

Expression, crystallization and preliminary X-ray analysis of human and rabbit 20α -hydroxysteroid dehydrogenases in complex with NADP(H) and various steroid substrates

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Progesterone plays an essential role in the maintenance of the pregnancy of most mammals. 20α -Hydroxysteroid dehydrogenase (20α -HSD) catalyses the inactivation of progesterone into its inactive form, 20α -hydroxyprogesterone, and could thus be involved in progesterone withdrawal and in the control of gestation. In this report, the purification and crystallization of recombinant human and rabbit 20α -HSDs (h 20α -HSD and rb 20α -HSD) are described, two highly homologous enzymes possessing, in addition to their common 20α -HSD activity, different activities and substrate specificities. Complete diffraction data sets have been collected for crystals of rb 20α -HSD in complex with NADP(H) and with either dihydrotestosterone (1.8 Å), progesterone (1.7 Å) or 4-androstenedione (1.8 Å). All these crystals belong to the monoclinic space group $P2_1$. A partial data set has also been collected for a crystal of h 20α -HSD ($P2_12_12_1$) in complex with NADP(H) and progesterone.

1. Introduction

Hydroxysteroid dehydrogenases (HSDs) play a major role in both the formation and inactivation of all five classes of mammalian steroid hormones. HSDs belong to two distinct protein superfamilies, namely the short-chain dehydrogenase/reductase (SDR; Persson *et al.*, 1991; Jornvall *et al.*, 1995) and the aldo-keto reductase (AKR; Bruce *et al.*, 1994) superfamilies. The AKR family includes all mammalian 3α -HSDs and 20α -HSDs characterized to date: human type 1 (Khanna *et al.*, 1995), human type 3 (Dufort *et al.*, 1996) and rat (Pawlowski *et al.*, 1991) 3α -HSDs, as well as human (Zhang *et al.*, 2000; Nishizawa *et al.*, 2000), rat (Mao *et al.*, 1994; Miura *et al.*, 1994), rabbit (Lacy *et al.*, 1993a,b) and bovine (Warren *et al.*, 1993) 20α -HSDs. It also includes human (Dufort *et al.*, 1999), mouse (Deyashiki *et al.*, 1995) and rat (Rheault *et al.*, 1999) type 5 17β -HSD. Several of these enzymes are multifunctional and are able to bind many different substrates with similar affinities. For example, the human type 3 3α -HSD (h 3α -HSD3; AKR1C2) possesses significant 20α - and 17β -HSD activities on progesterone (Prog) and androstenedione (4-dione), respectively, in addition to its high 3α -HSD activity on dihydrotestosterone (DHT; Dufort *et al.*, 1996). In contrast, human 20α -HSD (h 20α -HSD; AKR1C1) exerts a high 20α -HSD activity on Prog but negligible 3α -HSD and 17β -HSD activities (Zhang *et al.*, 2000), although it differs by only seven residues from

h 3α -HSD3 (Fig. 1). Mutagenesis of these residues indicates that the replacement of only one of them (Leu54 from the h 20α -HSD sequence by the less bulky Val as found in the h 3α -HSD3 sequence) is sufficient to give to 20α -HSD the substrate specificity and the activity of h 3α -HSD3 (Matsuura *et al.*, 1997). Interestingly, rabbit 20α -HSD (rb 20α -HSD; AKR1C5), which is also highly homologous to h 20α -HSD (Fig. 1), possesses strong intrinsic 3α -HSD and 17β -HSD activities despite the presence of a very large residue (Phe) at position 54 (Fig. 1). Therefore, the mechanisms by which HSDs are selectively active on one or a few steroid hormones are still not entirely understood.

20α -HSD activity has been found in a variety of tissues such as the thymus, ovary, testis, adrenal gland and placenta (Weinstein, 1977; Wiest, 1959; Matthijssen *et al.*, 1964; Armstrong & King, 1971; Pineda *et al.*, 1985; Nakajin *et al.*, 1989). In the placenta, where 17α -hydroxylase and 5α -reductase are absent, 20α -HSD is probably the only enzyme able to catalyze the inactivation of the progestins. Prog is essential for maintaining pregnancy and its catabolic transformation into 20α -hydroxyprogesterone (20α -OH-Prog) is associated with the termination of luteal and placental stage pregnancy (Armstrong & King, 1971). Indeed, it has been shown in the rat that 20α -HSD activity in the corpus luteum remains repressed throughout pregnancy and is induced 150-fold before parturition (Talwalker *et al.*, 1966; Strauss & Stambaugh, 1974). The

possibility exists that specific inhibitors of this enzyme may help maintain pregnancy before the luteal-placental shift in the steroidogenic and/or steroid-inactivating pathway(s).

To understand the differences between the 20 α -HSDs isolated from various species in their enzyme-substrate interactions and their functionality, crystallographic studies were initiated on human and rabbit enzymes in complex with a series of hormone substrates. The structural study of these enzymes will help us to understand their mode of action and to further investigate their exact role in hormone formation and inactivation. Here, we report the overproduction of the human and rabbit 20 α -HSDs in *E. coli* and their purification and crystallization in the presence of NADP(H) and various steroid substrates (Prog, DHT or 4-dione). Preliminary characterization of these different crystal complexes is also reported.

2. Materials and methods

2.1. Enzyme expression and purification

The DNA fragments containing the coding region of rb20 α HSD (Lacy *et al.*, 1993*a,b*) and h20 α -HSD (Zhang *et al.*, 2000) were introduced into pGEX-5X-1 and expressed as fusion proteins with glutathione-S transferase (GST). Fusion proteins were purified on glutathione Sepharose 4B and cleaved with Factor Xa (1 U per 100 μ g) for 4 d at 277 K. The digested proteins were then applied onto a DE52-cellulose column (Whatman) and the proteins passing through the column were collected. After reducing the volume to 1 ml using Centriprep YM-10 (Amicon), the eluted proteins were loaded onto Superdex 75 (Amersham Pharmacia Biotech) to obtain purified 20 α -HSDs. Fractions containing purified proteins were dialysed overnight against 10 mM Na₂PO₄ pH 7.0 containing 1 mM EDTA and 1 mM β -mercaptoethanol (buffer A), concentrated to about 14 mg ml⁻¹ by ultrafiltration (Centriprep YM-10, Amicon and Nanosep 10K, Pall Filtron Corporation) and stored at 277 K. Protein concentration was determined with the Bio-Rad Protein assay (Bio-Rad Laboratories) using BSA as a standard.

2.2. Assay of enzymatic activity

Determination of the enzymatic activities was performed using a procedure described previously (Luu-The *et al.*, 1995), with minor modifications. This procedure, which uses [¹⁴C]-labeled steroids, is much more sensi-

tive and accurate than the method used for other HSD studies, which is based upon the NADPH-NADP⁺ absorption difference at 340 nm (Lin *et al.*, 1992; Breton *et al.*, 1994). Briefly, the reactions were performed at 310 K with purified h20 α -HSD or rb20 α -HSD in a final volume of 1 ml containing 50 mM sodium phosphate pH 7.5, 20% glycerol, 1 mM EDTA, 1 mM NADPH and 0.1 μ M of the indicated [¹⁴C]-labeled steroid (Dupont Inc.). The steroids were dissolved in ethanol and added to the reaction buffer. Ethanol concentration was kept under 1% (v/v) to minimize its effect on protein stability. After various incubation times (0, 0.5, 1, 2 and 4 h), 200 μ l aliquots were taken and the steroids were extracted twice with 1 ml ether. The organic phases were pooled and evaporated to dryness. The steroids were solubilized in 50 μ l of dichloromethane and applied to Silica gel 60 thin-layer chromatography (TLC) plates (Merck) before separation by migration in a toluene-acetone (4:1) solvent system. Substrates and metabolites were identified by comparison with reference steroids,

revealed by autoradiography and quantified using the Storm 860 system (Molecular Dynamics).

2.3. Crystallization of the recombinant 20 α -HSDs and X-ray diffraction analysis

Prior to crystallization, a two- to threefold molar excess of cofactor (0.6 mM) and steroid substrate (0.6 mM) dissolved in ethanol was added to the enzyme sample (0.2 mM) and incubated for 16 h at 277 K. Ethanol concentration was kept under 1% (v/v) to minimize its effect on the protein. Crystals of the human and rabbit 20 α -HSDs were obtained by hanging-drop vapour diffusion. The SAMBA program (Audic *et al.*, 1997) was used to design balanced incomplete matrices of crystallization conditions (see Table 1). Crystals selected for cryocrystallographic experiments were incubated for a few days in crystallization buffer to which a cryoprotectant agent (12% ethylene glycol), the steroid, freshly dissolved in 100% ethylene glycol (10 μ M), and the cofactor had been

1	MDSKYQCVKL	NDGHFMPVLG	FGTYAPAEVP	KSKALEATKL	AIEAGFRHID	SAHLYNNEEQ	h20 α -HSD	
1	. . . P . F . R . A .	S I E M	I D Y F . K . . KE	rb20 α -HSD	
1 V H V	h3 α -HSD3	
1 P R E P R N R . V . V Y	h3 α -HSD1	
1 H P R V	h17 β -HSD5	
61	VGLAIRSKIA	DGSVKREDIF	YTSKLCWNSH	RPELVPALE	RSLKNLQLDY	VDLYLIHFPV	h20 α -HSD	
61 T T F S	D I T	rb20 α -HSD	
61 S	h3 α -HSD3	
61 T F F	Q . Q M . Q	S K L M	h3 α -HSD1	
61 S T F	N K A S M	h17 β -HSD5	
121	SVKPGEEVIP	KDENGKILFD	TVDLCAWTEA	VEKCKDAGLA	KSIGVSNFNR	RQLEMILNKP	h20 α -HSD	
121 A L V I	T H A I I	M	rb20 α -HSD	
121	M H L	h3 α -HSD3	
121 A L T P L V I S	V M C	h3 α -HSD1	
121 L L S	T V I	I T M	h17 β -HSD5	
181	GLKYKPCVNCQ	VECHPYFNQR	KLLDFCKSKD	IVLVAYSALG	SHREEPWVDP	NSPVLLEDPV	h20 α -HSD	
181 L . Q E	G P E Q S A L	rb20 α -HSD	
181	h3 α -HSD3	
181 L . S	H	T Q . H K L	h3 α -HSD1	
181 R S	Q . D K R	h17 β -HSD5	
241	LCALAKKHKR	TPALIALRYQ	LQGVVVLAK	SYNEQRIRQN	VQVFEPQLTS	EEMKAIDGLN	h20 α -HSD	
241 G Q Q I F T . K K E Q D V S	rb20 α -HSD
241	h3 α -HSD3	
241 Q E	I D V L	h3 α -HSD1
241 A D	h17 β -HSD5
301	RNVRYLTLDI	FAGPPNYPPS	DEY				h20 α -HSD	
301 F V	A D	rb20 α -HSD	
301	h3 α -HSD3	
301 Y V V M . F	h3 α -HSD1	
301 L H . F N S . S	h17 β -HSD5	

Figure 1

Alignment of the amino-acid sequences of some mammalian HSDs members of the AKR superfamily. Only amino acids differing from the human 20 α -HSD (h20 α -HSD) are shown. Percentage of identical amino acids compared with human 20 α -HSD are 80.8, 97.8, 82.7 and 87.9% for rabbit 20 α -HSD (rb20 α -HSD), human type 3 3 α -HSD (h3 α -HSD3), human type 1 3 α -HSD (h3 α -HSD1) and human type 5 17 β -HSD (h17 β -HSD5), respectively. Amino-acid sequences are given in the conventional single-letter code and are numbered on the left. Dots represent identical amino-acid residues.

added (see Table 2). Crystals were flash-cooled at 100 K in a nitrogen-gas stream. For the rb20 α -HSD crystals, X-ray diffraction oscillation images were collected on the F1 beamline at CHESS (Cornell High Energy Synchrotron Source), Ithaca, USA ($\lambda = 0.948 \text{ \AA}$) using an ADSC CCD detector with oscillations of 0.5 and 1 $^\circ$ for high- and low-resolution data, respectively. For the h20 α -HSD crystals, X-ray diffraction oscillation images were recorded on an R-AXIS IIC image-plate detector mounted on a Rigaku RU-200 rotating-anode generator. Data collections were analysed with the December 1999 version of the *XDS* software (Kabsch, 1993). Coordinates of the human type 3 3 α -HSD in complex with NADP $^+$ (Pierre Legrand *et al.*, unpublished results) were used as a search model in *AMoRe* (Navaza, 1994).

3. Results and discussion

3.1. Protein purification and preparation

Soluble active forms of human and rabbit 20 α -HSDs in fusion with glutathione S-transferase (GST-h20 α -HSD and GST-rb20 α -HSD) were highly expressed in *Escherichia coli* (Fig. 2; lane 1). When the bacteria were transferred at room temperature (about 293 K) after induction and then grown for a longer period of time (16 h), almost all the GST-h20 α -HSD and a large part of the GST-rb20 α -HSD fusion proteins were found in the soluble fraction obtained after the ultracentrifugation step (80 000g for 30 min) and thus were the major constituent of the total soluble protein (Fig. 2; lane 2). The fusion proteins were then purified by glutathione-Sepharose affinity chromatography and more than 25 mg of highly purified GST-20 α -HSDs (Fig. 2; lane 4) was obtained from 100 ml of bacterial culture. The factor Xa digested products (Fig. 2; lane 5) were applied onto an anionic exchange column to remove GST and the remaining uncleaved fusion protein. Finally, to obtain homogenous enzyme preparations, the purified 20 α -HSDs were subjected to size-exclusion chromatography. Typically, the complete purification procedure yielded 4–5 mg of highly purified 20 α -HSD (Fig. 2; lane 6) per 100 ml of bacterial culture.

3.2. Differential and specific activities of purified recombinant human and rabbit 20 α -HSDs

We characterized the purified 20 α -HSDs to determine if the recombinant enzymes expressed from *E. coli* cells retained the characteristic enzymatic properties of those

Table 1
Summary of the crystallization conditions.

	rb20 α -HSD	h20 α -HSD
Protein concentration (mg ml $^{-1}$)	14	14
Steroid	Prog, DHT or 4-dione	Prog
Cofactor	NADP $^+$	NADP $^+$
Crystallization temperature (K)	294	277
Composition of mother liquor	20–23% PEG 4000, 0.1 M HEPES pH 7.8–7.9, 0.2–0.4 M sodium acetate	25% PEG 4000, 0.1 M HEPES pH 7.5, 0.4 M sodium acetate, 0.01 M calcium chloride
Ratio of protein to mother liquor in the drop	2 μ l:1 μ l	1 μ l:1 μ l
Largest crystal size (mm)	0.2 \times 0.1 \times 1.0	0.45 \times 0.20 \times 0.05
Time to reach maximum size (months)	1	2–3

Table 2
Summary of data-collection statistics.

Data statistics for the last shell are given in parentheses.

	rb20 α -HSD			h20 α -HSD
	Prog + NADP $^+$	DHT + NADP $^+$	4-dione + NADP $^+$	Prog + NADP $^+$
Temperature (K)	100	100	100	297
Cryoprotecting agent	12% ethylene glycol	12% ethylene glycol	12% ethylene glycol	None
Space group	<i>P</i> 2 $_1$	<i>P</i> 2 $_1$	<i>P</i> 2 $_1$	<i>P</i> 2 $_1$ 2 $_1$ 2 $_1$
Unit-cell parameters				
<i>a</i> (\AA)	54.8	55.4	57.3	40.0
<i>b</i> (\AA)	84.2	84.4	84.7	84.5
<i>c</i> (\AA)	66.1	66.2	66.0	101.6
β ($^\circ$)	92.4	92.3	91.6	90
<i>Z</i> \dagger	2	2	2	2
Resolution range (\AA)	20.0–1.8 (1.9–1.8)	20.0–1.8 (1.9–1.8)	20.0–1.7 (1.73–1.7)	20.0–2.4 (2.49–2.4)
Estimated B_{overall} (\AA^2)	27.5	33.0	29.0	32.0
R_{merge} (%)	6.4 (23.5)	5.7 (19.1)	5.3 (29.6)	4.9 (11.7)
Completeness (%)	97.9 (94.2)	95.6 (91.2)	95.2 (92.0)	56.1 (54.2)
Redundancy	3.5	5.3	3.6	1.6

\dagger *Z* α is the number of molecules per asymmetric unit.

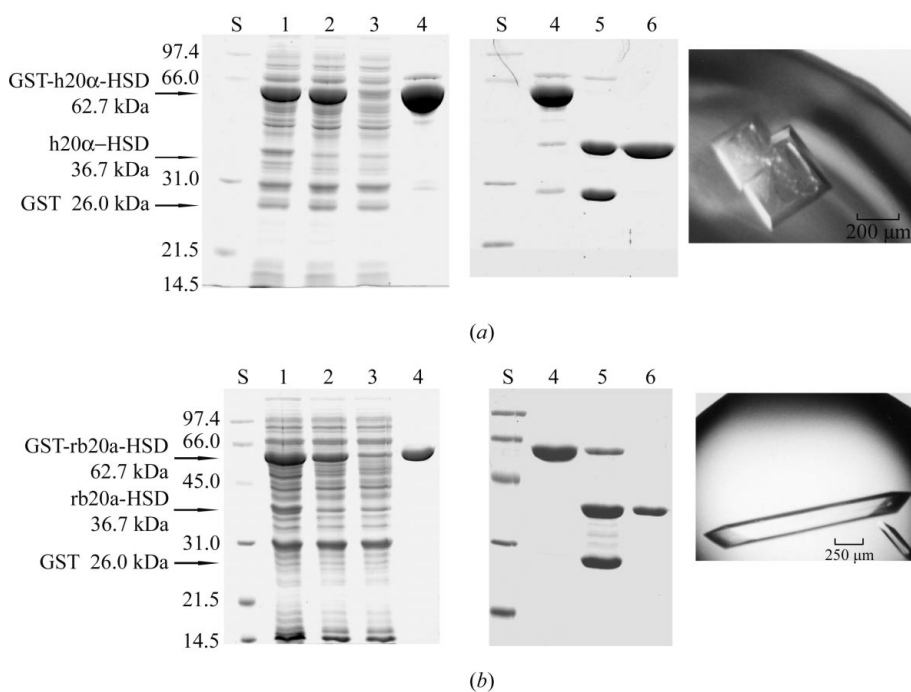


Figure 2
SDS-PAGE gels showing expression and purification of (a) human 20 α -HSD and (b) rabbit 20 α -HSD and photographs of their respective ternary complex crystals. Lane S, protein standards (the size of the protein standards is indicated on the left side of the gels); lane 1, total protein extract from cells expressing a GST fusion protein; lane 2, soluble proteins (after centrifugation for 30 min at 80 000g); lane 3, proteins unbound to the glutathione Sepharose 4B resin; lane 4, purified GST-20 α -HSD fusion protein; lane 5, GST-20 α -HSD cleaved with factor Xa; lane 6, purified 20 α -HSD. Photographs of typical crystals of h20 α -HSD or rb20 α -HSD in the presence of Prog and NADP $^+$ are presented on the right side of each panel.

expressed in intact stably transfected human embryonic kidney cells (HEK-293). In these cells, h20 α -HSD almost exclusively exerts a 20 α -HSD activity, catalyzing the reduction of Prog to 20 α -OH-Prog (Zhang *et al.*, 2000), whereas rb20 α -HSD, in addition to this 20 α -HSD activity, also possesses relatively high 3 α -HSD and 17 β -HSD activities that transform DHT into 5 α -androstane-3 α -17 β -diol (3 α -diol) and 4-dione into testosterone, respectively (V. Luu-The, unpublished results). As observed in HEK-293 cells stably transfected with 20 α -HSD, the predominant activity found for both enzymes is the 20 α -HSD activity, although a larger quantity of h20 α -HSD enzyme (about 4.7 times) is required to obtain a level of 20 α -HSD activity similar to that measured for the rb20 α -HSD (Fig. 3*a*). The relative activities for each enzyme are illustrated in Fig. 3*b*). For the recombinant rb20 α -HSD, strong 3 α - and 17 β -HSD activities corresponding to approximately 90 and 70% of the 20 α -HSD activity were found. As expected, only negligible 3 α -HSD activity was measured for h20 α -HSD, even though this enzyme is very homologous to the human type 3 3 α -HSD (Dufort *et al.*, 1996) which possesses comparable levels of 3 α -HSD and 20 α -HSD activities (Dufort *et al.*, 2001).

3.3. Crystallization of the recombinant 20 α -HSDs

After unsuccessful attempts to obtain crystals for both h20 α -HSD and rb20 α -HSD enzymes using the commercially available kits, we designed an incomplete matrix of conditions based on previously reported crystallization conditions for other members of the AKR superfamily (Bennet *et al.*, 1997; Ye *et al.*, 2000). The various sets of conditions for the screening matrix included varying pH values and various salts (ammonium, lithium and sodium), but most used PEG as precipitant. All crystallization trials were duplicated, being performed once at room temperature and once at 277 K. rb20 α -HSD crystals of good X-ray diffraction quality were finally obtained at both temperatures in the presence of PEG 4K, sodium acetate and HEPES buffer. h20 α -HSD crystals were only obtained at 277 K in the same crystallization buffer with the addition of calcium chloride (Table 1).

3.4. X-ray diffraction analysis

The data-collection statistics and unit-cell parameters are summarized in Table 2. All crystals of rb20 α -HSD complexes belong to the same space group (monoclinic $P2_1$). In

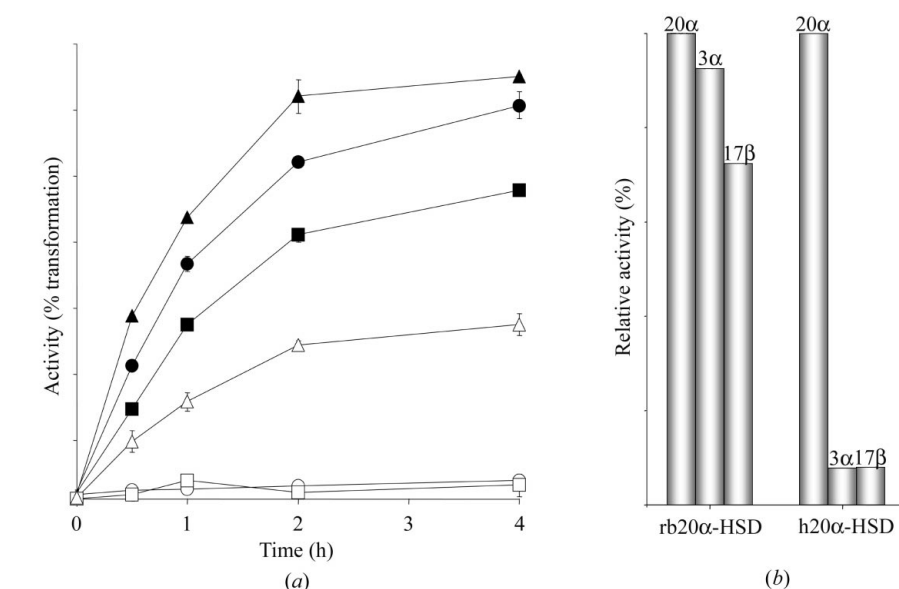


Figure 3

Comparison of the relative 20 α -HSD, 3 α -HSD and 17 β -HSD activities catalyzed by purified recombinant human and rabbit 20 α -HSDs. Purified recombinant h20 α -HSD and rb20 α -HSD were tested for their ability to transform Prog, DHT and 4-dione. Experimental conditions are described in §2. (a) We first determined the quantity of enzyme producing a maximum of 30% transformation after 4 h of incubation (h20 α -HSD, 0.6 μ g; rb20 α -HSD, 0.3 μ g). This amount of enzyme was then added to each reaction medium containing the [14 C]-labeled steroid and the reaction was followed for 4 h [h20 α -HSD: Prog (triangles), DHT (circles) or 4-dione (squares); rb20 α -HSD: Prog (filled triangles), DHT (filled circles) or 4-dione (filled squares)]. The data shown are means of duplicate measurements. (b) Relative 20 α -HSD, 3 α -HSD and 17 β -HSD activities catalysed by the rabbit and human 20 α -HSDs. Relative activities are calculated from the percentage of transformation of each substrate measured after 4 h of incubation with each enzyme.

contrast, the h20 α -HSD crystal belongs to the orthorhombic space group $P2_12_12_1$. At room temperature, the rb20 α -HSD crystals diffract to more than 2.4 \AA resolution but only for a short period of time (data not shown). The lifetime of these crystals was greatly improved when cryogenic conditions were used and near-complete diffraction data were collected at 100 K to 1.8 \AA resolution. Clear molecular-replacement solutions (correlation factor of 63.6% using diffraction data in the range 15.0–3.0 \AA) were found for rb20 α -HSD using the coordinates of h3 α -HSD3 in complex with NADP $^+$ (Pierre Legrand *et al.*, unpublished results) as a search model. For h20 α -HSD, the data completeness was not sufficient to proceed to molecular replacement.

In conclusion, recombinant human and rabbit 20 α -HSDs, which showed identical characteristics to those expressed in human HEK-293 cells in terms of substrate specificity and catalytic activities, have been expressed and purified from *E. coli*. Crystals of both proteins obtained in the presence of their preferred cofactor and steroid hormone substrate(s) have been analysed. This work will allow us to determine the crystallographic structure of these enzymes, the first examples for the 20 α -HSD enzyme family. Such studies will also provide the information needed to understand in detail

the differences in substrate specificity and catalytic activity between these two 20 α -HSDs and others of the aldo-keto reductase family such as human type 1 and type 3 3 α -HSD or type 5 17 β -HSD. Comparative study of the high-resolution structures of 20 α -HSDs and h3 α -HSD3 (Nahoum *et al.*, 2001) will allow us to determine the conformational difference between their active sites and to clarify the mechanisms by which these enzymes are selectively active on one or a few steroid hormones.

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