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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 (h20α-HSD and rb20α-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 rb20α-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 h20α-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.*, 1993*a*,*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 (h3α-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 (h20α-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 h3α-HSD3 (Fig. 1). Mutagenesis of these residues indicates that the replacement of only one of them (Leu54 from the h20α-HSD sequence by the less bulky Val as found in the h3α-HSD3 sequence) is sufficient to give to 20α-HSD the substrate specificity and the activity of h3α-HSD3 (Matsuura *et al.*, 1997). Interestingly, rabbit 20α-HSD (rb20α-HSD; AKR1C5), which is also highly homologous to h20α-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 20α-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 20α-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 rb20αHSD (Lacy *et al.*, 1993*a*,*b*) and h20α-HSD (Zhang *et al.*, 2000) were introduced into pGEX-5X-1 and expressed as fusion proteins with glutathione-S tranferase (GST). Fusion proteins were purified on glutathione Sepharose 4B and cleaved with Factor Xa (1 U per 100 µg) 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 Phamacia Biotech) to obtain purified 20α-HSDs. Fractions containing purified proteins were dialysed overnight against 10 m*M* Na_2PO_4 pH 7.0 containing 1 m*M* EDTA and 1 m*M* 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 [^{14}C]-labeled steroids, is much more sensitive 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 h20α-HSD or rb20α-HSD in a final volume of 1 ml containing 50 m*M* sodium phosphate pH 7.5, 20% glycerol, 1 m*M* EDTA, 1 m*M* NADPH and 0.1 µ*M* of the indicated [^{14}C]-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 µl 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 µl 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 20α-HSDs and X-ray diffraction analysis

Prior to crystallization, a two- to threefold molar excess of cofactor (0.6 m*M*) and steroid substrate (0.6 m*M*) dissolved in ethanol was added to the enzyme sample (0.2 m*M*) 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 20α-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

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  1 MDSKYQCVKL NDGHFMPVLG FGTYAPAEVP KSKALEATKL AIEAGFRHID SAHLYNNEEQ    h20α-HSD
  1 ..P.F.R.A. S....I.... ......E... ....M....I ..D....... ..YF.K..KE    rb20α-HSD
  1 .......... .......... .......V.. ......H... ...V......              h3α-HSD3
  1 ..P...R.E. .......... ......P... RNR.V.V... .......... ..Y.......    h3α-HSD1
  1 ....H..... .......... ......P... R.....V... .......... ..........    h17β-HSD5

 61 VGLAIRSKIA DGSVKREDIF YTSKLWCNSH RPELVRPALE RSLKNLQLDY VDLYLIHFPV    h20α-HSD
 61 .......... ..T....... .......TF. .......S.. D......... ....I....T    rb20α-HSD
 61 .......... .......... ......S... .......... .......... ..........    h3α-HSD3
 61 .......... .......... .......TFF Q.QM.Q.... S...K..... .....L...M    h3α-HSD1
 61 .......... .......... ......STF. .......... N...KA.... .......S.M    h17β-HSD5

121 SVKPGEEVIP KDENGKILFD TVDLCATWEA VEKCKDAGLA KSIGVSNFNR RQLEMILNKP    h20α-HSD
121 AL...V.I.. T..H..AI.. ...I...... M......... .......... ..........    rb20α-HSD
121 .......... .......... .......... M......... .........H .L........    h3α-HSD3
121 AL....TPL. ......VI.. ....S....V M......... .........C ..........    h3α-HSD1
121 .L.....LS. T.....VI.. I....T.... M......... .......... ..........    h17β-HSD5

181 GLKYKPVCNQ VECHPYFNQR KLLDFCKSKD IVLVAYSALG SHREEPWVDP NSPVLLEDPV    h20α-HSD
181 .......... ......L.Q. ...E.....G .......... ....PE...Q SA.......L    rb20α-HSD
181 .......... .......... .......... .......... .......... ..........    h3α-HSD3
181 .......... ......L..S .......... .....H.... TQ.HKL.... ..........    h3α-HSD1
181 .......... ........RS .......... .......... .Q.DKR.... ..........    h17β-HSD5

241 LCALAKKHKR TPALIALRYQ LQRGVVVLAK SYNEQRIRQN VQVFEFQLTS EEMKAIDGLN    h20α-HSD
241 .G......QQ .......... ....I..... .FT.K..KE. .Q........ .D..V..S..    rb20α-HSD
241 .......... .......... .......... .......... .......... ..........    h3α-HSD3
241 .........Q .......... .......... ........E. I......... .D..VL....    h3α-HSD1
241 .......... .......... .......... .......... .........A .D.......D    h17β-HSD5

301 RNVRYLTLDI FAGPPNYPFS DEY                                            h20α-HSD
301 ..F..V.AD. AI.H...... ...                                            rb20α-HSD
301 .......... .......... ...                                            h3α-HSD3
301 ..Y..VVM.F LMDH.D.... ...                                            h3α-HSD1
301 ..LH.FNS.S ..SH....Y. ...                                            h17β-HSD5
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Figure 1
Alignment of the amino-acid sequences of some mammalian HSDs members of the AKR superfamily. Only amino acids differing from the human 20α-HSD (h20α-HSD) are shown. Percentage of identical amino acids compared with human 20α-HSD are 80.8, 97.8, 82.7 and 87.9% for rabbit 20α-HSD (rb20α-HSD), human type 3 3α-HSD (h3α-HSD3), human type 1 3α-HSD (h3α-HSD1) and human type 5 17β-HSD (h17β-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 rb20α-HSD crystals, X-ray diffraction oscillation images were collected on the F1 beamline at CHESS (Cornell High Energy Synchrotron Source), Ithaca, USA (λ = 0.948 Å) using an ADSC CCD detector with oscillations of 0.5 and 1° for high- and low-resolution data, respectively. For the h20α-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 3α-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 20α-HSDs in fusion with glutathione S-transferase (GST-h20α-HSD and GST-rb20α-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-h20α-HSD and a large part of the GST-rb20α-HSD fusion proteins were found in the soluble fraction obtained after the ultracentrifugation step (80 000*g* 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 gluthathione-Sepharose affinity chromatography and more than 25 mg of highly purified GST-20α-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 20α-HSDs were subjected to size-exclusion chromatography. Typically, the complete purification procedure yielded 4–5 mg of highly purified 20α-HSD (Fig. 2; lane 6) per 100 ml of bacterial culture.

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

We characterized the purified 20α-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.

	rb20α-HSD	h20α-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 µl:1 µl	1 µl:1 µl
Largest crystal size (mm)	0.2 × 0.1 × 1.0	0.45 × 0.20 × 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.

	rb20α-HSD			h20α-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	$P2_1$	$P2_1$	$P2_1$	$P2_12_12_1$
Unit-cell parameters				
a (Å)	54.8	55.4	57.3	40.0
b (Å)	84.2	84.4	84.7	84.5
c (Å)	66.1	66.2	66.0	101.6
β (°)	92.4	92.3	91.6	90
Za†	2	2	2	2
Resolution range (Å)	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 $B_{overall}$ (Å^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

† *Za* is the number of molecules per asymmetric unit.

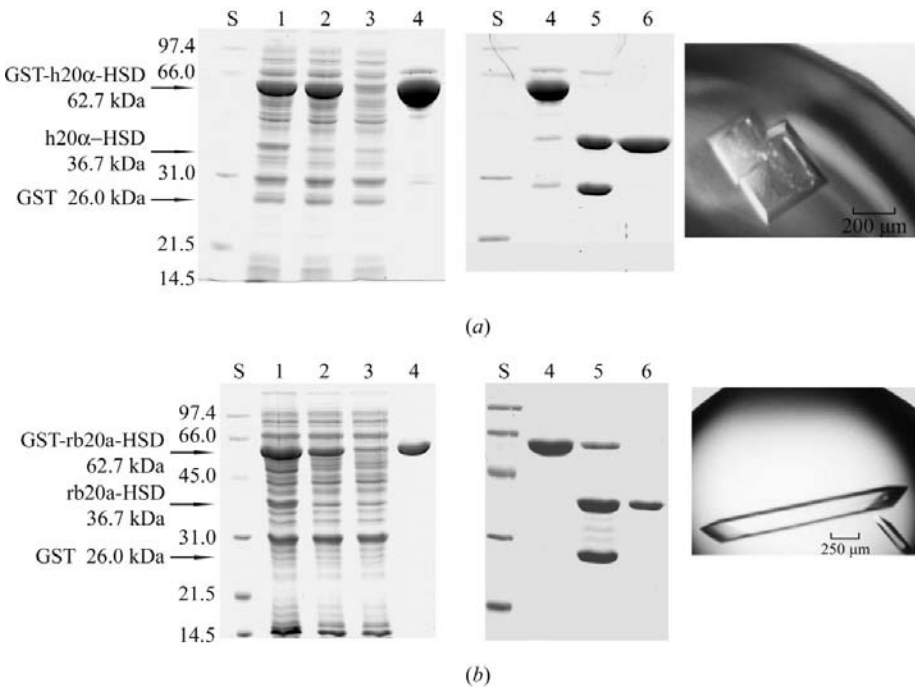

Figure 2
SDS–PAGE gels showing expression and purification of (*a*) human 20α-HSD and (*b*) rabbit 20α-HSD and photographs of their respective ternary complex crystals. Lane S, protein standards (the size of the protein standards is indicated on the left side of the gels); lane 1, total protein extract from cells expressing a GST fusion protein; lane 2, soluble proteins (after centrifugation for 30 min at 80 000*g*); lane 3, proteins unbound to the glutathione Sepharose 4B resin; lane 4, purified GST-20α-HSD fusion protein; lane 5, GST-20α-HSD cleaved with factor Xa; lane 6, purified 20α-HSD. Photographs of typical crystals of h20α-HSD or rb20α-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, h20α-HSD almost exclusively exerts a 20α-HSD activity, catalyzing the reduction of Prog to 20α-OH-Prog (Zhang *et al.*, 2000), whereas rb20α-HSD, in addition to this 20α-HSD activity, also possesses relatively high 3α-HSD and 17β-HSD activities that transform DHT into 5α-androstane-3α-17β-diol (3α-diol) and 4-dione into testosterone, respectively (V. Luu-The, unpublished results). As observed in HEK-293 cells stably transfected with 20α-HSD, the predominant activity found for both enzymes is the 20α-HSD activity, although a larger quantity of h20α-HSD enzyme (about 4.7 times) is required to obtain a level of 20α-HSD activity similar to that measured for the rb20α-HSD (Fig. 3*a*). The relative activities for each enzyme are illustrated in Fig. 3(*b*). For the recombinant rb20α-HSD, strong 3α- and 17β-HSD activities corresponding to approximately 90 and 70% of the 20α-HSD activity were found. As expected, only negligible 3α-HSD activity was measured for h20α-HSD, even though this enzyme is very homologous to the human type 3 3α-HSD (Dufort *et al.*, 1996) which possesses comparable levels of 3α-HSD and 20α-HSD activities (Dufort *et al.*, 2001).

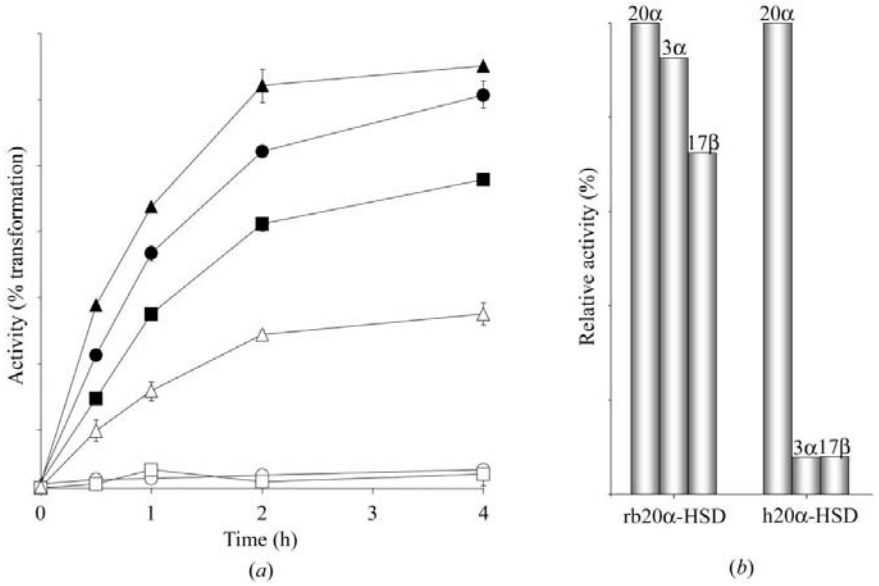

Figure 3
Comparison of the relative 20α-HSD, 3α-HSD and 17β-HSD activities catalyzed by purified recombinant human and rabbit 20α-HSDs. Purified recombinant h20α-HSD and rb20α-HSD were tested for their ability to transform Prog, DHT and 4-dione. Experimental conditions are described in §2. (*a*) We first determined the quantity of enzyme producing a maximum of 30% transformation after 4 h of incubation (h20α-HSD, 0.6 µg; rb20α-HSD, 0.3 µg). 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 [h20α-HSD: Prog (triangles), DHT (circles) or 4-dione (squares); rb20α-HSD: Prog (filled triangles), DHT (filled circles) or 4-dione (filled squares)]. The data shown are means of duplicate measurements. (*b*) Relative 20α-HSD, 3α-HSD and 17β-HSD activities catalysed by the rabbit and human 20α-HSDs. Relative activities are calculated from the percentage of transformation of each substrate measured after 4 h of incubation with each enzyme.

3.3. Crystallization of the recombinant 20α-HSDs

After unsuccessful attempts to obtain crystals for both h20α-HSD and rb20α-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. rb20α-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. h20α-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 rb20α-HSD complexes belong to the same space group (monoclinic $P2_1$). In contrast, the h20α-HSD crystal belongs to the orthorhombic space group $P2_12_12_1$. At room temperature, the rb20α-HSD crystals diffract to more than 2.4 Å 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 Å resolution. Clear molecular-replacement solutions (correlation factor of 63.6% using diffraction data in the range 15.0–3.0 Å) were found for rb20α-HSD using the coordinates of h3α-HSD3 in complex with NADP$^+$ (Pierre Legrand *et al.*, unpublished results) as a search model. For h20α-HSD, the data completeness was not sufficient to proceed to molecular replacement.

In conclusion, recombinant human and rabbit 20α-HSDs, which showed identical characterictics 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 20α-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 20α-HSDs and others of the aldo–keto reductase family such as human type 1 and type 3 3α-HSD or type 5 17β-HSD. Comparative study of the high-resolution structures of 20α-HSDs and h3α-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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