The 17β-HSD (17β-hydroxysteroid dehydrogenase) from the filamentous fungus Cochliobolus lunatus (17β-HSDcl) is a NADP(H)-dependent enzyme that preferentially catalyses the interconversion of inactive 17-oxo-steroids and their active 17β-hydroxy counterparts. 17β-HSDcl belongs to the SDR (short-chain dehydrogenase/reductase) superfamily. It is currently the only fungal 17β-HSD member that has been described and represents one of the model enzymes of the cP1 classical subfamily of NADPH-dependent SDR enzymes. A thorough crystallographic analysis has been performed to better understand the structural aspects of this subfamily and provide insights into the evolution of the HSD enzymes. The crystal structures of the 17β-HSDcl apo, holo and coumestrol-inhibited ternary complex, and the active-site Y167F mutant reveal subtle conformational differences in the substrate-binding loop that probably modulate the catalytic activity of 17β-HSDcl. Coumestrol, a plant-derived non-steroidal compound with oestrogenic activity, inhibits 17β-HSDcl (IC_{50} 2.8 μM; at 100 μM substrate (4-oestrone-3,17-dione)) by occupying the putative steroid-binding site. In addition to an extensive hydrogen-bonding network, coumestrol binding is stabilized further by π–π stacking interactions with Tyr122. A stopped-flow kinetic experiment clearly showed the coenzyme dissociation as the slowest step of the reaction and, in addition to the low steroid solubility, it prevents the accumulation of enzyme–coenzyme–steroid ternary complexes.

**Key words:** carbonyl reductase, Cochliobolus lunatus, 17β-hydroxysteroid dehydrogenase (17β-HSD), short-chain dehydrogenase/reductase (SDR), phyto-oestrogen.
oxidative and reductive directions [13]. 17β-HSDcl is fully functional as a dimer and shows an optimum pH of 7–8 for the interconversions of 4-oestrone-3,17-dione and 4-oestrone-17β-ol-3-one [15].

Substrate specificity studies revealed that 4-oestrone-3,17-dione and 4-oestrone-17β-ol-3-one is the best substrate pair, followed by 5α-androstane-3,17-dione/5α-androstane-17β-ol-3-one and 4-androstene-3,17-dione/4-androstene-17β-ol-3-one, whereas oestrone and oestradiol are less preferred substrates [20]. These steroids revealed no substrate inhibition at concentrations up to 200 μM (T. Lanišnik Rižner, K. Kristan and M. Brunsko Šegel; unpublished work). To date, steroids are still the best known substrates of this enzyme, and our recent data show also that phenanthrenequinone, a classical substrate of carbonyl reductases, is very poorly converted by 17β-HSDcl [21].

Analysis of the 17β-HSDcl amino acid sequence shows the presence of the characteristic sequence motifs of the SDR superfamily: the TGXXXGX29XG31 motif, implicated in coenzyme binding and found at the N-terminus, and the Y167XXXK171 pattern with Tyr167, Ser163 and additional Lys172 as the catalytic triad [15,22]. 17β-HSDcl is an NADP(H)-dependent enzyme [18] and belongs to the cP1 classical subfamily of the SDRs [23] in which the two negative charges of the 2-phosphate group of the coenzyme are compensated for by only one basic residue, Arg28, in the coenzyme-binding motif [19]. The amino acid sequence is highly similar to those of several fungal carbonyl reductases and also to SDR members (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/441/bj4410151add.htm for a sequence alignment) involved in the biosynthesis of mycostoxins, aflatoxins, sterigmatocystin and the fungal pigment melanin: versicolorin reductase from Aspergillus parasiticus (67% identity) and Emericella nidulans (65%); and 3HNR (13.8-trihydroxynaphthalene reductase) from Colletotrichum lagenarium (61%) and Magnaporthe grisea (3HNRmrg) (58%). Among the HSDs, the highest similarity has been shown for the E. coli 7α-HSD (37% identity), Brucella melitensis 7α-HSD (31%), human 17β-HSD type 4 (31%) and various mammalian 17β-HSDs type 8 (Mus musculus 30%, Rattus norvegicus 29%, Canis familiaris 29%, Sus scrofa 28% and Homo sapiens 28%) [15].

Despite the high amino acid similarity between 17β-HSDcl and 3HNR, these two enzymes have distinct roles and functions [24,25]. A participation of 17β-HSDcl in fungal secondary metabolism has been suggested [26], although its physiological role is currently not known. The characterization of a recently prepared C. lunatus knockout (N. Krajšev and T. Lanišnik Rižner, unpublished work) should soon lead to the determination of its function. 17β-HSDcl was the subject of a thorough kinetics analysis, which proposed an ordered Bi Bi reaction mechanism of the Theorell–Chance type [17]. To improve further our understanding of the catalytic events at the molecular level, as well as evolution of HSDs, in the present study, we have conducted a comprehensive structural analysis of 17β-HSDcl. We have solved the crystal structure of the apoenzyme, its active-site binary complex and a ternary complex with coumestrol, an inhibitor with an IC50 of 2.8 μM that provides insights into the structural determinants crucial for the binding of steroid substrates [27]. Additionally, two kinetic experiments were performed, with the focus on the accumulated reaction intermediates and the identification of the limiting step of the reaction rate.

**EXPERIMENTAL**

**Expression and purification of recombinant 17β-HSDcl**

17β-HSDcl was prepared as a GST (glutathione transferase)-fusion protein in E. coli JM107 cells. Protein purification, including GST-tag cleavage by thrombin, was carried out as described previously [15]. Protein concentrations were determined using the method of Bradford [28] with BSA as standard. The purity of the protein was checked by SDS/PAGE on 12% polyacrylamide gels with Coomassie Blue staining [29].

**Initial rate measurements of 17β-HSDcl activity in both directions**

Oxidation of 4-oestrone-17β-ol-3-one in the presence of NADP+ and reduction of 4-oestrone-3,17-dione in the presence of NADPH were monitored using a PerkinElmer Lambda 45 UV–visible spectrophotometer. The differences in NADPH absorbance were measured for 600 s at 340 nm and 25°C. The assays were carried out in 0.6 ml of 100 mM phosphate buffer (pH 8.0), with a 1% final concentration of N,N-dimethylformamide as co-solvent. An array of the time courses of the enzymatic reactions was measured at seven substrate and seven coenzyme concentrations, both ranging from 20 to 140 μM, in the presence of 0.5 μM 17β-HSDcl.

Measuring the first turnover in the 17β-HSDcl reaction with NADP+ and 4-oestrone-17β-ol-3-one

The first turnover of the 4-oestrone-17β-ol-3-one by the 17β-HSDcl–NADP+ complex was measured on a SF/PQ-53 stopped-flow apparatus (Hi-Tech Scientific). Aliquots (150 μl) of two solutions, one containing 800 μM NADP+ and 4.6 μM 17β-HSDcl, and the other containing 300 μM substrate 4-oestrone-17β-ol-3-one, were mixed at a volume ratio of 1:1 in the mixing

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**Figure 1** Reaction scheme and stopped-flow kinetics

(A) Reaction scheme for the reversible 4-oestrone-3,17-dione reduction by NADPH-dependent 17β-HSDcl. (B) Time course of the NADPH formation in the reaction of the 17β-HSDcl–NADP+ complex and the steroid substrate 4-oestrone-17β-ol-3-one, measured on stopped-flow apparatus in split sweep mode (10 + 200 s). The final concentrations during the measurement were 0.4 mM NADP+, 150 μM steroid and 4.6 μM enzyme. The thin line represents the fitted curve. Inset: progress of the reaction until its completion.
chamber of the stopped-flow apparatus. The difference in NADPH absorbance was measured in a split run for 10 s and for the next 200 s, at 25 °C in 100 mM phosphate buffer (pH 8.0), with a 1.5 % final concentration of N,N-dimethylformamide as co-solvent. Analysis of the progress curves, measured in triplicate, was carried out by fitting the coefficients of the differential equations characteristic for the Theorell–Chance mechanism, to the experimental points. The reaction scheme was drawn on the web-server at http://enzo.cmm.ki.si/ and the progress curve data along with initial values of the rate constants were submitted for the analysis.

Crystallization and data collection

Crystals of the apo and Y167F mutant forms of 17β-HSDcl were obtained according to a protocol described previously [30]. Crystals of the triclinic holo form were obtained by using the hanging-drop vapour-diffusion method. A 1 μl sample of the enzyme solution (14 mg/ml in 0.1 M Tris/HCl, 0.05 M NaCl and 5 mM NADPH, pH 7.0) was mixed with an equal amount of the hanging-drop solution. The crystals grew over nearly 2 months at a temperature of 293 K.

In addition, crystals of the 17β-HSDcl holo form belonging to the monoclinic system were obtained in less than 1 week following the same protocol as described above with a precipitant solution composition of 30 % (w/v) PEG 2000 MME (monomethyl ether) and 0.1 M KCNS (pH 7.0). Monoclinic crystals of the holoenzyme were used for the inhibition studies. The crystals were soaked for 24 h in a solution with the following composition: 30 % (w/v) PEG 2000 MME, 0.1 M KCNS, 5 % (v/v) DMSO, 2 mM NADPH and 2 mM coumestrol. A complete dataset from the native apo 17β-HSDcl crystal up to 1.5 Å (1 Å = 0.1 nm) resolution was acquired at the XRD1 beamline at ELETTRA synchrotron, following the same procedures for data collection and data reduction already described in our previous study [30].

A 1.9 Å dataset from the apo Y167F mutant crystal was collected at the ID 23-2 beamline at the ESRF (European Synchrotron Radiation Facility) synchrotron (X-ray wavelength of 0.8726 Å). A complete 1.9 Å dataset from a crystal of the holo triclinic crystal form was collected at the beamline ID 14-4 at the ESRF (X-ray wavelength of 0.939 Å). Finally, a dataset for the ternary complex was collected at the XRD1 beamline at ELETTRA up to a 2.5 Å resolution (X-ray wavelength of 1.0 Å) and using crystallization solution containing 15 % (v/v) ethylene glycol as a cryoprotectant. All datasets were collected at cryogenic temperature (100 K). All of the data, except those for the apo form, were indexed and integrated using the Mosflm and Mosflm packages [31], and then scaled and merged according to Scala [32]. Statistics of data collection and data reduction are reported in Table 1.

Structure solution and refinement

The structure solution of the apo and Y167F mutant forms of 17β-HSDcl have been reported previously [30]. Crystal structures of the other crystal forms were solved by Patterson search procedure using Phaser (holo form) and MOLREP (coumestrol ternary complex) software packages [32]. The refined apo structure was used as the search model. For all of the structures, the same refinement protocol was used. A first simulating annealing refinement cycle was performed, followed by positional restrained minimization and B-factor refinement, as implemented in the CNS software package [33]. Strict NCS (Non-Crystallographic Symmetry) restraints were initially applied when appropriate and then relaxed in the following refinement cycles. Cycles of model rebuilding with the program Coot [34] were then alternated with TLS (translation, libration, screw-rotation) refinement, using the REFMAC software [32]. Medium-weighted NCS restraints were used only for the ternary complex structure throughout the refinement, leaving the substrate-binding loop (residues 200–230) free of NCS restraints. Water and ligands were introduced once the protein model could not be further improved and refined without NCS restraints. Ligand geometries and topologies for REFMAC were obtained by using the PRODRG web-server (http://davapc1.bioch.dundee.ac.uk/prodrg/).

All of the final models were subjected to a final geometry and refinement quality check using the following software packages: Procheck [32], NQ-flipper web-server (https://flipper.services.came.sbg.ac.at/cgi-bin/flipper.php) and SFCheck [32], and eventually re-refined according to their indications. The selected refinement parameters and quality indicators are reported in Table 1. The PDB codes of the structures deposited are 3IS3 (apo), 3ITD (Y167F mutant), 3QWF (holoenzyme) and 3QWI (coumestrol-inhibited ternary complex).

RESULTS AND DISCUSSION

First turnover measurements reveal that coenzyme release is the rate-limiting step of the reaction

We have already provided evidence [17–19,35] that the reaction between 17β-HSDcl follows a Theorell–Chance mechanism (see reactions 1 and 2) which presumes that the ternary EAB complex does not accumulate [25,36]:

\[ E + A \xrightleftharpoons{\kappa_{-1}} B + EA \xrightarrow{\kappa_k} EP + Q \xrightarrow{\kappa_{-2}} E + P \]  (1)

\[ E + A \xrightarrow{\kappa_{-1}} B + EA \xrightarrow{\kappa_{-2}} Q + EP \xrightarrow{\kappa_k} E + P \]  (2)

where E represents the enzyme, A, P, B and Q are NADPH and NADP+, and the oxidized and reduced substrates respectively.

The linear dependence of the plots (see Supplementary Figures S2A and S2B, lower panels, at http://www.BiochemJ.org/bj441/bj4410151add.htm) showing the initial rates against steroid concentration clearly pinpoints to the lack of an EAB complex accumulation. On the other hand, the dependence of the initial rates on coenzyme concentration, inferred from progress curves within each of the first seven panels in Supplementary Figures S2(A) and S3(B), would result in plots with curves with a trend parallel to the x-axis. Therefore, even at the lowest coenzyme concentration used (20 μM), the enzyme is saturated with the coenzyme. Overall, these findings corroborate the good affinity of 17β-HSDcl for either the reduced or oxidized form of the coenzyme.

To obtain direct experimental evidence for the rate-limiting step in the enzymatic reaction of 17β-HSDcl, we measured the initial rates of the reaction using a stopped-flow apparatus. This experiment recorded the changes in the redox state of the coenzyme when the reaction was started by the addition of the steroid substrate to the solution of the enzyme–coenzyme complex under saturating conditions. If the concentration of the initial enzyme–coenzyme complex is within a suitable concentration range for the determination method, the rate-limiting step of the complete reaction dictates the shape of the progress curve measured. The concave downwards curve obtained with a sloped asymptote, as clearly seen in Figure 1(B), reveals that the
exchange of a new coenzyme molecule after the first conversion is the slowest event during the reaction.

The data from the first turnover measurements were processed in a combined analysis using some of our previously published values for the model characteristic rates and dissociation constants. The values from [35] are listed along with the updated ones in Table 2. For the evaluation, we used a numerical integration solver [37], which has recently been implemented as a web-server application [38].

### The substrate-binding loop accounts for a large part of the 17β-HSDcl conformational variability

The crystal structures of 17β-HSDcl in its apo form as well as bound to NADPH and the coumestrol-inhibited ternary complex have been determined. The protein backbone encompassing residues 14–270 of 17β-HSDcl was continuously traced inside the electron density in all of the cases considered here, with an expected poorer 2Fo-Fc map for the flexible substrate-binding loop in the holo form and in the ternary complexes. The N-terminal residues showed poorer electron density, and for all of the structures determined, the chain backbone could not be traced for the first nine residues. The residues from 10 to 14 show structural plasticity, and they can assume two possible conformations. All of the refined structures showed overall good final geometries, as indicated by the quality descriptors reported in Table 1.

The 17β-HSDcl structure has a central seven-stranded parallel β-sheet that is flanked by six α-helices, three on each side (Figure 2A); there are two more small α-helices, α1 and α2, located in the substrate-binding loop, which is a helix-loop-helix domain that acts as a flexible lid that covers the active site. In all of the crystal structures described in the present paper, the general fold and shape is conserved with approximate overall monomer dimensions of 55 Å × 48 Å × 46 Å. The RMSDs (root mean square deviations) calculated with ProFit (http://www.bioinf.org.uk/software/profit/) on 254 Cα atoms between the apo form and the holo monomers span between 0.6 and 0.9 Å, depending on the subunit considered: values of 0.65 Å were obtained when comparing the apo form and the ternary complex. The omission of 31 residues spanning the substrate-binding loop (residues 200–230) in the structure comparisons almost halved the RMSDs between the apo and the holo forms, and lowered the RMSDs by 0.6 and 0.9 Å, depending on the subunit considered: values of 0.30 Å were obtained when comparing the apo form and the ternary complex. Taken together, these data clearly show an almost identical structure, although they also reveal that the substrate-binding loop accounts for a large part of the conformational variability of 17β-HSDcl.

### The interactions between monomers through the Q-interface are tighter than those through the P-interface

Crystallographic analysis showed that 17β-HSDcl is organized as a homotetramer, independent of the crystal form considered, although 17β-HSDcl is a homodimer in its functionally active form [13,15]. This is not unusual, as SDR enzymes often exchange of a new coenzyme molecule after the first conversion is the slowest event during the reaction.

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show a common crystallographic tetrameric arrangement despite homodimeric functional forms. SDR crystallographic tetramers show a 222 symmetry with P, Q and R dyads, according to the SDR crystal structures convention [11] (Figure 2B and see Supplementary Figure S3 at http://www.BiochemJ.org/bj/441/bj4410151add.htm). The Q axis associates monomers through αE and αF helix interactions (Q-interface), and the P axis involves the C-terminal residues, including αG, βG and their connecting loop, and N-terminal residues (P-interface). Subunit interactions through the R axis, which is orthogonal to both the P and Q axes, are generally scarce. It was shown that the Q-interface is responsible for the dimerization of 17β-HSDcl in solution [39,40] and that the four-helix bundle formed upon dimerization is probably responsible for maintaining the active-site residues in the correct, catalytically active, conformation. Our crystallographic study confirms that the catalytic residues, Tyr167 and Lys171, are part of helix αF that is involved in homodimer formation. However, the total interface area of the Q-interface of the holo crystal structure (3602 Å²) is not much greater than that of the P-interface (3356 Å²), and both of these are well above the 1600 Å² indicated by Janin and Chothia [41] as the minimum value for potential functionally relevant protein interfaces. Moreover, interface structural dissection showed that, despite the Q-interface generally having slightly better parameters than the P-interface for functionally relevant surface interactions, these two interfaces are not dissimilar when considering their general features (see Supplementary Table S1 at http://www.BiochemJ.org/bj/441/bj4410151add.htm). Nonetheless, the monomers interacting through the Q-interface are in tighter contact than those bound through the P-interface. The gap volume between the interfacing surfaces is much less for the Q-interface (2261 Å³) than for the P-interface (5046 Å³) which is in agreement with the lower number of water molecules and the protein–water interactions characterizing the former. Furthermore, a higher percentage of atoms, tightly interacting, participate to the Q-interface. Together, all of these data agree with the observed functional relevance of the Q-interface.

It is interesting to note that the 17β-HSDcl homologue, 3HNR from M. grisea (3HNRmg), shows different oligomerization patterns in solution [39,40], but not in the crystal. 3HNRmg in solution is functional as a tetramer, involving both P- and Q-interfaces. These data raise the question of why the structurally similar 17β-HSDcl and 3HNRmg adopt different oligomerization states? A comparison of the 17β-HSDcl and 3HNRmg (PDB code 1G0O) [42] P-interfaces shows a remarkable sequence identity and structural similarity in the C-terminal residues: a large and deeply buried Trp557 (17β-HSDcl numbering), Phe261 stacking with the same residue from a neighbouring monomer, and a salt
bridge between Asp^{266} and Lys^{61}. A further inspection of the 3HNRmg P-interface shows instead a marked difference in the N-terminal region that extensively participates in the P-interface interactions. Such extended interactions are almost absent from 17β-HSDcl. The involvement of the N-terminal residues in the 3HNRmg P-interface results in a much larger interface area (4510 Å²) when compared with its equivalent interface in 17β-HSDcl, and is probably responsible for the energetically favoured shift of the oligomerization. Interestingly, the Q-interface in 3HNRmg does not substantially differ from its equivalent in 17β-HSDcl (3450 Å² for 3HNRmg), probably playing a similar role as hypothesized for fungal HSD [39].

NADPH forms a complex interaction network

In the apo structure, the coenzyme-binding site is filled by a network of water molecules, with the entire cavity encompassing the adenine site and the catalytic Tyr^{167}.

In the coenzyme-bound binary complex, a well-defined electron density was observed (Figure 2C) where a NADPH molecule could be easily fitted and refined. NADPH in holo 17β-HSDcl adopts an extended shape, with the nicotinamide ring in the syn conformation, in agreement with the stereochemistry of the B-face 4-pro-S hydride transfer in catalysis. Upon binding, the NADPH coenzyme establishes several polar and ionic interactions with the 17β-HSDcl protein, as shown in Figure 3(A). The adenine ring is lodged inside a protein cleft that is partially exposed to the solvent. The phosphate group in position 2 of the adenine ribose forms several hydrogen bonds with protein residues and water molecules in analogy to what has been observed for Arg^{28}, the basic residue that is responsible for the NADP(H) selectivity of 17β-HSDcl. Adenine ribose assumes a C2-endo conformation and interacts through its 3-hydroxy group with Gly^{21}, one of the conserved residues of the coenzyme-binding site in the SDR superfamily. The nicotinamide ribose also adopts a C2-endo conformation and forms essential hydrogen bonds through its hydroxy groups, with the side chains of the catalytic Tyr^{167} and Lys^{71}. The nicotinamide ring is buried inside the catalytic pocket.

As well as these polar interactions, the NADPH coenzyme is involved in several hydrophobic interactions. The nicotinamide ring is ‘sandwiched’ between the side chain of Met^{217} and Pro^{197}, which is one of the most conserved residues in the SDR enzymes.

The active site is hosted in a hydrophobic pocket and it is covered by a flexible lid

The active site is located at the bottom of a mostly hydrophobic pocket, with Tyr^{167} and Lys^{71} interacting with NADPH, and Ser^{153} situated in a turn between βE and αF (Figure 3B). The substrate-binding loop partially shields the active site from the bulk solvent. No significant positional differences are observed in the catalytic triad among the different crystallographic structures of the present study. Also, the mutation of Tyr^{167} to phenylalanine does not have any appreciable effect on the overall geometry of the active site as witnessed by the Y167F mutant crystal structure.

The catalytic pocket encompasses a free volume of ~320 Å³, and it is filled in the apo, Y167F and holo forms by glycerol molecules and PEG fragments both present in the crystallization cocktail. In the coumestrol-inhibited ternary complex, two ethylene glycol molecules are situated at the entrance of the cavity. Overall, the catalytic pocket shows a clear affinity for hydroxylated organic molecules, which occupy the putative substrate site in the apo and the holo forms.

In a previous study based on kinetic and thermodynamic data [35], it was hypothesized that Tyr^{212} and His^{164} control the open/closed conformation of 17β-HSDcl. In the present study, our crystallographic data show that Tyr^{212} that is located at the C-terminus of helix αI, belonging to the substrate-binding loop, is indirectly linked through a water bridge to His^{164}, which is a residue just before the N-terminal of helix αF (Figure 3D). This interaction, involving Tyr^{212} OH and His^{164} Ne2, is present in both the apo and holo forms, but not in the ternary complex where it is replaced by a direct, but geometrically less favoured, hydrogen bond between the same atoms. These findings are somewhat surprising, considering that the holo and apo forms are the most conformationally distant states as discussed in below.

Analysis of the structural determinants responsible for the different substrate specificities of 17β-HSDcl and fungal 3HNRs was reported in a previous study [14,21]. Indeed, the superimposition of the coumestrol-inhibited 17β-HSDcl crystal structure with that of a ternary complex of 3HNRmg inhibited by pyroquilon shows that coumestrol would clash with Trp^{243} of 3HNRmg. In 17β-HSDcl, the back side of the inhibitor-binding site is probably defined by Met^{217}, which leaves more space for the correct steroid accommodation.

Crystal structures of 17β-HSDcl are snapshots along the reaction pathway

A general mechanism for carbonyl reduction with the SDR enzymes [43] involves proton transfer from the catalytic tyrosine residue to the carbonyl oxygen, coupled with hydride transfer from the nicotinamide ring to the C(sp³) of the carbonyl group. For the oxidation case, the reverse hydride/proton transfer occurs. The catalytic lysine residue should lower the pKₐ of the tyrosine residue, which makes the proton transfer easier and the catalytic serine residue should stabilize the reaction intermediates at the reduction centre. This general mechanism requires a tyrosine residue as a proton/donor acceptor, and proton shuttle to the bulk solvent to re-establish the catalytically competent triad. It has been hypothesized [44] that a buried water molecule and a conserved asparagine residue (Asn^{127} in 17β-HSDcl) proximal to the catalytic lysine residue take part in the proton shuttling from the catalytic lysine residue to the bulk solvent, as observed in apo alcohol dehydrogenase from Drosophila lebanonensis [36]. A proton-relay system has also been suggested for 3HNRmg, where a structurally conserved buried water can bridge the catalytic lysine residue and the conserved asparagine residue [45]. In the present study, we have produced snapshots of 17β-HSDcl in three different states of the enzyme action according to the proposed reaction mechanism. The apoenzyme crystal structure shows a catalytic triad that is well exposed to the solvent molecules that in turn populate both the active site and the coenzyme-binding channel. The holoenzyme and the inhibited ternary complex structures show a quite different water arrangement, with fewer solvent molecules in the active site, and with Lys^{71} having no contacts with any solvent molecules. Only two water molecules are conserved in all but one of the monomers of the holoenzyme structure: the water molecule that bridges His^{164} and Tyr^{212}, and a second water in contact with Asn^{154} and Ser^{153}. In the ternary inhibited complex, only two of three coumestrol-inhibited active sites are populated with solvent molecules. In monomer A, the water molecule bridging Asn^{154} and Ser^{153} in the holoenzyme has also been found, whereas in monomer D a water molecule is engaged in hydrogen bonds with the Tyr^{212} OH and the O3 of coumestrol. Therefore, in contrast with 3HNRmg, in 17β-HSDcl the proton-relay system probably involves His^{164}, Tyr^{212}, Asn^{154} and at least two conserved water molecules. The two individual water molecules found in the coumestrol crystal structure suggest the involvement of a solvent water in the catalytic
The substrate-binding loop of fungal 17β-hydroxysteroid dehydrogenase

Figure 3 NADPH-binding site and hydrogen-bond network and substrate-binding loop flexibility

(A) LIGPLOT representation of the hydrogen-bond network involving the coenzyme in the 17β-HSDcl holo form. (B) Selected interactions of coumestrol with residues belonging to the active-site pocket. Residues forming the catalytic triad are coloured yellow, hydrogen bonds are represented by red broken lines. (C) Superposition of the apo and holo forms of 17β-HSDcl. The substrate-binding loop region of the apo and holo forms are coloured cyan and magenta respectively. Arg28, Asp203, Met204 and Tyr212 are shown in the ball-and-stick representation, as is the coenzyme. (D) Superposition of the apo, holo and ternary forms. Colours for the apo and holo forms are as in (C); the ternary complex is coloured green. The coenzyme and His164, Tyr163, Lys171 and Tyr212 are shown in ball-and-stick representation. A water molecule belonging to the holo form and bridging Tyr212 and His164 is also shown.

proton/hydride-transfer step. However, the substrate would be closer to Tyr167 and to the coenzyme, leaving no room for a nearby solvent molecule (Figure 3B). Thus it appears that the proton shuttling follows the product release after the reduction or oxidation step, as previously proposed for Drosophila alcohol dehydrogenase [43]. Moreover the progressive water depletion that occurs as the reaction progresses could affect the intrinsic pKₐ of Tyr167 as a consequence of the increasing shielding of the catalytic centre from the bulk solvent. Our hypothesis does not contradict the reaction mechanisms described in reactions 1
and 2. A labile enzyme–NADPH–substrate complex is rapidly converted into products in a mechanism that is supported by the stopped-flow experiments described in the present paper, which agrees well with fast proton shuttling following the oxidoreduction step.

**The driving-force for coumestrol binding is π–π interactions between Tyr212 and the coumarin moiety**

The crystal structure of the ternary complex inhibited by coumestrol was also determined. The $2F_{o} - F_{c}$ and $F_{c} - F_{o}$ omit maps showed coumestrol to be present in three out of four possible catalytic pockets (Figure 2D). Its correct position and orientation in the catalytic pockets was established after exploring different orientations of the rigid and asymmetric coumestrol moiety, followed by crystallographic refinement. The model of the present paper was the best that would fit the $2F_{o} - F_{c}$ map together with the lowest $F_{o} - F_{c}$ peak level. The crystallographic analysis shows that the coumestrol occupancy in monomer C was too low to be included in the final crystallographic model. Moreover, the electron-density maps and the refined $B$-factors of the coumestrol suggested an uneven occupation of the other three sites that was not refined nor investigated further. Unequal occupation of the monomers’ active sites is due to packing effects, which differently hinder the accessibility of the diverse active sites as well as the mobility of the different substrate-binding loops, thus modulating the effective affinity of the different active sites.

Coumestrol occupies the putative steroid-binding site inside the catalytic pocket, thus confirming previous studies [27]. This inhibitor shows an interaction pattern common with the enzyme, which involves Tyr$^{127}$ and Asn$^{154}$ (Figure 3B). The driving force for binding appears to be a π–π stacking between the aromatic side chain of Tyr$^{127}$ and the aromatic coumarin moiety (see Table 3 for selected distance values). Coumestrol is ‘sandwiched’ between Tyr$^{127}$ and Gly$^{199}$ with a water bridging its carbonyl to Gly$^{199}$ peptide N, and a hydrogen bond between the same carbonyl and Asn$^{154}$ N32. Additionally, there is also stacking between the hydroxy group in position 3 and the carbonyl of the carboxyamide group of NADPH, resulting in an antiparallel dipole orientation. These interactions appear to be more effective within subunit A, with coumestrol being closer to the nicotinamide moiety. Two hydrogen bonds with two crystallization waters are also observed in all of the coumestrol-occupied monomers. Finally, there is a weak interaction between hydroxy group 3 and $S\delta$ of Met$^{204}$, more relevant in monomer A, with coumestrol pushed slightly towards the coenzyme.

If such an interaction pattern is conserved for steroid substrates, an explanation for their weak binding might arise from steroids not being planar molecules and lacking an extended aromatic system. The relatively strong π–π stacking that is observed between coumestrol and the Tyr$^{127}$ side chain is not possible with steroids, which instead might establish weaker hydrophobic interactions. Moreover, all of the steroids tested as substrates of $17\beta$-HSDcl lack hydrogen-bond donor/acceptor-interacting abilities with Asn$^{154}$, as seen in our inhibited ternary complex. This structural data conforms well with the kinetics results of the weak binding of steroids to the holoenzyme. Indeed, we did not observe bound steroids in any of our attempts to determine the crystal structure of the product/substrate-bound ternary complex, not even when we used the inactive Y167F mutant.

Our structural data agree well with the two kinetics experiments that pinpointed the dissociation of the coenzyme as the rate-determining step of the entire catalytic cycle. First, the direct determination of high coenzyme affinities by native tryptophan fluorescence suggests that the coenzyme dissociation rates are in the range of the enzyme turnover number [35]. Secondly, the concave downwards curve of the initial turnover recording obtained by the rapid kinetics experiment reveals that the proton and hydride transfers during the reaction occur faster than the subsequent exchange of the used coenzyme molecule by a new one.

**The binding of NADPH affects the conformation of the substrate-binding loop**

The substrate-binding loop, which can be roughly defined as a domain that includes residues from 199 to 238, is organized into two almost orthogonal helices that also include several turns and coil subregions. This loop shows an intrinsic flexibility, witnessed by a poor-quality electron-density map and large $B$-factors. The NADPH coenzyme binding induces a conformational transition in the α1 helical region. Two short $3_{10}$-helices in the apo form rearrange as a full α-helix in the holo form. This transition involves Asp$^{203}$ and Met$^{204}$, and is triggered by the establishment of a hydrogen bond between Thr$^{202}$ and the pyrophosphate moiety of NADPH and the formation of a salt bridge between Arg$^{28}$ and Asp$^{203}$ through their side chains (N$\delta$ and O$\nu$). The effect of this different interaction network is 2-fold: region 203–208 assumes a proper α-helix character, capped by a $3_{10}$-helix turn, and the Met$^{204}$ moves closer to the NADPH nicotinamide ring with a Cα shift of 3.8 Å. As a result of this conformational transition, Tyr$^{212}$ that is important for substrate orientation, moves further away from the active site by ~1.4 Å (Figures 3C and 3D).

The binding of the inhibitor does have smaller and local effects on the loop conformation: Tyr$^{212}$ is pushed towards the catalytic site by ~1 Å with rotation of the aromatic ring of ~40° (Figure 3D). In monomer C, where the inhibitor is absent, the position and orientation of Tyr$^{212}$ is substantially coincident with the position assumed in the inhibitor-free holo form.

Structural rearrangements of the substrate-binding loop upon NADPH coenzyme binding can be globally depicted as the movement of a rigid domain. Following the DynDom approach [46], comparisons of the apo and the holo forms revealed a hinge-bending motion of a rigid region that include residues 201–227, with the hinges defined by residues 200–204 and 227–228 respectively, with an average rotation of the rigid region of ~18°. Similar conclusions can be reached by inspecting the error-scaled DDM (distance-difference matrix) calculated with the ESCET

<table>
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<th>Interaction</th>
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<td>Coumarin</td>
<td>Tyr$^{127}$ (side chain)</td>
</tr>
<tr>
<td>Coumarin</td>
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<td>C3-D3</td>
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*Water molecule not present in sites A and B.
† Geometrical requirements for stacking not fulfilled.
program [47]. Regions that are involved in the conformational changes are identified comparing the apo and holo structures, by calculating the differences between equivalent interatomic distances in the two models (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/441/bj4410151add.htm). This analysis shows that the regions that include residues 27–29, 49–55 and 201–227 experience Cα movements that are greater than 1 Å.

The DynDom analysis when comparing the holo form and the coustemol ternary complex shows no rigid domain movement. Accordingly, DDM calculated on the holo form and the ternary complex (see Supplementary Figures S4C and S4D), show that the Cα displacement affects only the central part of the substrate-binding loop. These findings support our hypothesis of minor and local structural rearrangements upon inhibitor binding.

The comparisons of the apo and holo enzyme structures show remarkable structural changes, and it is realistic that a large activation energy is required for coenzyme dissociation. On the other hand, dissociation of the enzyme–NADPH–substrate complex and the release of the product implicate a minor reorganization of the enzymatic system, as seen by our crystal structures and kinetics data, presumably with a lower activation barrier. A substrate-binding loop motion has previously been postulated in the SDR enzyme mechanism [11] and this has been mainly associated with a ‘closure’ of the loop on to the catalytic pocket to protect the reaction centre from the bulk solvent [43]. The present study depicts more subtle conformational differences in this loop in 17β-HSDc1. NADPH binding implicates a ‘closure’ movement of the outer part of the loop, whereas the inner part assumes a more open conformation. This is plausible, as residues 210–220 act as a flexible flap that can control the entrance of the substrate to the active site. The comparison of the holoenzyme with the ternary complex shows that it is only the central part of the loop that experiences this shrinking movement, thus closing the access to the active site (Figure 3D, and see Supplementary Figure S4C). Therefore a more complex situation occurs along the reaction pathway, as a likely consequence of the involvement of Tyr212 in the active site. A previous kinetics study established that the mutation of Tyr212 to alanine slightly decreased the interconversion rates between substrates and products [35]. Indeed, our structural data show that the Tyr212 position is shifted inwards and rotated around Cβ–Cγ upon substrate/inhibitor binding, to accommodate the ligand. This observation suggests that, in 17β-HSDc1, the dynamics of the substrate-binding loop are not just related to a solvent-shielding role, but also to a specific and functionally relevant action.

AUTHOR CONTRIBUTION

Alberto Cassetta designed and performed the crystallographic study. Ivet Krsančova participated in the crystallographic analysis. Katja Kristan and Mojca Brunske Svecelj expressed and purified the protein, and carried out the kinetics experiments. Doriano Lamba participated in the design of the crystallographic study and in the interpretation of the structural data. Tea Lanisnik Rizner initiated the project, and designed and supervised the biochemical studies. Jure Stojan initiated, designed and analysed the kinetics data. Alberto Cassetta co-ordinated the work and, together with Mojca Brunske Svecelj and Jure Stojan wrote the paper. All authors contributed to revising and improving the paper before submission.

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SUPPLEMENTARY ONLINE DATA

Insights into subtle conformational differences in the substrate-binding loop of fungal 17$\beta$-hydroxysteroid dehydrogenase: a combined structural and kinetic approach

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*Istituto di Cristallografia – UOS Trieste, Consiglio Nazionale delle Ricerche, Area Science Park-Basovizza, S.S. 14 km 163.5, I-34149 Trieste, Italy, †Structural Biology Laboratory, Sincrotrone Trieste S.C. p.A., Area Science Park-Basovizza, S.S. 14 km 163.5, I-34149 Trieste, Italy, and ‡Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, SI-1000 Ljubljana, Slovenia

Figure S1  Sequence alignment

Sequence alignment of fungal 17$\beta$-HSDcl with selected fungal and mammalian homologous enzymes (ESPrift web server; http://esprift.ibcp.fr/ESPrift/ESPrift). The secondary structure (top) and residue accessibilities (bottom) are shown (accessible, blue; intermediate, cyan; buried, white). UniprotKB accession numbers: 17$\beta$-HSDcl, O93874; Ver1, P50161; Ver_red, Q00791, 3HNRmg, Q8J2N1; 3HNRcl, Q9HFV6; 4HNRmg, Q92506.

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Structural co-ordinates have been deposited in the PDB for 17$\beta$-hydroxysteroid dehydrogenase of Cochliobolus lunatus under PDB codes 3IS3 (apo), 3ITD (Y167F mutant), 3QWF (holoenzyme) and 3QWI (coumestrol-inhibited ternary complex).

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Figure S2  Reaction progress curves

(A) Progress curves of NADPH disappearance as a consequence of 4-oestrene-3,17-dione reduction by NADPH in the presence of 0.5 \( \mu \)M 17\( \beta \)-HSDcl. (B) Progress curves for NADPH production as a consequence of 4-oestrene-17\( \beta \)-ol-3-one oxidation by NADP\(^+\) in the presence of 0.5 \( \mu \)M 17\( \beta \)-HSDcl. Initial concentrations of NADPH/NADP\(^+\) were in all panels between 20 and 140 \( \mu \)M and the concentrations of the steroid substrate were: panels 1, 20 \( \mu \)M; panels 2, 40 \( \mu \)M; panels 3, 60 \( \mu \)M; panels 4, 80 \( \mu \)M; panels 5, 100 \( \mu \)M; panels 6, 120 \( \mu \)M; panels 7, 140 \( \mu \)M. The lower-right-hand panels show initial rates as determined from progress curves in panels 1–7.
Figure S3  Quaternary structure

Solvent-accessible surface of the crystallographic 17β-HSDcl tetramer together with the P and Q reference two-fold axes. Interacting surface residues are coloured according to their different localizations (P-interface, blue; Q-interface, red).
Figure S4  Distance-difference matrices

Error-weighted DDMs calculated on $\alpha_C$ for different $17\beta$-HSDcl forms. All of the DDMs were calculated for the same region of residues 15–270. Secondary-structure elements are indicated (bottom panels) by black (strand) or white (helix) rectangles respectively. White background indicates rigidly moving regions, as determined by the ESCET program. For every matrix, the upper triangle reports the absolute difference in Å. The blue and red colours indicate a shortening or lengthening of the distances respectively. The lower triangles describe the differences in distances in terms of $\sigma$ with respect to the positional uncertainties (the same colour coding as for the absolute differences). (A) Comparison between the apo and the holo forms shows that residues in the regions 200–208 and 218–226 experience a general shortening of their intramolecular $\alpha_C-\alpha_C$ distances, whereas residues 212–218 tend to widen their intramolecular contacts. (B) Comparison of the apo and the coumestrol-inhibited ternary forms. Only a shortening of the distances involving regions 200–208 and 218–226 are apparent. (C) Comparison of the holo form and the coumestrol-inhibited ternary complex. The region 212–218 experiences a shortening of the $\alpha_C-\alpha_C$ distances, resulting in a movement towards the catalytic pocket. (D) The same as (C), but for the inhibitor kaempferol (A. Cassetta, I. Krastanova and D. Lamba, unpublished work).
Table S1  Quaternary structure interface parameters

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†Values obtained by using the PROTORP web server: http://www.bioinformatics.sussex.ac.uk/protorp/.
‡Values obtained by using the WAP web server: http://dicsoft2.physics.iisc.ernet.in/wap/.