = 205.86 Å, $\alpha = \beta = 90°$, $\gamma = 120°$
Data limit (Å)	1.80	2.66
No. of reflections	282268 (9060)	432762 (11626)
No. of unique reflections	57969 (4734)	44712 (1309)
Completeness (%)	90.1 (73.8)	98.9 (95.0)
Multiplicity	4.87 (1.91)	9.68 (8.88)
$I/\sigma(I)$	12.37 (2.41)	6.84 (2.02)
Data > 1σ (%)	89.56 (68.61)	91.04 (70.50)
R_{merge}†	0.069 (0.357)	0.143 (0.475)
Mosaicity	0.56	0.58

† $R_{merge} = \sum_h \sum_i |I(h) - I(h)_i| / \sum_h \sum_i I(h)$, where $I(h)$ is the mean intensity after rejections.

using glutathione Sepharose 4B and Q-Sepharose columns. After loading the sample on the glutathione Sepharose 4B column, thrombin protease was used to cleave the desired protein from the fusion product in a similar manner as in Cantin *et al.* (in preparation). The enzyme was stored at 277 K in 10 m*M* potassium phosphate pH 7.0, 1 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT). Prior to crystallization, 0.06% β-octyl glucoside (β-OG), 1 m*M* $NADP^+$ and 25 μ*M* testosterone were added to the enzyme sample. This sample was concentrated to form the ternary complex 3α-HSD–$NADP^+$–testosterone in a Centricon concentrator at 4500*g* in an RC-5 centrifuge, giving a final concentration of 22 mg ml^{-1}. Protein concentration was calculated by the method of Bradford (1976); *i.e.* measured by absorption at 595 nm, using BSA (bovine serum albumin) as the standard. The optical density was determined using a Beckman DU-70 spectrophotometer.

2.2. Crystallization

Crystallization was carried out by the vapour-diffusion method with hanging drops at room temperature (McPherson, 1982). The reservoir contained 1 ml of solution including the precipitant (polyethylene glycol 4000), salt (ammonium acetate) and sodium citrate at pH 5.6. Equal volumes of protein and reservoir solution (3 µl) were mixed to initiate the crystallization.

2.3. X-ray diffraction analysis

Data sets were collected at 100 K from a single crystal of the ternary complex 3α-HSD–$NADP^+$–testosterone and were recorded on an R-AXIS IIC image-plate detector on a Rigaku RU-200 rotating-anode generator. Data frames were recorded while the crystal was oscillated through 1° steps. The diffraction data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

2.4. Molecular replacement

The molecular-replacement calculations were performed with a 4 Å resolution cutoff using the program *EPMR* (Kissinger *et al.*, 1999). The search model used was the refined 2.5 Å resolution structure of the rat 3α-hydroxysteroid dehydrogenase in complex with testosterone and $NADP^+$ (Bennett *et al.*, 1997; PDB entry 1afs; Berman *et al.*, 2000).

3. Results and discussion

3.1. Protein sample preparation

A homogeneous enzyme preparation was purified using fast protein liquid chromatography (FPLC) (Fig. 1; Cantin *et al.*, in preparation). The procedure of Zhu *et al.* (1996, 1999) was used to saturate the enzyme with a high concentration of testosterone and $NADP^+$.

3.2. Crystal growth

The sparse-matrix screening method (Jancarik & Kim, 1991) was used for preliminary screening. When the ternary crystals obtained after three weeks were washed several times with the reservoir solution, dissolved and subjected to SDS–PAGE, a single band was observed indicating integrity of the protein (Fig. 1). Through preliminary screening, many small cubic shaped crystals were obtained in the ammonium acetate/sodium citrate/PEG 4K solution. Based on the above-described preliminary result, an additional refinement was carried out to increase the crystal volume and quality. The refinement included variation of the ammonium acetate concentration, pH, PEG and protein concentration and temperature. Two crystal forms have been obtained under similar conditions that varied only in the ammonium acetate concentration (Table 1). The higher quality form I crystals were obtained at 15 mg ml^{-1} in the presence of 0.38 *M* ammonium acetate, 26%(*w*/*v*) PEG 4K, 1.5% MPD (2-methyl-2,4-pentanediol) and 0.1 *M* sodium citrate pH 5.6. These elongated crystals appeared after 1–2 d at room temperature and grew to typical dimensions of 0.06 × 0.18 × 0.72 mm in three weeks (Fig. 2*a*). The ammonium acetate concentration for the form II crystals was 0.16 *M*. These cubic shaped crystals appeared after 1–2 d at room temperature and grew to typical dimensions of 0.07 × 0.30 × 0.30 mm in three weeks (Fig. 2*b*).

3.3. Data collection and molecular replacement (MR)

The data-collection statistics and unit-cell parameters are given in Table 1. Crystal form I was used for molecular-replacement

Figure 1
Coomassie-stained SDS–PAGE showing human 3α-hydroxysteroid dehydrogenase from purification or from dissolved crystals. Lane *M*, molecular-weight markers (from top): 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa; lane 1, purified human 3α-HSD; lane 2, protein sample from dissolved crystals of human 3α-HSD ternary complex.

calculations owing to its higher resolution. An initial attempt at finding an MR solution using *X-PLOR* with Patterson correlation refinement (Brünger, 1990) was unsuccessful. A sequential search was performed for the two subunits in the asymmetric unit using *EPMR* (Kissinger *et al.*, 1999). 5289 reflections in the resolution range 15–4 Å were used. Each evolutionary search was carried out over 50 generations using a population size of 300. In the first run, a solution was found for one of the molecules in the asymmetric unit with a correlation coefficient of 0.257 (R factor = 0.546). The partial structure contribution of the first solution was calculated and added to the structure-factor calculations during the search for the second subunit. A solution was found with a correlation coefficient for the combined solutions of 0.433 (R factor = 0.477).

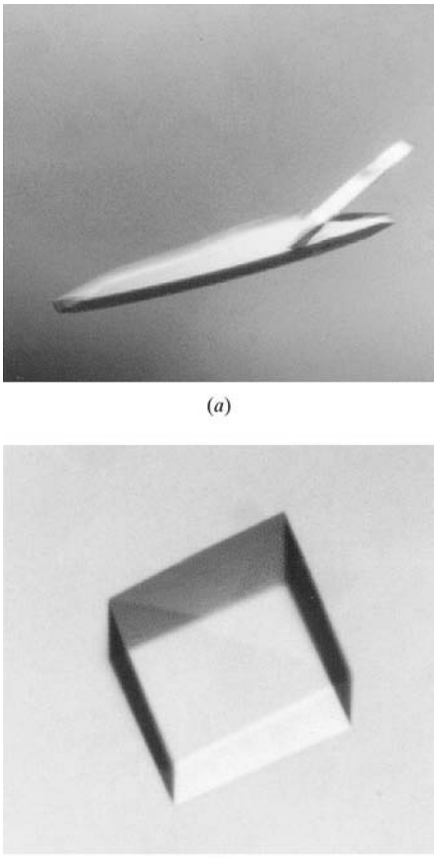

Figure 2
Two human 3α-HSD–NADP$^+$–testosterone ternary complex crystal forms. (*a*) Form I crystals belong to the $P2_1$ space group and diffract to 1.6 Å. (*b*) Form II crystals belong to the $R32$ space group and diffract to 2.6 Å.

The rat 3α-HSD was crystallized in the primitive orthorhombic space group $P2_12_12$, which is quite different from the two space groups studied here. Both the rat and human enzymes are monomers in solution with both substrate and cofactor buried deeply within the molecule. Crystal packing is not expected to have a direct effect on the conformation of the active site, as in both the rat and both human forms crystal packing contacts are far removed from the active site. The solution of the high-resolution ($P2_1$) structure is expected not only to reveal the exact geometry of the active site but also to provide clues to the structural basis for the multiple substrate specificity of 3α-HSD.

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