**Abstract**

17β-hydroxysteroid dehydrogenase from the filamentous fungus *Cochliobolus lunatus* (17β-HSDcl) is an NADP(H) dependent enzyme that preferentially catalyses oxidoreduction of estrogens and androgens. It bears sequence similarity to fungal ketoreductases, to bacterial 7α-HSD and even to human 17β-HSD types 4 and 8. A structure-based model of 17β-HSDcl with a docked coenzyme NADPH and the substrate androstenedione, was built previously, based on the crystal structure of a homologous fungal ketoreductase, which also belongs to the short-chain dehydrogenase-reductase superfamily. To validate the homology built model and to gain further insight into the structure and function of this model enzyme, we initiated the production and purification of the wild type enzyme on a multi-milligram scale for its X-ray structural determination.

The protein coding sequence of the 17HSDcl gene was cloned into the pGEX expression vector as a fusion protein with glutathione-S-transferase (GST). The recombinant protein was expressed in *Escherichia coli*, purified to homogeneity by affinity chromatography on Glutathione Sepharose and recovered, following thrombin cleavage, as recombinant 17β-HSDcl. The purified enzyme was about 95% homogenous, as revealed by SDS PAGE. Activity surveillance at 4°C and 21°C showed adequate protein stability only at the lower temperature. The optimal conditions for crystallization of 17β-HSDcl (apo form) were established. Crystals appeared as well shaped bi-pyramids, displayed I4122 space group symmetry and diffracted to a resolution of 1.7Å. The unit cell parameters were determined to be a = b = 67.17Å and c = 266.90Å. Phasing was successfully performed by Patterson search techniques. The crystallographic refinement and the modelling of solvent molecules are now in progress.

**Keywords:** 17β-hydroxysteroid dehydrogenase; crystallization; fungi; HSD; SDR; *Cochliobolus lunatus*

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**Introduction**

17β-hydroxysteroid dehydrogenase from the filamentous fungus *Cochliobolus lunatus* (17β-HSDcl) is an NADP(H) dependent enzyme that preferentially catalyses oxidoreduction of estrogens and androgens (Lanišnik Riţner et al., 1999). It is the only fungal HSD that has been cloned and purified so far (Lanišnik Riţner et al., 1999). 17β-HSDcl belongs to the short-chain dehydrogenase/reductase (SDR) superfamily and contains the sequence characteristics of this family—a T-G-X-X-G-X-G motif found at the N-terminus, close to the dinucleotide binding site, and a Y-X-X-X-K motif as part of the active site (Lanišnik Riţner et al., 1999; Kallberg et al., 2002). 17β-HSDcl possesses a high percentage of sequence identity to fungal carbonyl reductases involved in the biosynthesis of aflatoxin and the fungal pigment melanin, including versicolorin reductase (Ver1) from *Aspergillus parasiticus* (66%), versicolorin reductase (VerA) from *Emericella nidulans* (65%), 1,3,8-trihydroxynaphtalene reductase (3HNR) from *Magnaporthe grisea* (58%) and other fungi and tetrahydroxynaphtalene reductase (4HNR) from *Magnaporthe grisea* (45%). Even though to a lesser extent 17β-HSDcl also bears sequence similarity to *Escherichia coli* 7α-HSD (35%) and to human 17β-HSD types 4 and 8 (30%
and 29% respectively) (Lanišník 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-4H-benzopyran-4-one (DDBO) to 4,5-dihydroxy-2H-benzopyran-2-one (DBO), an artificial substrate of 3HNR and 4HNR from Magnaporthe grisea and other fungi (Thompson et al., 1997; 1998; Lanišník Rižner et al., 2000). Furthermore, tricyclazole, a specific inhibitor of 3HNR and 4HNR, does not inhibit 17β-HSDcl (Lanišník Rižner et al., 2000). We have recently identified the 3HNR gene in C. lunatus; it has been cloned and expressed and the product purified to homogeneity (Lanišník Rižner and Wheeler, 2003). This enzyme converts DDBO and, to a lesser extent, scytalone, the natural substrate of 4HNR, to 1,3,6,8-tetrahydroxy naphthalene (1,3,6,8-tetraHN) (Lanišník Rižner and Wheeler, 2003). These results are consistent with the findings of Thompson et al. who reported that 3HNR from M. grisea oxidizes DDBO to DBO much more readily than scytalone to 1,3,6,8-tetraHN (Thompson et al., 1997; 1998). Thus, in spite of high sequence similarity, 17β-HSDcl is not a 3HNR or 4HNR, and the specificity towards steroids makes the elucidation of its physiological role intriguing. Nevertheless, its very high similarity to SDR members with unknown 3D-

structure, such as fungal carbonyl reductases (Ver1, VerA) and human 17β-HSD type 8, allows the 17β-HSDcl to be exploited as an alternative model enzyme of the SDR superfamily.

Recombinant fungal 17β-HSDcl was expressed as a GST fusion protein in Escherichia coli and purified to homogeneity (Lanišník Rižner et al., 1999). We have reported a structure-based homology model of 17β-HSDcl with the docked coenzyme NADPH and substrate androstenedione, using as template the crystal structure of 3HNR from M. grisea (Lanišník Rižner et al., 2000). 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 enzyme on a multi-milligram scale for determining its X-ray structure. In this paper we report the purification, thermal stability studies, crystallization, X-ray diffraction analysis and phasing of 17β-HSDcl.

**Methods**

**Protein purification**

17β-HSDcl was expressed in Escherichia coli and purified as previously described (Lanišník Rižner et al., 1999). Recombinant 17β-HSDcl, at concentrations ranging from 1.5 to 2.5 mg/ml, was kept in PBS buffer pH 7.3 and stored at –80°C. For crystallization trials, the enzyme was concentrated to 20 mg/ml by ultrafiltration through YM membrane at 4000 g at 4°C, using Centricon 10 concentrators (Amicon, USA).

**SDS page**

Homogeneity of the proteins was checked by SDS PAGE. The samples (4, 10 or 15 μg) were denatured in Laemmli sample buffer (5 min at 90°C) and then applied to the gel (12% or 15% acrylamide). Proteins were visualized by Coomassie blue staining.

**Western blot analysis**

A total of 4 μg of recombinant 17β-HSDcl, 17β-HSDcl-GST fusion protein and GST were submitted to SDS-PAGE. After transfer, Western blot analysis was performed using mouse antibodies against GST diluted 1:5000 and incubated overnight. The secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse polyclonal antibodies, were diluted 1:2000 and incubated for 2 hours. Proteins were detected by enhanced chemiluminescence.
Assays for stability of recombinant 17β-HSDcl

Oxidation of 4-estrene-17β-ol-3-one in the presence of NADP\(^+\) catalyzed by 17β-HSDcl was followed photometrically at 340 nm, in 100 mM phosphate buffer, pH 8.0 at 25º C as described (Kristan et al., 2003). In all experiments 1% DMF was present to enhance the substrate solubility.

To check the enzyme stability, we measured the activity (initial rates) after incubations at 4º C and 21º C for several days. Enzyme activities were expressed as the percentage of initial enzyme activity, and as a mean of two independent measurements with a standard deviation.

Crystallization

Initial crystallization trials of 17β-HSDcl (apo form) were based on a matrix sampling approach (Jancarik et al., 1991), using the hanging drop vapour-diffusion method and exploring temperature (21º C and 4º C), pH (5–9) and % w/v range (10–30) of the precipitant (PEG 6000) as described for the crystallization of 3HNR from Magnaporthe grisea (Andersson et al., 1996a; 1996b; 1997; Liao et al., 2001).

Results and Discussion

Protein purification

The protein coding sequence of 17HSDcl was cloned into the pGEX expression vector as a fusion protein with GST at its N-terminus. The recombinant fusion protein GST-17β-HSDcl was expressed in Escherichia coli strain JM107, purified to homogeneity by affinity chromatography on Glutathione Sepharose and recovered, after thrombin cleavage, as recombinant 17β-HSDcl. The yield of production was approximately 5 mg of protein / l of bacterial culture. The recombinant protein was about 95% homogenous, as assessed by SDS PAGE (Fig. 1). The 17β-HSDcl enzyme preparation was not contaminated with GST, since GST specific antibodies recognized only purified GST and GST-17β-HSDcl fusion protein, but not purified 17β-HSDcl (Fig. 2).

Stability of the recombinant 17β-HSDcl

The thermal stability of the purified recombinant enzyme was evaluated. The enzymatic activity was measured after incubation at 4º C and 21º C, the temperatures generally employed for crystallization trials. The activity did not change substantially when incubating the protein at 4º C for 7 days. When the protein was incubated at 21º C the activity was lost already after 48 hours (Fig. 3). Additionally, we tested for putative proteolysis of the recombinant enzyme by the small amount of thrombin still present in the protein solution. Figure 4 clearly shows the absence of digestion products after two weeks incubation of 17β-HSDcl at 4º C and complete unspecific digestion after 48 hours and one-week incubation at 21º C indicating thrombin still needs to be removed from the protein solution. However, the reported procedure has proved to be useful for the production of at least 95% homogenous recombinant.
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III: Reductases

Crystallization

Preliminary crystallization conditions were established using the hanging-drop vapour-diffusion method. The most promising results were obtained from 0.1 M Tris•NaOH pH 8.0, 20% PEG 6000 precipitant solution. Unfortunately, analysis of the diffraction pattern of several crystals revealed that these were poorly diffracting and to be highly mosaic. Diffraction quality and well shaped bi-pyramidal crystals were eventually obtained under optimized conditions at 4°C, by mixing equal volumes (2 μl) of the protein stock solution, 10 mg/ml in 50 mM TrisHCl pH 7.0, 105 mM NaCl, with a reservoir solution (2 μl) containing 0.1 M Tris•NaOH pH 8.0, 20% PEG 6000 and 20% glycerol. Typical 17β-HSDEL crystals are shown in Figure 5; they reached their maximum dimensions over a 30–40 day period. Crystals belong to the tetragonal space group I4122 with unit cell parameters a = b = 67.17 Å and c = 266.90 Å.

Table 1. Crystal parameters, data collection and processing statistics (values in parentheses are for the highest resolution shell).

<table>
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<th>Parameter</th>
<th>Value</th>
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<td>Detector</td>
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<tr>
<td>Space group</td>
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<tr>
<td>Unit-cell parameters</td>
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<tr>
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<td>c (Å)</td>
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<td>No. of unique reflections I ≥ 0</td>
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<tr>
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<td>&lt;I/σ(I)&gt; of measured data</td>
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<tr>
<td>R&lt;sub&gt;xim&lt;/sub&gt; (%)</td>
<td>11.7 (54.6)</td>
</tr>
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</table>

<sup>†</sup>R<sub>xim</sub>(I)= \frac{\sum_{hkl} |I_{hkl}| - <I_{hkl}>}{\sum_{hkl} |I_{hkl}|} with <I_{hkl}> mean intensity of the multiple I<sub>hkl</sub> observations from symmetry-related reflections.

**Figure 4. SDS PAGE of 17β-HSDEL after incubations at 4°C and 21°C.** Purified recombinant 17β-HSDEL was kept in PBS buffer pH 7.3 at A) 4°C for two weeks or B) 21°C for 24 hours, 48 hours or one week. Homogeneity was checked using 15% SDS-polyacrylamide gel. A) Lane 1, pre-stained molecular weight marker (26.6, 36.6, 48, 58, 84, 116 and 180 kDa, respectively); lane 2, 17β-HSDEL (10 μg); lane 3, 17β-HSDEL (15 μg). B) Lane 1, molecular weight marker (20, 25, 37, 50, 75, 100, 150 and 250 kDa, respectively), lane 2, 17β-HSDEL after incubation for 24 hours (15 μg), lane 3, 17β-HSDEL after incubation for 48 hours (15 μg) and lane 4, 17β-HSDEL after incubation for one week (15 μg).

**Figure 5. Well shaped bi-pyramidal diffraction quality crystals of 17β-HSDEL.**
Data collection
A complete data set at 1.7 Å resolution was collected at beam-line XRD-1 of the Italian Synchrotron facility ELETTRA (Trieste, Italy), using a MarCCD detector (marUSA Inc., USA). The crystal was directly flash-cooled in a stream of cold nitrogen gas at 100K, using an Oxford Cryosystem cooling device (Oxford Cryosystem Ltd., UK). There was no need for transfer to a cryoprotecting solution, due to the high glycerol concentration (20%) already present in the mother solution. X-ray diffraction images were then indexed, integrated and subsequently scaled using the programs DENZO e SCALEPACK (Otwinowski et al., 1997); the CCP4 package (CCP4, 1994) was used for data reduction. Crystal and data collection statistics are summarized in Table 1.

Phasing
Since 58% of the amino acids in 17β-HSDcl are identical to those in 3HNR, whose 3D structure has been determined (Liao et al., 2001), the most appropriate method for determining the 17β-HSDcl structure is Patterson-search. The PDB entry 1G0N was chosen as the suitable search model. A unique solution for 17β-HSDcl with a correlation factor of 0.68 and an R factor of 0.35 was obtained using the AMoRe package (Navaza et al., 1994) in space group I4_122 and data between 15.0 and 3.5 Å. Graphical inspection of the crystal packing with the program O (Jones et al., 1991) confirmed the correctness of the solution. Mutations according to the 17β-HSDcl amino acid sequence were then introduced. Current efforts are focused on the crystallographic refinement with the CNS package (Brunger et al., 1998) and on modelling the solvent molecules.

Conclusion
Recombinant 17β-HSD from the filamentous fungus Cochliobolus lunatus was expressed in E. coli and purified in the amounts and purity required for structural and functional studies. The enzyme was crystallized, and well shaped bi-pyramidal crystals diffracted out to 1.7Å using synchrotron radiation. The crystal structure of 17β-HSDcl was solved by Patterson search methods. The partially refined crystallographic structure of the apo-enzyme was superimposed on a previously built homology model showing an r.m.s.d. of 0.88Å, based on backbone atoms of the residues 14–267 (Fig. 6), strongly supporting our proposed rationale on the structure-function relationship studies (Kristen et al., 2003).

Acknowledgments
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References
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