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Purification, crystallization and preliminary X-ray diffraction results of human 17β-hydroxysteroid dehydrogenase type 5

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17β-Hydroxysteroid dehydrogenases (17β-HSDs) catalyze the last step in the biosynthesis of all androgens and estrogens, thus playing a pivotal role in sex-hormone metabolism. Human 17β-HSD type 5 (17β-HSD5) catalyzes hydride transfer at the 17β-hydroxy position, but possesses high sequence homology to 3α-hydroxysteroid dehydrogenases (3α-HSD). Two crystal forms of 17β-HSD5 in an enzyme–testosterone–NADP ternary complex have been obtained under different crystallization conditions. A form I crystal obtained at pH 8.5 diffracted to 1.32 Å. It belonged to space group $P2_1$, with unit-cell parameters a = 47.41, b = 77.16, c = 48.67 Å, β = 116.32°. Form II crystals obtained at pH 6.5 diffracted to 2.0 Å and belonged to space group $P6_3$, with unit-cell parameters $a = b$ = 110.58, c = 56.89 Å.

1. Introduction

Members of the 17β-hydroxysteroid dehydrogenase family (17β-HSDs) catalyze hydride transfer between 17β-hydroxy- and 17-ketosteroid pairs in a positional and stereospecific manner, leading in the last step to the synthesis of active androgens and estrogens. Both androgens and estrogens possess their highest activity in the 17β-hydroxy form. These enzymes therefore play important roles in regulating the biological activity of sex hormones. Specifically, they convert the relatively inactive hormones E_1 (estrone), A-dione (androstenedione) and 5α-androstenedione to their potent forms E_2 (estradiol), T (testosterone) and DHT (5α-dihydrotestesterone), respectively (Geissler *et al.*, 1994; Luu-The *et al.*, 1995; Labrie *et al.*, 1996).

11 types of 17β-HSD have been reported from human, rat and mouse tissues (Dufort *et al.*, 1999; Fomitcheva *et al.*, 1998; Luu-The *et al.*, 1989; Nokelainen *et al.*, 1998; Peltoketo *et al.*, 1988; Sha *et al.*, 1997; Su *et al.*, 1999; Wu *et al.*, 1993). They share very low sequence identity (about 20%). The enzymes are dependent on nicotinamide adenine dinucleotide [NAD(H)] or its phosphorylated form [NADP(H)]. The different subtypes of 17β-HSD have their own substrate specificity toward estrogens or androgens.

Human 17β-HSD type 5 principally catalyzes the conversion of androst-4-ene-3,17-dione (4-dione) to T with NADPH as its cofactor and is considered to be an androgenic enzyme. The enzyme is implicated in the development of hormone-dependent prostate cancer (Labrie *et al.*, 1985, 2000). Therefore, the structure–function relationship study of 17β-HSD5 will provide us with a basis for understanding the enzyme mechanism and for designing inhibitors.

On the other hand, human 17β-HSD5 also possesses 3α-HSD activity (Dufort *et al.*, 1999), *e.g.* the conversion of DHT to 3α-diol (androstane-3α,17β-diol). It shares a high sequence identity with other 3α-HSDs as well as 20α-HSD. Sequence analysis shows that it belongs to the aldo–keto reductase family like other 3α-HSDs (Jez *et al.*, 1997), while most 17β-HSDs belong to the short-chain dehydrogenase/reductase family. Therefore, human 17β-HSD5 has also been named 3α-HSD2 or AKR1C3 (Khanna *et al.*, 1995; Lin *et al.*, 1997; Jez *et al.*, 1997). The structure–function relationship study of 17β-HSD5 will help us understand how the enzyme is regulated to switch between those activities. Here, we report the purification, crystallization and preliminary X-ray study of 17β-HSD5.

2. Materials and methods

The media glutathione Sepharose 4B, Blue Sepharose 6B and *Escherichia coli* strain BL21 were purchased from Amersham Pharmacia (QC, Canada). The chemicals, including K_2HPO_4, KH_2PO_4, NaCl, trizma base [tris-(hydroxymethyl)aminomethane], decyl maltoside, DTT (dithiothreitol), EDTA (ethylenediaminetetraacetate), MES [2(*N*-morpholino) ethanesulfonic acid], NADP (β-nicotinamide adenine dinucleotide), β-OG (β-octyl-glyco-

side) and PMSF (phenylmethanesulfonyl fluoride), were from Sigma (ON, Canada). PEGs (polyethylene glycol 4K and 8K) and matrix-screen kits were from Hampton Research Inc. (CA, USA). IPTG (isopropyl-β-D-thiogalactopyranoside) and thrombin were from ICN (QC, Canada). Centricon 30 was from Millipore (Canada), ^{14}C-4-dione was an NEN Life-Science product (MA, USA) and silica-gel TLC plates were from VWR Canlab (QC, Canada). Blue Sepharose chromatography was performed on an ÄKTA system (Amersham-Pharmacia). V_{max} data were quantified and analysed using a Storm860 analysis system and *ImageQuant* (Molecular Dynamics, Sunnyville, CA, USA). Buffer blue-A contains 20 m*M* Tris pH 7.5, 10% glycerol, 0.5 m*M* EDTA and 1 m*M* DTT; buffer blue-B contains 2 *M* NaCl in buffer blue-A. 17β-HSD5 stock solution contains 10 m*M* K_2HPO_4/KH_2PO_4 pH 7.0, 1 m*M* EDTA, 1 m*M* DTT, 0.05% decyl-maltoside, 1.2 m*M* NADP.

2.1. Protein expression

The *E. coli* cells expressing human 17β-HSD5 were kindly supplied by the group of Dr V. Luu-Thé. Details are given in Dufort *et al.* (1999).

To express the protein, a cell starter was grown to saturation in LB media by shaking at 310 K overnight. Next morning, 50 ml starting cells were added to 1 l LB media and shaken for 2–3 h until OD_{600nm} reached 0.8. The induction was started by adding IPTG to 50 μ*M* and incubation continued at 310 K for at least 3 h. The cells were then collected by centrifugation and resuspended in 20 ml buffer containing 40 m*M* Tris pH 7.5, 20% glycerol and 0.5 m*M* EDTA. They were stored at 193 K. Before purification, the cell stock was dissolved in 0.8% β-OG, 1 m*M* NADP, 1 m*M* DTT and 0.1 m*M* PMSF and then disrupted by five cycles of freezing (1 min in dry-ice cooled ethanol) and thawing (1 min in a water bath at 310 K) in aliquots of 0.5 ml. The supernatant was collected for protein purification after centrifugation.

2.2. Purification

The supernatant of the cell homogenate (from 800 ml of culture) was first applied to a 10 ml self-packed glutathione Sepharose column. The impurities were removed with six volumes of PBS. 17β-HSD5 eluted after overnight cleavage with 1200 units of thrombin at 277 K. The 17β-HSD5 eluate (approximately 20 mg of protein) was diluted with two volumes of buffer blue-A, applied onto a 24 ml Blue Sepharose 6B column and eluted with an NaCl gradient. The gradient consisted of three steps: (i) from buffer blue-A to 40% buffer blue-B in 48 ml, (ii) remaining at 40% buffer for 10 ml and (iii) reaching 100% buffer blue-B in 48 ml. 17β-HSD5 eluted at an NaCl concentration of approximately 0.8 *M*. A Centricon 30 was used for exchanging buffer from elution conditions to 17β-HSD5 stock solution (three cycles of 1:200 dilution) as well as for concentrating protein to 20–25 mg ml^{-1} for crystallization.

2.3. Activity assay

Assays were performed in a buffer containing 0.1 *M* Tris pH 7.5, 1 mg ml^{-1} BSA, 200 μ*M* cofactor NADPH and ^{14}C-4-dione substrate at a concentration in the range 2.5–60 μ*M*. At each different substrate concentration, reactions were carried out at 310 K for periods of 0, 1, 3 and 10 min. The reactions were initiated by adding purified 17β-HSD5 and stopped by extracting the steroids with 2 ml diethyl ether on ice. The amount of enzyme added to the reaction was adjusted to produce products below 10% of the input substrate. The steroids were then applied onto a thin-layer chromatographic plate and the substrates and products were separated by migrating in a mixture of 80% toluene and 20% acetone. The results were quantified by *ImageQuant* software on a Storm860 analysis system and treated according to the double-reciprocal curve.

2.4. Crystallization

Crystallization tests were performed by the hanging-drop vapour-diffusion method, starting with matrix-screening kits I and II. The hanging drops consist of 2 μl 20 mg ml^{-1} protein solution and the same volume of reservoir solutions. Two crystal forms, one cube-shaped and the other rod-shaped, were observed in different crystallization conditions. The reservoir for form I crystals contained 0.1 *M* Tris pH 8.5, 30% PEG 4K and 0.2 *M* $MgCl_2$. The reservoir of crystal form II contained 0.1 *M* sodium cacodylate pH 6.5, 20% PEG 8K and 0.2 *M* magnesium acetate. Testosterone was added to the drop to a final concentration of 0.5 m*M* in both conditions.

A complete data set from each crystal form has been collected at the X8C beamline at the National Synchrotron Light Source, Brookhaven National Laboratory, NY, USA. The diffraction images were collected with a Quantum-4 CCD detector. The wavelength was 0.9511 Å. The oscillation angle was 1.0°, the distance from the crystal to the detector was 100 and 150 mm for forms I and II, respectively, and the temperature was 100 K. Crystals were soaked briefly in mineral oil as a cryoprotectant.

3. Results and discussion

17β-HSD5 has been previously reported to be a highly labile protein; only 10% of the 17β activity can be recovered after homogenization (Dufort *et al.*, 1999). We have tested different methods of cell disruption. Using freeze–thaw disruption in the presence of 0.8% β-OG and cofactor considerably improved the recovery and specific activity of the protein.

17β-HSD5 purified by GST column showed a single band on SDS–PAGE, native gel and IEF gel. However, 17β-HSD5 could not be crystallized at this point. We tested the protein stability by verifying its activity and molecular mass over 10 d after purification. Both results showed that it degraded at room temperature in a few hours and in a few days at 277 K. For further purification, the protein was applied onto a Blue Sepharose 6B column; 17β-HSD5 eluted from this column was stable at room temperature for weeks (data not shown). The purified enzyme catalyzes the 17β reduction with a specific activity of 5.8 $\pm$ 0.2 nmol min^{-1} mg^{-1}. The stabilization of the active enzyme was critical for crystallization.

Two crystal forms have been obtained from different crystallization conditions. Form I crystals grown at pH 8.5 were cube shaped. A complete data set was collected at

Table 1
Data-processing statistics.

Values in parentheses refer to the highest resolution shell.

Crystal type	Form I	Form II
No. of images	180	75
Space group	$P2_1$	$P6_3$
Unit-cell parameters (Å, °)	a = 47.41, b = 77.16, c = 48.67, β = 116.32	a = b = 110.58, c = 56.89
Mosaicity (°)	0.49	0.50
Resolution range (Å)	50.0–1.32 (1.37–1.32)	50.0–2.0 (2.12–2.0)
No. of measured reflections	478255	207946
No. of unique reflections	73320	27053
Redundancy	3.4	3.9
R_{sym}† (%)	8.3 (55.9)	15.9 (52.8)
Completeness (%)	98.8 (99.5)	99.5 (98.5)
$\langle I/\sigma(I)\rangle$	13.5 (2.0)	9.1 (2.3)

† $R_{sym} = \sum|I - \sigma(I)|/\sum\sigma(I)$.

1.32 Å resolution. Based on systematic absences from the diffraction data, the crystal was determined to belong to space group $P2_1$, with unit-cell parameters $a = 47.41$, $b = 77.16$, $c = 48.47$ Å, $\beta = 116.32^\circ$ (Table 1). Form II crystals (pH 6.5) were rod-shaped. A complete data set was collected at 2.0 Å. These crystals belong to space group $P6_3$, with unit-cell parameters $a = b = 110.58$, $c = 56.89$ Å (Table 1). The assignments of these two space group were further supported by molecular-replacement results. Both data sets were obtained using flash-freezing with mineral oil as cryoprotectant.

Data were processed using the *DENZO* and *SCALEPACK* package (Otwinowski & Minor, 1997). The results are summarized in Table 1.

V_M for the form I crystal was calculated to be 2.2 $Å^3\ Da^{-1}$, with a solvent content of 45%. V_M for the form II crystal was calculated to be 2.1 $Å^3\ Da^{-1}$, with a solvent content of 42% (Matthews, 1968). In both cases, there is only one molecule in the asymmetric unit.

Structure determination of the 17β-HSD5 is under way using molecular replacement with rat 3α-HSD as the starting model (Bennett *et al.*, 1997). In the 17β-HSD family, structures of human 17β-HSD1 in complex with different cofactors and substrates have been solved (Ghosh *et al.*, 1995; Azzi *et al.*, 1996; Breton *et al.*, 1996) and functional analyses by mutagenesis and kinetic studies have been carried out on the basis of the structural knowledge (Han *et al.*, 2000; Huang *et al.*, 2001). The comparison of the structures of human 17β-HSD type 1 and type 5 will help us to better understand the mechanism of substrate recognition of the members of the 17β-HSD family and facilitate inhibitor design for the therapy of breast and prostate cancers (Labrie *et al.*, 2000).

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