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Crystallization, X-ray diffraction analysis and phasing of 17β-hydroxysteroid dehydrogenase from the fungus *Cochliobolus lunatus*

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17β-Hydroxysteroid dehydrogenase from the filamentous fungus *Cochliobolus lunatus* (17β-HSDcl) is an NADP(H)-dependent enzyme that preferentially catalyses the oxidoreduction of oestrogens and androgens. The enzyme belongs to the short-chain dehydrogenase/reductase superfamily and is the only fungal hydroxysteroid dehydrogenase known to date. 17β-HSDcl has recently been characterized and cloned and has been the subject of several functional studies. Although several hypotheses on the physiological role of 17β-HSDcl in fungal metabolism have been formulated, its function is still unclear. An X-ray crystallographic study has been undertaken and the optimal conditions for crystallization of 17β-HSDcl (apo form) were established, resulting in well shaped crystals that diffracted to 1.7 Å resolution. The space group was identified as $I4_122$, with unit-cell parameters $a = b = 67.14$, $c = 266.77$ Å. Phasing was successfully performed by Patterson search techniques. A catalytic inactive mutant Tyr167Phe was also engineered, expressed, purified and crystallized for functional and structural studies.

1. Introduction

Hydroxysteroid dehydrogenases (HSDs) are enzymes that are essential for the biosynthesis of hormonally active steroids and that are responsible for their pre-receptor action regulation (Penning, 1997, 2003). 17β-Hydroxysteroid dehydrogenase (17β-HSD) modulates the biological potency of androgens and oestrogens by converting the inactive 17-keto steroids into their active 17-hydroxy forms or *vice versa* (Mindnich *et al.*, 2004). To date, 12 different types of 17β-HSD have been described that differ in their localization and enzymatic behaviour (Mindnich *et al.*, 2004); they have been found in primitive microorganisms such as bacteria, yeast and fungi, as well as in all vertebrates. Moreover, several 17β-HSD human types have been related to the development of pathologies (Mindnich *et al.*, 2004) such as pseudo-hermaphroditism, polycystic kidney disease and Alzheimer's disease and various hormone-dependent types of cancer.

17β-HSDs belong to one of two protein superfamilies: the short-chain dehydrogenase/reductases (SDRs) or the aldo-ketoreductases (AKRs). Enzymes belonging to the SDR superfamily (Kallberg *et al.*, 2002) show a large sequence divergence, but share some common characteristics: they have a molecular mass in the range 25–35 kDa, they are non-metalloenzymes, they are functional as oligomers (dimers or tetramers) and they require NAD(P)(H) as a cofactor. Several sequence motifs are conserved among all members of the superfamily and despite the low level (15–30%) of amino-acid sequence identity, structural studies have shown that SDR members share a remarkable folding similiarity.

17β-Hydroxysteroid dehydrogenase from the filamentous fungus *Cochliobolus lunatus* (17β-HSDcl) is an NADP(H)-dependent enzyme that preferentially catalyses the oxidoreduction of oestrogens and androgens (Lanišnik Rižner *et al.*, 1999). It is the only fungal HSD that has been cloned and purified to date (Lanišnik Rižner *et al.*, 1999). The 17β-HSDcl sequence contains the characteristic signature motifs of the SDR superfamily: a TG*XXX*G*X*G pattern found at the N-terminus as part of the dinucleotide-binding site and a Y*XXX*K pattern as part of the active site (Kallberg *et al.*, 2002; Lanišnik Rižner *et al.*, 1999). 17β-HSDcl possesses a high percentage of sequence identity to fungal carbonyl reductases involved in the biosynthesis of

Table 1
Crystal parameters, data-collection and processing statistics for the apo form and the Tyr167Phe mutant of 17β-HSDcl.

Values in parentheses are for the highest resolution shell.

	Native protein	Tyr167Phe mutant
X-ray source	XRD1, ELETTRA (Trieste, Italy)	
Wavelength (Å)	1.00	1.20
Detector	MAR 345	MAR CCD165
Crystal dimensions (µm)	300 × 200 × 200	100 × 80 × 80
Space group	$I4_122$	$I4_122$
Unit-cell parameters		
a (Å)	67.14	67.79
b (Å)	67.14	67.79
c (Å)	266.75	268.24
Z	16	16
V_M (Å^3 Da^{-1})	2.6	2.7
Solvent (%)	52.3	53.5
Mosaicity (°)	0.39	0.49
Resolution range (Å)	30.0–1.7 (1.73–1.70)	30.0–2.50 (2.59–2.50)
No. of observed reflections, $I \geq 0$	257988	132994
No. of unique reflections, $I \geq 0$	34324	11039
Completeness (%)	99.6 (99.8)	99.1 (100)
Redundancy	7.5 (6.4)	12.0 (7.3)
$\langle I/\sigma(I)\rangle$ of measured data	11.3 (3.8)	17.1 (3.7)
R_{sym}† (%)	8.8 (51.4)	10.8 (41.1)

† $R_{sym}(I) = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I\rangle|/\sum_{hkl}\sum_i |I_{hkl,i}|$, where $\langle I_{hkl}\rangle$ is the mean intensity of the multiple $I_{hkl,i}$ observations from symmetry-related reflections.

aflatoxins and the fungal pigment melanin, including versicolorin reductase from *Aspergillus parasiticus* (67%), versicolorin reductase from *Emericella nidulans* (65%), 1,3,8-trihydroxynaphtalene reductase (3HNR) from *Colletotrichum lagenarium* (61%) and *Magnaporthe grisea* (58%) and tetrahydroxynaphtalene reductase (4HNR) from *M. grisea* (51%). To a lesser extent, 17β-HSDcl also shares sequence similarity to *Escherichia coli* 7α-HSD (37%) and to human 17β-HSD types 4 and 8 (31%) (Lanišnik Rižner *et al.*, 1999).

It is worthy of note that 17β-HSDcl differs from fungal carbonyl reductases and does not convert 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one (DDBO), an artificial substrate of several fungal 3HNRs and 4HNRs, to 4,5-dihydroxy-2*H*-benzopyran-2-one (DBO) (Thompson *et al.*, 1997, 1998). Furthermore, tricyclazole, a specific inhibitor of 3HNR and 4HNR, does not inhibit 17β-HSDcl (Lanišnik Rižner *et al.*, 2001). We have recently identified the *3HNR* gene in *Cochliobolus lunatus* and reported its cloning, heterologous expression, protein purification and biochemical characterization (Lanišnik Rižner & Wheeler, 2003). This enzyme converts DDBO and, to a lesser extent, scytalone, the natural substrate of 4HNR, to 1,3,8-trihydroxynaphatalene (Lanišnik Rižner & Wheeler, 2003). These results are consistent with the findings of Thompson *et al.* (1998), who reported that 3HNR from *M. grisea* oxidizes DDBO to DBO much more readily than it does scytalone to 1,3,6,8-tetrahydroxynaphthalene (Thompson *et al.*, 1997, 1998). Thus, in spite of the high sequence similarity, 17β-HSDcl is not a 3HNR or 4HNR and its specificity towards steroids makes the elucidation of its physiological role intriguing. On the basis of sequence homology and structural similarity, several possible functional implications have been considered for 17β-HSDcl (Lanišnik Rižner *et al.*, 2001): participation in the biosynthesis of mycotoxins, involvement in the melanin-biosynthetic pathway, a possible role in fungal steroid signalling and possible participation in a fungal detoxification system.

A structure-based homology model of 17β-HSDcl with the docked cofactor NADPH and the substrate androstenedione has already been proposed (Lanišnik Rižner *et al.*, 2000). In order to validate the homology-built model and to gain further insights into the structure and function of this model enzyme, we have initiated the production and purification of the wild-type 17β-HSDcl enzyme on a multi-milligram scale in order to determine its X-ray structure. Moreover, its very high similarity to SDR members with unknown three-dimensional structures, such as fungal carbonyl reductases and human 17β-HSD type 8, allows 17β-HSDcl to be exploited as an alternative model enzyme of the SDR superfamily.

2. Methods and results

2.1. Cloning, overexpression and purification of 17β-HSDcl

The protein-coding sequence of *17HSDcl* was cloned into the pGEX expression vector as a fusion protein with GST at its N-terminus (Lanišnik Rižner *et al.*, 1999). The recombinant fusion protein GST-17β-HSDcl was expressed in *E. coli* strain JM107, purified to homogeneity by affinity chromatography on glutathione Sepharose and recovered, after thrombin cleavage, as recombinant 17β-HSDcl (Lanišnik Rižner *et al.*, 1999). The yield of production was approximately 5 mg of protein per litre of bacterial culture. The recombinant protein was about 95% homogenous as assessed by SDS–PAGE: the samples (10 or 15 µg) were denatured in Laemmli sample buffer (5 min at 363 K) and then applied onto the gel (12 or 15% acrylamide). Proteins were visualized by Coomassie blue staining.

The 17β-HSDcl enzyme preparation was not contaminated with GST; GST-specific antibodies recognized only purified GST and GST-17β-HSDcl fusion protein, but not purified 17β-HSDcl.

Recombinant 17β-HSDcl, at concentrations ranging from 1.5 to 2.5 mg ml^{-1}, was kept in PBS buffer pH 7.3 and stored at 193 K. For crystallization trials, the enzyme was concentrated to 10–20 mg ml^{-1} by ultrafiltration through YM membrane at 4000*g* at 277 K using Centricon 10 concentrators (Amicon, USA).

The 17β-HSD Tyr167Phe mutant was generated by the Quick ChangeQ site-directed mutagenesis kit (Stratagene). The forward primer 5′ CCA AAG CAC TCG CTG **TTC** TCT GGG TCC AAG GGC G 3′ and the reverse primer 5′ CGC CCT TGG ACC CAG A**GA A**CA GCG AGT GCT TTG G 3′ were hybridized to template DNA consisting of the construct pGEX-17β-HSDcl. Mutagenesis reactions followed the standard protocol described by Stratagene. The designed mutation was verified by DNA sequencing. The Tyr167Phe mutant was expressed in *E. coli* as previously described for wild-type 17β-HSD.

Figure 1
Well shaped bipyramidal diffraction-quality crystals of wild-type 17β-HSDcl (apo form).

2.2. Crystallization

Initial crystallization trials of 17β-HSDcl (apo form) were based on the sparse-matrix sampling approach (Jancarik & Kim, 1991) using the hanging-drop vapour-diffusion method, exploring variations in temperature (294 and 277 K) and pH (5–9) and using PEG 6000 as the precipitant in the range 10–30% (*w*/*v*) as described for the crystallization of 3HNR from *M. grisea* (Andersson, Jordan, Schneider, Valent *et al.*, 1996; Andersson, Jordan, Schneider & Lindqvist, 1996; Andersson *et al.*, 1997; Liao *et al.*, 2001). The most promising lead condition was found to be 0.1 *M* Tris pH 8.0, 20% PEG 6000. Unfortunately, analysis of the diffraction patterns of several crystals revealed them to be poorly diffracting and highly mosaic. Diffraction quality and well shaped bipyramidal crystals were eventually obtained under optimized conditions at 277 K by mixing equal volumes (2 µl) of reservoir solution [0.1 *M* Tris pH 8.0, 20%(*w*/*v*) PEG 6000 and 20%(*v*/*v*) glycerol] and protein stock solution (10–20 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.0, 105 m*M* NaCl). Typical 17β-HSDcl crystals are shown in Fig. 1; they reached maximum dimensions over a period of 30–40 d. The same crystallization conditions were applied to the Tyr167Phe mutant of 17β-HSDcl, except that the protein concentration was 7.3 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.0, 105 m*M* NaCl. Crystals grew over 3–7 d, showing the same morphology as observed for the native enzyme but with consistently smaller dimensions (see Table 1).

2.3. Data collection, processing and structure solution

A complete data set at 1.7 Å resolution was collected from a native 17β-HSDcl crystal at beamline XRD-1 (Bernstorff *et al.*, 1995) of the Italian synchrotron facility ELETTRA (Trieste, Italy) using a MAR345 imaging-plate detector (MAR Research, Germany). The crystal was directly flash-cooled in a stream of cold nitrogen gas at 100 K using an Oxford Cryosystems cooling device (Oxford Cryosystems Ltd, UK). There was no need for prior crystal transfer to a cryoprotecting solution. X-ray diffraction images were indexed, integrated and subsequently scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997); the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) was used for data reduction. Crystal and data-collection statistics are summarized in Table 1.

The structure of 17β-HSDcl was determined by Patterson search techniques (data to 3.5 Å) using the monomer of 3HNR (PDB code 1g0n; Liao *et al.*, 2001) as a search model (58% amino-acid sequence identity) after removal of all solvent, cofactor and inhibitor molecules. A unique solution for 17β-HSDcl was obtained only when assuming the space group to be $I4_122$. A final correlation coefficient of 0.68 and an *R* factor of 0.35 were obtained using the *AMoRe* package (Navaza, 2001). Graphical inspection of the crystal packing with the program *O* (Jones *et al.*, 1991) confirmed the correctness of the solution. Data collection, processing and phasing of the Tyr167Phe mutant were the same as for the native 17β-HSDcl. The coordinates of the partial refined structure of the native 17β-HSDcl were then used as a search model, which resulted in a final correlation coefficient of 0.76 and an *R* factor of 0.32. Model rebuilding and further refinement are under way for both crystal structures.

The crystallization and structure determination of the 17β-HSDcl Tyr167Phe mutant in complex with a variety of substrates and substrate analogues will also be attempted, leading to a structure-based elucidation of the enzyme mechanism of action and of its substrate-binding specificities.

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