A novel 17β-hydroxysteroid dehydrogenase in the fungus *Cochliobolus lunatus*: new insights into the evolution of steroid-hormone signalling

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17β-Hydroxysteroid dehydrogenase (17β-HSD) from the filamentous fungus *Cochliobolus lunatus* (17β-HSDcl) catalyses the reduction of steroids and of several *o*- and *p*-quinones. After purification of the enzyme, its partial amino acid sequence was determined. A PCR fragment amplified with primers derived from peptide sequences was generated for screening the *Coch. lunatus* cDNA library. Three independent full-length cDNA clones were isolated and sequenced, revealing an 810-bp open reading frame encoding a 270-amino-acid protein. After expression in *Escherichia coli* and purification to homogeneity, the enzyme was found to be active towards androstenedione and menadione, and was able to form dimers of M_r 60000. The amino acid sequence of the novel 17β-HSD demonstrated high homology with fungal carbonyl reductases, such as versicolorin reductase from *Emericella nidulans* (*Aspergillus nidulans*; VerA) and *Asp. parasiticus* (Ver1), polyhydroxynaphthalene reductase from *Magnaporthe grisea*, the product of the *Brn1* gene from *Coch. heterostrophus* and a reductase from *Colletotrichum lagenarium*, which are all members of the short-chain dehydrogenase/reductase superfamily. 17β-HSDcl is the first discovered fungal 17β-hydroxysteroid dehydrogenase belonging to this family. The primary structure of this enzyme may therefore help to elucidate the evolutionary history of steroid dehydrogenases.

Key words: carbonyl reductase, *Cochliobolus lunatus*, evolution, fungi, 17β-hydroxysteroid dehydrogenase.

INTRODUCTION

17β-Hydroxysteroid dehydrogenases (17β-HSDs) are involved in the biosynthesis and modulation of the biological action of steroid hormones in steroidogenic [1,2], as well as in peripheral, tissues [3–5]. Several mammalian 17β-HSDs from different species have been identified and cloned ([6–14], and references therein). These enzymes belong to two protein families, the short-chain dehydrogenase/reductase (SDR) and the aldo–keto reductase family [14]. To date, eight different types of 17β-HSD have been found and seven of them belong to the SDR family [14–16], whose members catalyse diverse biochemical reactions [17]. Most of the proteins are 25–28 kDa non-metalloenzymes, and function as multimers, usually dimers [14]. They share approx. 25% of overall sequence identity [18,19]. Despite the low similarity, six typical protein domains and the typical SDR motifs, i.e. the coenzyme-binding site, Gly-Xaa-Xaa-Xaa-Gly-Xaa-Gly (where Xaa denotes 'any amino acid'), in the N-terminal region (domain A) and the catalytic site, Tyr-Xaa-Xaa-Xaa-Lys (domain D), are conserved among its members [18,19]. For certain of the SDR members, e.g. 3α,20β-HSD from *Streptomyces hydrogenans* [20,21], mammalian 17β-HSD type 1 [22,23], dihydropteridine reductase [24], carbonyl reductase [25] and 7α-HSD from *Escherichia coli* [26], the three-dimensional structures have been determined. It has been shown that protein folding is highly conserved despite a low sequence identity [14].

The SDR family is expanding very quickly [17,27]. While the bacterial members 3α,17β-HSD from *Comamonas testosteroni* [28,29] and 3α,20β-HSD from *Streptomyces hydrogenans* [21,30] are very well characterized, so far no fungal HSD has been identified within this family. Since the members of the SDR family are thought to share a common ancestor [31], our interest was to obtain further data about the evolution of these enzymes, especially of fungal HSDs. In fungi, 17β-HSD activity was first detected in the filamentous fungi *Cochliobolus lunatus* [32] and *Cylindrocarpon radicicola* [33] and, later, in further fungal classes [34]. The enzymes from *Coch. lunatus* and *Cylind. radicicola* have been purified and characterized [33,35]. They have quite different characteristics, since the enzyme from *Coch. lunatus* favours reduction (e.g. of 4-androstene-3,17-dione) using NADPH [35], whereas the latter preferentially catalyses oxidative reactions using NAD^+ [33]. The 17β-HSD from *Coch. lunatus* has additional substrate specificity towards non-steroidal substrates, such as *o*- and *p*-quinones, e.g. menadione [36]. Such a property has not been observed until now in fungi, although for mammalian and bacterial HSDs the ability to convert carbonyl compounds, in addition to steroids, into their respective products (see the Materials and methods section) has been reported [37,38]. *Coch. lunatus* 17β-HSD (17β-HSDcl) is thus the first known fungal pluripotent HSD. Since this fungus performs the biosynthesis of mammalian-like steroid hormones [39] and also contains steroid-binding proteins [40], this 17β-HSD is of especial importance in studying the evolution of HSDs and the steroid-hormone signalling system.

This paper describes the cDNA isolation, analysis of the amino acid sequence and bacterial expression of a novel 17β-HSD from *Coch. lunatus*.

Abbreviations used: GST, glutathione S-transferase; HSD, hydroxysteroid dehydrogenase; 17β-HSDcl, 17β-HSD from *Cochliobolus lunatus*; SDR, short-chain dehydrogenase/reductase; TFA, trifluoroacetic acid.

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The cDNA sequence of 17β-HSDcl has been deposited in the GenBank database under the accession number AF069518.

MATERIALS AND METHODS

Fungal species

Coch. lunatus m118 from the Strain Collection of the Friedrich Schiller University of Jena was grown as described previously [32].

Peptide sequencing

17β-HSDcl was purified to homogeneity as described previously [35]. For N-terminal sequencing, the purified protein (30 μg) was further separated from minor contaminants with SDS/PAGE and transferred to a PVDF membrane using a semi-dry transfer unit (Semi-Phor Te 70 Hoefer Scientifical, San Francisco, CA, U.S.A.) as described previously [41]. The transferred proteins were stained by Coomassie Blue and the band of interest was excised. The N-terminal sequence was determined on an Applied Biosystems apparatus 140 A by on-line analysis of phenylthiohydantoin-modified amino acids. For peptide sequencing, minor contaminants were removed from the purified 17β-HSDcl (50 μg) by HPLC (Kontron, Neufahrn, Germany) using a C4 Nucleosil 300 column (100 mm $\times$ 2.1 mm) with the solvents A [0.1 % trifluoroacetic acid (TFA) in water] and B (0.08 % TFA, 60 % CH_3CN, 20 % propan-2-ol in water). A linear gradient with an A:B ratio of 99:1 to 1:99 was performed at a flow rate of 0.1 ml/min and the A_{215} was measured. The fraction containing pure 17β-HSDcl was concentrated in a Speed Vac apparatus or by Filtron concentrators (Pall, Biosupport Division, Dreieich, Germany). The digestion of the protein was performed overnight at room temperature using endoproteinases Lys-C (from Wako, Tokyo, Japan) and Glu-C (sequencing grade; Boehringer Mannheim, Mannheim, Germany). The ratio of protein to protease was 1:20. With Glu-C, the digestion was performed in 100 mM Tris/HCl buffer, pH 8.2, with Lys-C in 100 mM Tris/HCl buffer, pH 9.0, containing 1 mM EDTA. After digestion, the peptides were separated by HPLC (Kontron) using an Aquaphore Phenyl PH300 column (300 mm $\times$ 2.1 mm; Brownlee ABI, Weiterstadt, Germany) and the solvents A and B described above. The following gradient was used: from 1–10 % B in 1 min, from 10–20 % B in 5 min, from 20–50 % B in 60 min, from 50–80 % B in 15 min and from 80–95 % B in 5 min at a flow rate of 0.1 ml/min [41]. The samples were immediately frozen and stored at -80 °C. The single peptides were applied to Polybrene coated-glass-fibre filters and sequenced on an ABI 140A sequencer, as described above.

RNA isolation and cDNA synthesis

The fungus was grown for 42 h as described previously [35], filtered, washed, frozen in liquid nitrogen and ground in a mortar. Total RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform method [42]. The mRNA was separated from total RNA by Dynabeads Oligo$(dT)_{25}$ (Dynal, Oslo, Norway) using the magnetic-separation technique. The mRNA (5 μg) was transcribed to cDNA with the Moloney-murine-leukaemia-virus reverse transcriptase (Stratagene, Heidelberg, Germany).

PCR amplification

Degenerate primers were designed on the basis of the partial amino acid sequence and used for DNA amplification. PCR reactions contained about 20 ng of DNA template, 75 pmol of degenerate forward and reverse primers, 0.2 mM of each dNTP, the reaction buffer [20 mM Tris/HCl (pH 8.4)/50 mM KCl/1.5 mM $MgCl_2$] and 1 unit of *Taq* polymerase (Gibco BRL, Cergy-Pontoise, France). The reactions were performed in a thermal cycler (Perkin–Elmer Gene Amp 2400; Courtaboeuf, France) in 20-μl volumes. The mixture was denatured at 94 °C for 5 min, after which thirty cycles were performed as follows: 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s. The PCR products were confirmed on a 1 % (w/v) agarose gel and cloned into the vector pCR2.1 (TA Cloning Kit, InVitrogen, Leek, The Netherlands).

The PCR reactions for the amplification of the screening probe were carried out with specific oligonucleotide primers. The conditions were the same as those described above, except that the concentration of each of the primers was 15 pmol/20 μl reaction volume.

Construction and screening of a cDNA library

The *Coch. lunatus* cDNA library was constructed in a λUNI-ZAP XR vector (ZAP cDNA Synthesis Kit, Stratagene) according to the manufacturer's recommendations. It was packaged using the ZAP-cDNA Gigapack Gold III extract and titred in the XL1-Blue MRF′ bacterial strain (Stratagene). A library of 6×10^9 plaque-forming units/ml was obtained after the first amplification; 6×10^5 plaque-forming units of recombinant phage were screened with a probe of 531 bp. The probe was labelled with [^{32}P]dCTP using magenta polymerase and random primers (Random Primer Labeling Kit, Stratagene). Positive clones were plaque-purified. An *in vivo* excision of the pBluescript plasmid with the full-length cDNA insert was done according to the Stratagene protocols using the ExAssist helper phage (Stratagene).

Plasmid inserts were sequenced using T3 and T7 primers. Similarity searches were performed with BLAST [43] or FASTA [44], and phylogenetic analyses with ClustalW [45] and PHYLIP packages (J. Felsenstein, Department of Genetics, University of Washington, WA, U.S.A.).

Protein expression and purification

The DNA coding sequence of 17β-HSDcl was amplified by PCR using the cDNA as template and oligonucleotide primers that added *Bam*HI and *Hin*dIII restriction sites at the 5′- and 3′-ends respectively. The primers were as follows: forward, 5′-TTT TGG ATC CAT GCC ACA CGT AGA GAA CGC ATC CGA G-3′; and reverse, 5′- AAA AAA GCT TTT ATG CGG CAC CAC CAT CTA GAG TGA GAA C-3′. The PCR product was digested with *Bam*HI and *Hin*dIII and cloned into the pGex vector (Pharmacia, Orsay, France). The pGex-17β-HSDcl vector was transferred into the *E. coli* strain JM107. Cells were then grown in Luria–Bertani medium containing 50 μg/ml ampicilin at 37 °C in a rotary shaker until D_{600} reached 1.2. Expression was induced by isopropyl β-D-thiogalactoside at a final concentration of 0.5 mM and the incubation was continued for 4 h. Preparation of cell extracts, purification of glutathione S-transferase (GST)-fusion protein by affinity binding to glutathione–Sepharose, cleavage with thrombin and determination of protein expression and purification by SDS/PAGE were performed as described previously [46].

Enzyme activity measurement

17β-HSD activity of the expressed enzyme was measured with 15 μg of protein and 120 pmol of 4-androstene-3,17-dione (Amersham International, Braunschweig, Germany) in 100 mM phosphate buffer, pH 8.0, with 0.1 mM NADPH as the cofactor. Separation of product (testosterone) from substrate (4-androstene-3,17-dione) was performed on a reverse-phase C_{18}

HPLC column, as described previously [10]. The conversion of the quinone menadione (Sigma, Deisenhofen, Germany) into menadiol was measured with 30 μg of enzyme in 50 mM Tris/HCl, pH 7.0, 20% (v/v) glycerol and 0.1 mM NADPH. The reaction was monitored by the decrease of the NADPH absorption at 340 nm, as described previously [35].

Protein determination

Protein concentration was determined by using the bicinchoninic acid method with BSA as the standard, as described previously [35].

Estimation of molecular mass

The molecular mass of the native recombinant 17β-HSDcl protein (after thrombin cleavage) was estimated by gel filtration on a TSH G4000 SWxl column (TosoHaas, Zettachring, Germany) fitted to a BioSprint HPLC apparatus (PerSeptive, Wiesbaden-Nordenstadt, Germany). 300 μg of the protein (100 μl) was analysed at a flow rate of 1.0 ml/min in 100 mM NaH_2PO_4/150 mM NaCl, pH 7.0. A_{280} was monitored. Fractions (250 μl) were collected for the measurements of enzymic activity with 4-androstene-3,17-dione. The gel-filtration column of 7.8 mm internal diameter and 300 mm length was calibrated using carbonic anhydrase (29 kDa), alcohol dehydrogenase (150 kDa) and BSA (66 kDa; Sigma).

RESULTS AND DISCUSSION

Partial amino acid sequence and PCR amplification of fragments of 17β-HSDcl cDNA

Purified 17β-HSDcl was subjected to amino acid sequencing of the N-terminus and the proteolytic fragments. Non-overlapping peptides (170 residues in length) analysed by BLAST similarity searches [43], revealed that the novel protein belongs to the SDR superfamily. Because of the high similarities to fungal keto reductase from *Aspergillus parasiticus* [47] and the tetrahydroxynaphthalene reductase from *Magnaporthe grisea* [48], we were able to ascribe the peptides to the putative full amino acid sequence of the novel enzyme. With the partial amino acid sequence information, it was possible to design degenerate primers, amplify fragments covering in total 531 bp of the 17β-HSD cDNA, and subsequently use the whole region as a probe for cDNA-library screening.

Screening of the cDNA library of *Coch. lunatus*

A cDNA library was prepared from a 42-h-old culture of *Coch. lunatus* that possessed 17β-HSD activity, and this was screened with the 531-bp probe. Several positive clones were found in the first round of screening, and selected for further screening by dilution. Three of the positive clones were subjected to *in vivo* excision of the pBluescript phagemid and sequenced.

cDNA and amino acid sequence of 17β-HSD from *Coch. lunatus*

The three clones were identical, except for the last bases at the furthermost 3′-sequence. The consensus cDNA sequence covers a 1 kbp region, and consists of a 57 bp 5′-non-coding region and a 177 bp 3′-non-coding region. An open reading frame of 810 bp was identified, coding for a protein of 270 amino acids, with a calculated molecular mass of 28 kDa (Figure 1). This is in good agreement with the molecular mass determined by matrix-assisted laser desorption ionization–time-of-flight MS for the purified protein [36]. All sequenced peptides were identical with the amino acid sequence deduced from the cDNA, and are underlined in Figure 1. For the purified 17β-HSDcl, we could not identify the initial methionine, as well as the following four amino acids of the deduced sequence (designated by the arrow in Figure 1). These amino acids were probably removed by post-translational processing, as suggested by Vidal-Cros et al. [48].

As shown in Figure 1, the primary protein structure of 17β-HSDcl contains all of the domains commonly identified in members of the SDR family (boxed in Figure 1), including the most important ones, i.e. the coenzyme-binding and active-site domains. The coenzyme-binding site with the conserved glycines (Gly-Ser-Gly-Arg-Gly-Ile-Gly) was found at positions 25–31 in domain A. Several residues in the same domain confer coenzyme selectivity to the SDR proteins [29]. In the case of NADPH-dependent enzymes, two well-conserved basic amino acids form electrostatic interactions with the ribose 2′-phosphate moiety of NADPH. In the NADPH-preferring mouse lung carbonyl reductase, these residues are Lys-17 and Arg-39 [25]. In NADH-dependent enzymes, however, an aspartate side chain replaces these basic side chains [25]. We have shown previously [35] that 17β-HSDcl is an NADPH-preferring enzyme. Indeed, one conserved basic residue is located at position 28 in the amino acid sequence, although the second amino acid cannot be assigned unambiguously. A threonine preceding the Gly-Xaa-Xaa-Xaa-Gly-Xaa-Gly motif is conserved throughout the SDR enzyme family. This residue probably interacts with the amino-acid backbone in the βD strand, thus stabilizing the protein–coenzyme interactions [29]; it is also found in 17β-HSDcl. The catalytic site is located in domain D, at amino acid positions 167–171. The sequence Tyr-Ser-Gly-Ser-Lys contains the conserved amino acids tyrosine and lysine, which have already been shown by mutagenesis experiments with different members of the SDR family to be essential for catalysis [49].

Sequence similarity

The amino acid sequence of 17β-HSDcl was found to be highly similar with four fungal carbonyl reductases (Table 1) involved in the biosynthesis of the mycotoxins, aflatoxin and sterigmatocystin, and the fungal pigment melanin. Versicolorin reductase from *Emericella nidulans* and *Asp. parasiticus* catalyse the conversion of versicolorin into sterigmatocystin [47,50], whereas tetrahydroxynaphthalene reductase from *Mag. grisea* reduces 1,3,6,8-tetrahydroxynaphthalene to scytalone and 1,3,8-trihydroxynaphthalene to vermelone [48]. A similar function was proposed for the product of the *Brn1* gene from *Coch. heterostrophus* [51] and the reductase from *Colletotrichum lagenarium* [51], which only catalyse the reduction of 1,3,8-trihydroxynaphthalene [51,52]. All these fungal carbonyl reductases have been shown to belong to the SDR family [47,48,51].

Unexpectedly, 17β-HSDcl also revealed a high similarity to the SDR from the Norway spruce (*Picea abies*) and other members of this gene family. Among only HSDs, the highest similarity was found with 7α-HSD from *E. coli* and human 17β-HSD type 4 (Table 1).

Activity of expressed protein

To verify the identity of the cloned cDNA, we expressed 17β-HSDcl in *E. coli*. As illustrated in Figure 2, the GST–17β-HSDcl fusion protein has an M_r of 54000, as shown by SDS/PAGE. Almost-pure fusion protein was eluted from glutathione–Sepharose with 10 mM glutathione. The pure 17β-HSDcl (28 kDa) was obtained after cleavage of GST-fusion protein

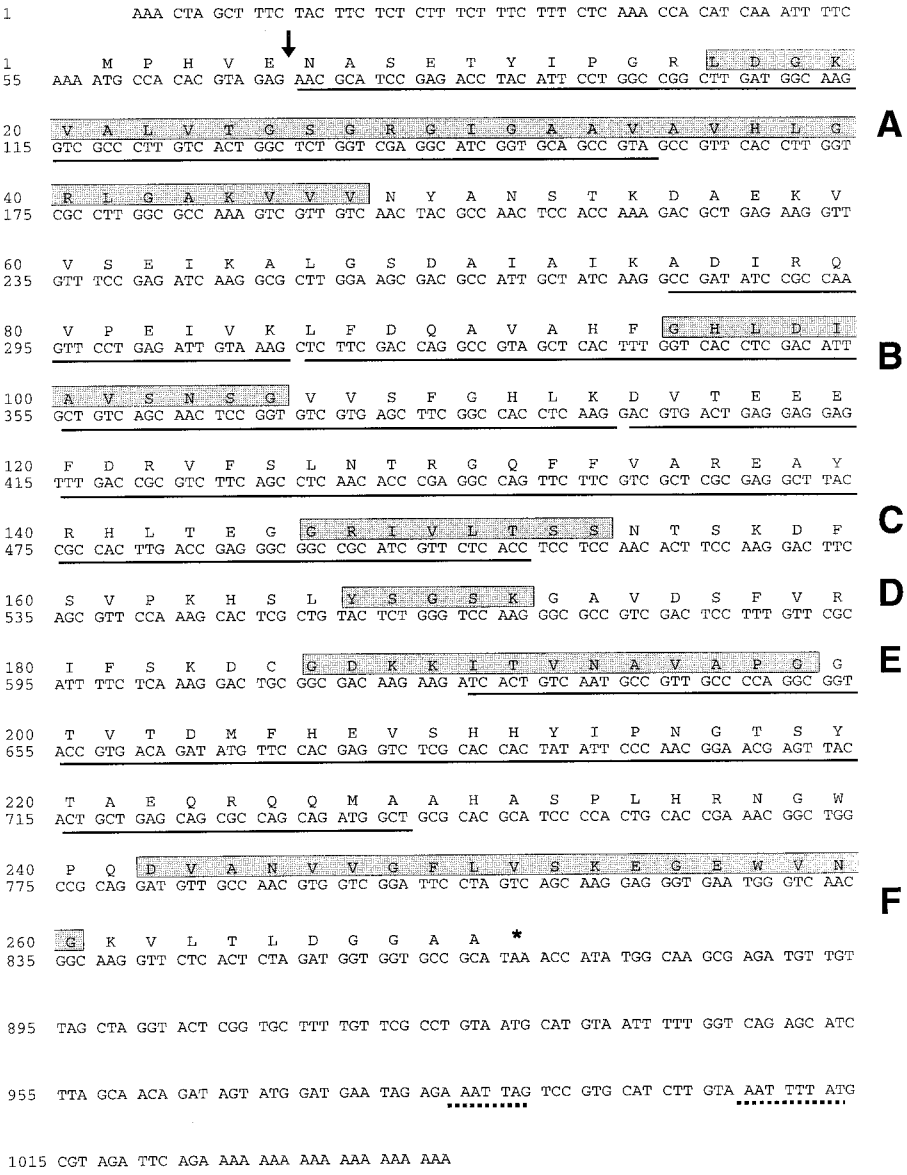

Figure 1 cDNA and protein sequence of 17β-HSDcl

Deduced amino acids are given above the nucleotide sequence. The arrow points to the putative post-translational cleavage site. Amino acid sequences determined by Edman degradation are underlined. Boxed amino acids represent domains of the SDR gene family. The stop codon is depicted by an asterisk and putative polyadenylation signals are underlined with dotted lines.

bound to glutathione–Sepharose with thrombin. The level of expression in *E. coli* and the degree of purification were monitored by activity measurements using 4-androstene-3,17-dione as a substrate and NADPH as a cofactor. Purified 17β-HSDcl converted androstenedione (356 pmol/min per mg protein) and menadione (21.5 nmol/min per mg protein), thus confirming that the novel cDNA codes for 17β-HSD.

Although with SDS/PAGE the recombinant 17β-HSDcl shows an M_r of 28000, under native conditions this enzyme is able to form dimers of M_r 60000 (Figure 3), and is enzymically active. Minor impurities had no measurable activity towards 4-androstene-3,17-dione. Dimerization of the enzyme has also been observed during the purification of 17β-HSDcl from *Coch. lunatus* [35]. The expression of 17β-HSDcl in *E. coli* presented in the present study provided properly folded enzyme, which is suitable for further structural and functional studies.

Functional aspects

The amino acid sequence of 17β-HSDcl has the highest homology with fungal carbonyl reductases involved in the biosynthesis of mycotoxin and fungal pigment (59–67% identity in a 255–267 amino-acid overlap). Such a high homology might indicate a common enzymic mechanism, as suggested by Baker [53]. A protein from the vaccinia virus with a 35% sequence identity with human 3β-HSD has the same biological activity [54]. On the other hand, a high similarity does not automatically indicate functional identity: proteins with about 65% sequence identity

Table 1 Amino acid similarities

Amino acid sequences were compared using BLAST and FASTA software [43,44].

Protein	Abbreviation	% Similarity with 17β-HSDcl	Species	Accession number
17β-HSD	17β-HSDcl	100	*Cochliobolus lunatus*	AF069518
Versicolorin reductase	Ver1	67	*Aspergillus parasiticus*	P50161
Versicolorin reductase	VerA	65	*Emericella nidulans*	L27825
Tetrahydroxynaphthalene reductase	ThnR	61	*Magnaporthe grisea*	S41412
Trihydroxynaphthalene reductase	THR1	61	*Colletotrichum lagenarium*	D83988
Trihydroxynaphthalene reductase	Brn1	59	*Cochliolobus heterostrophus*	AB001564
SDR	SDR1	37	*Picea abies*	Q08632
7α-HSD	Hdha	36	*Escherichia coli*	P25529
3-Ketoacyl acyl carrier protein reductase	fabG	34	*Bacillus subtilis*	Z99112
Glucose 1-dehydrogenase	DHG1	28	*Bacillus megatherium*	P39482
17β-HSD type 4	HSD17B4	31	*Homo sapiens*	P51659

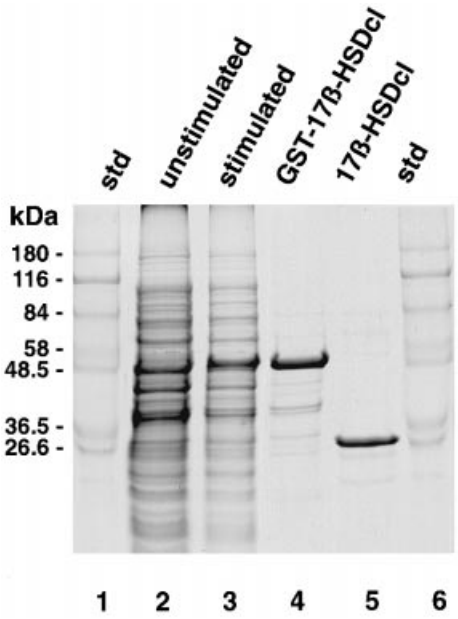

Figure 2 Expression and purification of 17β-HSDcl

SDS/PAGE and Coomassie Blue staining, showing different steps of 17β-HSDcl expression and purification. *E. coli* was transfected with the pGEX-17β-HSDcl vector (lane 2); after treatment with isopropyl β-D-thiogalactoside, a band of 54 kDa corresponding to the fusion protein GST–17β-HSDcl appeared (lane 3). This protein was adsorbed on to the affinity matrix glutathione–Sepharose. A part of the matrix was treated with glutathione to elute the bound protein and verify its molecular mass and activity (lane 4). The rest of the matrix was incubated with thrombin to cleave the 17β-HSDcl from the fusion protein (lane 5). Std, molecular-mass standards (lanes 1 and 6) (Fluka, Buchs, Switzerland).

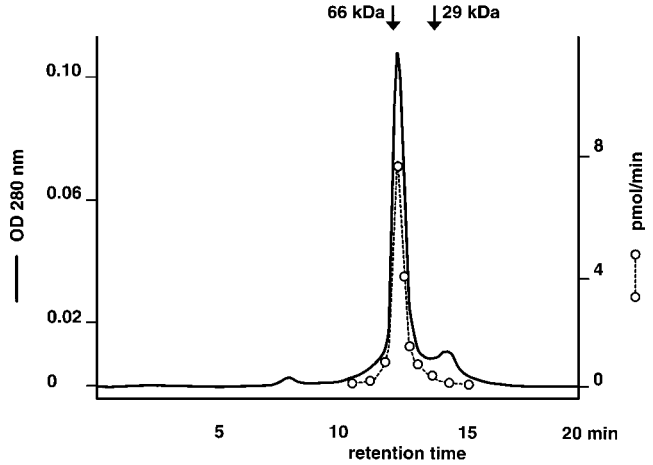

Figure 3 Estimation of native molecular mass by gel filtration

Recombinant purified 17β-HSDcl was analysed on a TSK G4000 SW gel-filtration column and enzymic activity measured with 4-androstene-3,17-dione. Positions of standards, carbonic anhydrase and BSA (29 kDa and 66 kDa respectively), are depicted. Activity values are shown only for samples with measurable conversion.

with rat 3α-HSD do not show the same enzyme activity [55]. Moreover, despite a high homology (56% sequence identity) between the fungal carbonyl reductases involved in the biosynthesis of mycotoxin and fungal pigment, versicolorin reductase from *Asp. parasiticus* and tetrahydroxynaphthalene reductase from *Mag. grisea* metabolize quite different natural substrates. The only similarity between these enzymes is that they both catalyse a reduction step in the dehydroxylation of polyketide-derived polyphenols [48]. Polyketide-derived compounds are also most probably the natural substrates for 17β-HSDcl.

Evolution of 17β-HSD

In view of the functional and amino acid similarity of 17β-HSDcl to the human 17β-HSDs, we performed phylogenetic analyses of these steroid-metabolizing enzymes (Figure 4). The branch comprising oxidative human 11β-HSD type 2 (HSD11B2) and rat 17β-HSD type 6 (HSD17B6) contains human hydroxybutyrate dehydrogenase. This branch has been reported to contain retinol dehydrogenases [53], which show a high homology with the rat 17β-HSD type 6 [12]. Another branch contains the reductive human 17β-HSD type 1 (HSD17B1) and the bacterial acetoin reductase (Budc) and is rooted close to the 11β-HSD type 1 (HSD11B1). Because 17β-HSDcl is similar to enzymes participating in the fungal synthesis of melanin, we examined the phylogenetic position of human sepiapterin reductase (Spre) involved in tetrahydrobiopterin metabolism. However, both this

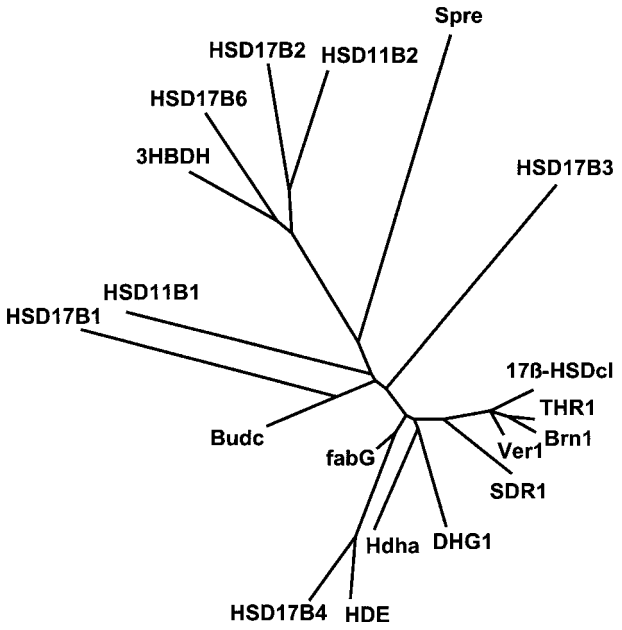

Figure 4 Phylogenetic tree of 17β-hydroxysteroid dehydrogenases

The branches of the tree are proportional to distances in amino acid sequence similarities. 17β-HSDcl has the accession number AF069518; subsequent accession numbers for proteins are indicated in parentheses. Brn1, trihydroxynaphthalene reductase of *Coch. heterostrophus* (AB001564); Budc, acetoin dehydrogenase from *Klebsiella terrigena* (Q04520); DHG1, glucose 1-dehydrogenase of *Bacillus megatherium* (P39482); fabG, 3-ketoacyl carrier protein reductase of *B. subtilis* (Z99112); 3HBDH, human 3-hydroxybutyrate dehydrogenase (M93107); HDE, hydratase–dehydrogenase–epimerase of *Can. tropicalis* (P22414); Hdha, 7α-hydroxysteroid dehydrogenase of *E. coli* (P25529); HSD11B1, human 11β-hydroxysteroid dehydrogenase type 1 (P28845); HSD11B2, human 11β-hydroxysteroid dehydrogenase type 2 (2134657); HSD17B1, human 17β-hydroxysteroid dehydrogenase type 1 (M36263); HSD17B2, human 17β-hydroxysteroid dehydrogenase type 2 (L11708); HSD17B3, human 17β-hydroxysteroid dehydrogenase type 3 (U05659); HSD17B4, human 17β-hydroxysteroid dehydrogenase type 4 (P51659); HSD17B6, rat 17β-hydroxysteroid dehydrogenase type 6 (U89280); SDR1, SDR-type oxidoreductase of *P. abies* (Q08632); Spre, human sepiapterin reductase (P35270); THR1, trihydroxynaphthalene reductase of *Coll. lagenarium* (D83988); Ver1, versicolorin reductase of *Asp. parasiticus* (P50161).

enzyme and the 17β-HSD type 3 (HSD17B3) are located the furthest distance from the common root. 17β-HSDcl is located in another branch that also contains bacterial 7α-HSD (Hdha) and human 17β-HSD type 4 (HSD17B4). Furthermore, the hydratase–dehydrogenase–epimerase of *Candida tropicalis* (HDE) is included in this branch. This enzyme shares 55% homology with the human 17β-HSD type 4 [53], but has no activity towards steroids (J. Adamski, unpublished work). The whole branch is rooted close to bacterial 3-ketoacyl carrier protein reductase (fabG), and is remarkably close in relationship to the primordial, evolutionarily distinct species.

Mammalian HSDs of the present day might have evolved by gene duplication of an ancestral HSD, followed by sequence divergence [17,53]. A broad substrate specificity towards steroids and other substrates was suggested for this ancestral protein of the SDR family [53]. Since 17β-HSDcl has been shown to convert quinones in addition to steroids, the enzyme is a good candidate to be a eukaryotic ancestor of the SDR family. The sequence of this enzyme, the first discovered fungal HSD member of the SDR family, might therefore help to elucidate the precise evolutionary history of steroid dehydrogenases.

The work was supported by Deutsche Forschungsgemeinschaft (grant AD 127/1-1 to J.A.), the Ministry of Science and Technology of Slovenia (grant J1-7426 to M.Ž.-M.) and by fellowships of the Federation of European Biochemical Societies and of the Slovenian Science Foundation (to T.L.R.). The skillful technical assistance of M. Marušič is gratefully acknowledged.

REFERENCES

1 Stewart, P. M. and Sheppard, M. C. (1992) Mol. Cell. Endocrinol. **83**, C13–C18
2 Roy, A. K. (1992) Proc. Soc. Exp. Biol. Med. **199**, 265–272
3 Millewich, L., Garcia, R. L. and Gerrity, L. W. (1985) Metabolism **34**, 938–944
4 Martel, C., Rheaume, E., Takahashi, M., Trudel, C., Couet, J. and Luu-The, V. (1992) J. Steroid Biochem. Mol. Biol. **41**, 597–603
5 Labrie, F., Simmard, J., Luu-The, V., Palletier, G. and Belghmi, K. (1994) Bailliere's Clin. Endocrinol. Metab. **8**, 451–477
6 Jarabak, J. (1969) Methods Enzymol. **15**, 746–752
7 Puranen, T., Poutanen, M., Ghosh, D., Vihko, P. and Vihko, R. (1997) Mol. Endocrinol. **11**, 77–86
8 Wu, L., Einstein, M., Geissler, W. M., Chan, H. K., Elliston, K. O. and Andersson, S. (1993) J. Biol. Chem. **268**, 12964–12969
9 Andersson, S., Geissler, W. M., Wu, L., Davis, D. L., Grumbach, M. M., New, M. I., Shwarz, H. P., Blethen, S. L., Mendonca, B. B., Bloise, W. et al. (1996) J. Clin. Endocrinol. Metab. **81**, 130–136
10 Adamski, J., Husen, B., Marks, F. and Jungblut, P. W. (1992) Biochem. J. **288**, 375–381
11 Adamski, J., Normand, T., Leenders, F., Monte, D., Begue, A., Stehelin, D., Jungblut, P. W. and de Launoit, Y. (1995) Biochem. J. **311**, 437–443
12 Biswas, M. G. and Russell, D. W. (1997) J. Biol. Chem. **272**, 15959–15966
13 Blomquist, C. H. (1995) J. Steroid Biochem. Mol. Biol. **55**, 515–524
14 Penning, T. M. (1997) Endocr. Rev. **18**, 281–305
15 Fomitcheva, J., Baker, M., Anderson, E., Lee, G. Y. and Aziz, N. (1998) J. Biol. Chem. **273**, 22664–22671
16 Nokelainen, P., Peltoketo, H., Vihko, R. and Vihko, P. (1998) Mol. Endocrinol. **12**, 1048–1059
17 Jornvall, H., Persson, B., Krook, M., Atrian, S., Gonzales-Duarte, R., Jefrey, J. and Ghosh, D. (1995) Biochemistry **34**, 6003–6013
18 Krozowski, Z. (1992) Mol. Cell. Endocrinol. **84**, C25–C31
19 Krozowski, Z. (1994) J. Steroid Biochem. Mol. Biol. **51**, 125–130
20 Ghosh, D., Weeks, C. M., Grochulski, P., Duax, W. L., Erma, M., Rimsay, R. L. and Orr, J. C. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 10064–10068
21 Ghosh, D. and Warwrzak, Y. (1994) Structure **2**, 629–640
22 Breton, R., Housset, D., Mozza, C. and Fontecillo-Camps, C. J. (1996) Structure **4**, 905–915
23 Azzi, A., Rehse, P. H., Zhu, D. W., Campbell, R. L., Labrie, F. and Lang, S. X. (1996) Nature Struct. Biol. **3**, 665–668
24 Varughese, K. I., Skinner, M. M., Whiteley, J. M., Matthews, D. A. and Xuong, N. H. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6080–6084
25 Tanaka, N., Nonaka, T., Nakanishi, M., Deyashiki, Y., Hara, A. and Mitsui, Y. (1996) Structure **4**, 33–45
26 Tanaka, N., Nonaka, T., Tanabe, T., Yoshimoto, T., Tsuru, D. and Mitsui, Y. (1996) Biochemistry **35**, 7715–7730
27 Duax, W. L. and Ghosh, D. (1997) Steroids **62**, 95–100
28 Schultz, R. M., Groman, E. V. and Engel, L. L. (1977) J. Biol. Chem. **252**, 3775–3783
29 Oppermann, U. C. T., Filling, C., Berndt, K. D., Persson, B., Benach, J., Ladenstein, R. and Jornvall, H. (1997) Biochemistry **36**, 34–40
30 Feller, K. and Trager, L. (1987) FEMS Microbiol. Lett. **48**, 183–188
31 Baker, M. (1994) Steroids **59**, 248–258
32 Plemenitaš, A., Žakelj-Mavrič, M. and Komel, R. (1988) J. Steroid Biochem. **29**, 371–372
33 Itagaki, E. and Iwaya, T. (1988) J. Biochem. **103**, 1039–1044
34 Lanišnik, T., Žakelj-Mavrič, M. and Belič, I. (1992) FEMS Microbiol. Lett. **99**, 49–52
35 Lanišnik Rižner, T., Žakelj-Mavrič, M., Plemenitaš, A. and Zorko, M. (1996) J. Steroid Biochem. Mol. Biol. **59**, 205–214
36 Lanišnik Rižner, T., Zorko, M., Peter-Katalinič, J., Strupat, K. and Žakelj-Mavrič, M. (1996) in Enzymology and Molecular Biology of Carbonyl Metabolism, vol. 6 (Weiner, H., Lindahl, R., Crabb, D. W. and Flynn, T. G., eds.), pp. 569–577, Plenum Press, New York
37 Oppermann, U. C. T., Netter, K. J. and Maser, E. (1993) in Enzymology and Molecular Biology of Carbonyl Metabolism, vol. 4 (Weiner, H., Lindahl, R., Crabb, D. W. and Flynn, T. G., eds.), pp 379–390, Plenum Press, New York
38 Maser, E. (1995) Biochem. Pharmacol. **49**, 421–440
39 Kastelic-Suhadolc, T., Plemenitaš, A. and Žigon, D. (1994) Steroids **59**, 357–361
40 Kastelic-Suhadolc, T. and Lenasi, H. (1993) FEMS Microbiol. Lett. **108**, 121–126
41 Thole, H. H., Maschler, I. and Jungblut, P. W. (1995) Eur. J. Biochem. **15**, 510–516

42 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
43 Altschul, S. F., Maden, T. L., Schaffer, A. A., Zhang, J., Miller, W. and Lipman, D. J. (1997) Nucleic Acids Res. **25**, 3389–3402
44 Pearson, W. R. and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2444–2448
45 Thomson, J. D., Higgins, D. G. and Gibson, T. J. (1994) Comput. Appl. Biosci. **10**, 19–29
46 Leenders, F., Tesdorph, J. G., Markus, M., Engel, T., Seedorf, U. and Adamski, J. (1996) J. Biol. Chem. **271**, 5438–5442
47 Skory, C. D., Chang, P. K., Cary, J. and Linz, J. E. (1992) Appl. Environ. Microbiol. **58**, 3527–3537
48 Vidal-Cros, A., Viviani, F., Labesse, G., Boccaro, M. and Gaudry, M. (1994) Eur. J. Biochem. **219**, 985–992
49 Chen, Y., Jiang, J. C., Lin, Z. G., Lee, W. R., Baker, M. E. and Chang, S. E. (1993) Biochemistry **32**, 3342–3346
50 Brown, D. W., Yu, J. H., Kelkar, H. S., Fernandes, M., Nesbitt, T. C., Keller, N. P., Adams, T. H. and Leonard, T. J. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 1418–1422
51 Shimizu, K., Tanaka, C. and Tsuda, M. (1997) J. Gen. Appl. Microbiol. **43**, 145–150
52 Keller, N. P. and Hohn, M. (1997) Fungal Genetics and Biology **21**, 17–29
53 Baker, M. E. (1995) BioEssays **18**, 1, 63–70
54 Baker, M. E. (1992) J. Steroid Biochem. Mol. Biol. **41**, 301–308
55 Qin, K. and Cheng, K. C. (1994) Biochemistry **33**, 3223–3228

Received 14 August 1998/19 October 1998; accepted 11 November 1998