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ARTICLE *in* THE JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY · MARCH 2011

Impact Factor: 3.63 · DOI: 10.1016/j.jsbmb.2011.03.019 · Source: PubMed

Journal of Steroid Biochemistry & Molecular Biology 129 (2012) 92–98

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

The role of Ala231 and Trp227 in the substrate specificities of fungal 17β-hydroxysteroid dehydrogenase and trihydroxynaphthalene reductase: Steroids *versus* smaller substrates

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ARTICLE INFO

Article history:
Received 3 February 2011
Received in revised form 4 March 2011
Accepted 12 March 2011

Keywords:
Curvularia lunata (teleomorph: *Cochliobolus lunatus*)
Trihydroxynaphthalene reductase
17β-Hydroxysteroid dehydrogenase
4-Estrene-3,17-dione
4-Estrene-17β-ol-3-one
2,3-Dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one
9,10-Phenanthrenequinone

ABSTRACT

17β-Hydroxysteroid dehydrogenase and trihydroxynaphthalene reductase from the fungus *Curvularia lunata* (teleomorph: *Cochliobolus lunatus*; 17β-HSDcl and 3HNR, respectively) are two homologous short-chain dehydrogenase/reductase proteins that are 58% identical and have 86% similar amino acids. The minor differences in their substrate-binding regions are believed to be crucial for their substrate specificities. 3HNR shows high affinity for substrates with two rings, like trihydroxynaphthalene and 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one (DDBO), while 17β-HSDcl can accommodate ligands with four rings, like steroids. In the present study, we examined the role of Ala231 in 17β-HSDcl and Trp227 in 3HNR, as the potential key amino acids in the determination of substrate recognition based on size. We constructed Ala231Trp 17β-HSDcl and Trp227Ala 3HNR mutant proteins and used spectrophotometric analyses to compare their catalytic activities with those of the wild-type enzymes, for oxidation of 4-estrene-17β-ol-3-one and DDBO and for reduction of 4-estrene-3,17-dione and 9,10-phenanthrenequinone (PQ). The Ala231Trp side-chain substitution in 17β-HSDcl abolished and decreased (by 14.6-fold) the initial rates for steroid oxidation and reduction, respectively, while the initial rate for PQ reduction was increased 5.6-fold. The bulky Trp227Ala side-chain substitution in 3HNR enabled oxidation of 4-estrene-17β-ol-3-one, increased the initial rates for reduction of 4-estrene-3,17-dione and PQ by 4.5-fold and 1.5-fold, respectively, while the initial rate for DDBO oxidation was decreased 4.1-fold. Our TLC analysis and docking simulations also support these findings. Our study thus confirms the important roles of Ala231 in 17β-HSDcl and Trp227 in 3HNR, for the selection between larger and smaller substrates.

Article from a special issue on steroids and microorganisms.

1. Introduction

Curvularia lunata (teleomorph: *Cochliobolus lunatus*) is a dark pigmented filamentous fungus that resides primarily in the soil [1]. It is a known plant and human pathogen, and thus it is a particularly interesting target for the development of novel fungicides and antimycotics [2–4]. In the present study, our attention was focused on two enzymes from this fungus: 17β-hydroxysteroid dehydrogenase (17β-HSDcl), with an unknown physiological function and trihydroxynaphthalene reductase (3HNR), which is involved in 1,8-dihydroxynaphthalene-melanin biosynthesis. 17β-HSDcl appears not to be essential for fungal survival or pathogenicity (our unpublished data), however it has biotechnological potential for the stereospecific biotransformation of steroids at position C-17. 3HNR is an interesting novel drug target [5,6], as melanin is a known virulence factor that protects pigmented fungi against environmental stress and host defense mechanisms, and thus has an important role in their pathogenicity [7]. Both of these enzymes, 17β-HSDcl and 3HNR, belong to the short-chain dehydrogenase/reductase superfamily [5,8]. They are 58% identical and have 86% similar amino-acid residues, and consequently they have a similar backbone organization and catalytic site amino-acid arrangement [9]. Homology-built models have been constructed for 17β-HSDcl and 3HNR based on the crystal structure of 3HNR from *Magnaporthe grisea* [6,10,11].

17β-HSDcl catalyses oxidoreductions of estrogens and androgens at position C-17, while no significant conversions at positions C-3 and C-20 have been observed [10]. Among all of the tested steroids, 4-estrene-3,17-dione was converted with highest affinity

Abbreviations: 3HNR, trihydroxynaphthalene reductase; 17β-HSDcl, 17β-hydroxysteroid dehydrogenase from fungus *Cochliobolus lunatus*; DBO, 4,5-dihydroxy-2*H*-benzopyran-2-one; DDBO, 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one; PQ, 9,10-phenanthrenequinone; TLC, thin-layer chromatography.

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doi:10.1016/j.jsbmb.2011.03.019

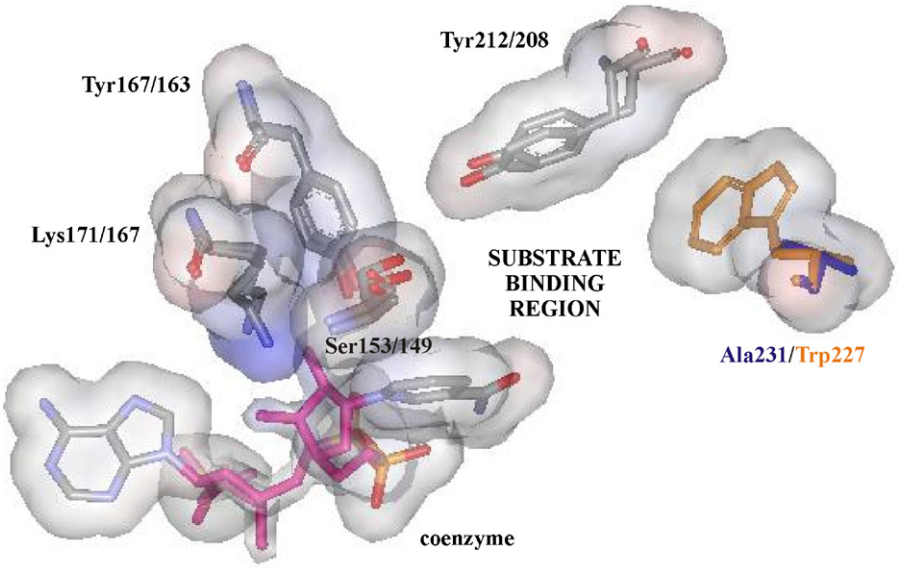

Fig. 1. View of the superimposed active sites of 17β-HSDcl and 3HNR. The catalytic amino acids of Ser153/149, Tyr167/163 and Lys171/167, and the amino acids Tyr212/208 and Ala231 (blue) and Trp227 (orange) are shown for 17β-HSDcl and 3HNR, respectively.

[12]. Further substrate specificity studies have revealed that 17β-HSDcl preferentially catalyses 17-keto reduction of 3,17-keto steroid substrates with a flexible (saturated) ring A [13]. 3HNR catalyses the reduction of 1,3,8-trihydroxynaphthalene to vermelone, which is then further dehydrated to 1,8-dihydroxynaphthalene, and finally 1,8-dihydroxynaphthalene-melanin is formed by enzymatic polymerization [14]. 1,3,8-Trihydroxynaphthalene is the physiological substrate of 3HNR, however it is not stable in aerated solutions and thus inappropriate for in vitro enzymatic assays. The alternative nonphysiological substrate of 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one (DDBO) has been synthesized and its oxidation by 3HNR has been characterized kinetically [6,15,16]. In addition to DDBO, 3HNR catalyses the conversion of steroids to some extent; however, 17β-HSDcl has no activity toward DDBO [9,10]. Although the majority of the carbonyl reductases reduce 9,10-phenanthrenequinone (PQ) [17], its reduction by 17β-HSDcl and 3HNR have not yet been examined.

The amino-acid residues responsible for the substrate specificity of 17β-HSDcl have already been studied. Inspection of a model of the three-dimensional structure of 17β-HSDcl indicated that Val161 and Tyr212 might interact with the C-19 methyl groups of androgens and thus prevent their correct accommodation within the 17β-HSDcl active site. The Val161Gly and Tyr212Ala mutant proteins of 17β-HSDcl were constructed in an attempt to increase the specificity for androgens. Increased initial rates for the conversion of androgens were indeed seen for both of these 17β-HSDcl mutant proteins, which confirmed the limiting role of Val161 and Tyr212 for the turnover of androgens [18]. To determine the importance of His230 and Ala231 in the substrate specificity of 17β-HSDcl, His230Ala and Ala231Ser mutant proteins were prepared. These investigations indicated that His230 and Ala231 do not affect the 17β-HSDcl specificity for different steroids [19]. Although site-directed mutagenesis has not yet been performed with 3HNR from *C. lunata*, other studies have demonstrated that 3HNR prefers ligands with two rings (e.g. 1,3,8-trihydroxynaphthalene, DDBO), while the active site of 17β-HSDcl can accommodate ligands with four-ring systems (e.g. the steroids). As suggested by homology-built models, these different substrate specificities can be explained by differences in their substrate-binding sites [9].

The substrate-binding regions of 17β-HSDcl and 3HNR differ in the loop that is positioned just before the catalytic αF helix (residues 153–157 of 3HNR) and in the substrate-binding loop (residues 198–227 of 3HNR). The 17β-HSDcl substrate-binding region has an alanine at the 231 position, while 3HNR has a tryptophan at the corresponding 227 position (Fig. 1). We hypothesized that with 17β-HSDcl, the small side-chain residue of Ala231 will allow the accommodation of larger substrates, on the other hand the bulkier Trp227 residue of 3HNR will prevent their binding, while potentially providing additional interactions for smaller substrates, like 1,3,8-trihydroxynaphthalene and DDBO. To better understand the substrate specificities and the active sites of both of these enzymes, which should shed light on their evolution and help in the further design of inhibitors, we here examined the roles of Ala231 in 17β-HSDcl and Trp227 in 3HNR.

2. Materials and methods

2.1. Site-directed mutagenesis

The mutant proteins were prepared using Quick Change II Site-Directed Mutagenesis kit (Stratagene) and the pGex-17β-HSDcl and pGex-3HNR expression vectors. The following primers were used (only forward primers shown, with the mutations introduced underlined):

- 17β-HSDcl, Ala231Trp: 5′-GCAGATGGCTGCGCACTGGTCCCCAC-TGCACCG-3′
- 3HNR, Trp227Ala: 5′-CGAGTACGCCTGCACAGCGTCTCCCCACA-ACCG-3′

The complete coding regions of the mutated cDNAs were confirmed by sequencing.

2.2. Expression and purification of the recombinant proteins

Recombinant 17β-HSDcl and 3HNR wild-type and mutant proteins were prepared as glutathione S-transferase (GST)-fusion proteins in *Escherichia coli* BL21 cells (wild-type) and *E. coli*

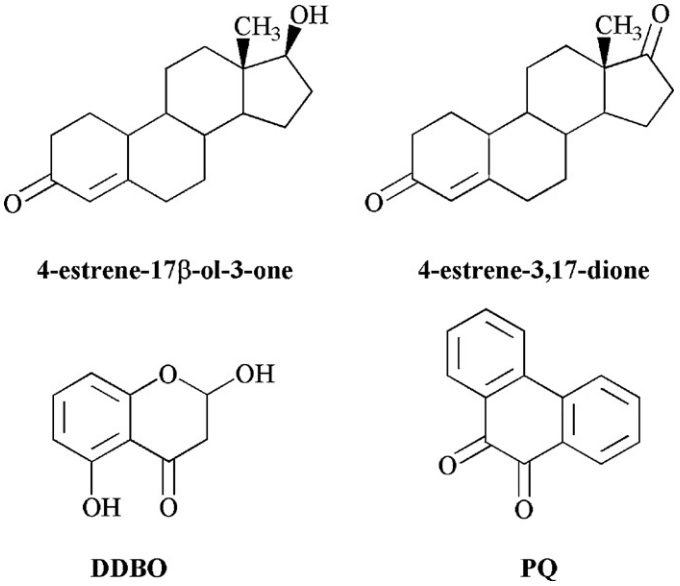

Fig. 2. The substrates of the wild-type and mutant 17β-HSDcl and 3HNR proteins used in this study. DDBO, 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one; PQ, 9,10-phenanthrenequinone.

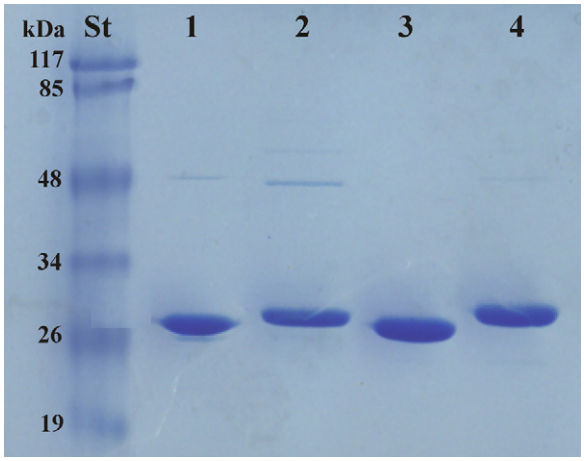

Fig. 3. Characterisation of the recombinant proteins by SDS-PAGE with staining with Coomassie blue. Five μg protein was applied in each lane of 12% polyacrylamide gels. St, standard; lane 1, wild-type 17β-HSDcl; lane 2, wild-type 3HNR; lane 3, Ala231Trp 17β-HSDcl mutant; lane 4, Trp227Ala 3HNR mutant.

BL21-CodonPlus(DE3)-RIL cells (mutants). They were purified by affinity binding to glutathione-Sepharose, followed by cleavage with thrombin, as described previously [8]. Protein concentrations were determined according to Bradford [20], with bovine serum albumin as standard. The purity of proteins was checked by SDS-PAGE on 12% polyacrylamide gels with Coomassie blue staining [21].

2.3. Determination of enzymatic activities

2.3.1. Spectrophotometric assay

The oxidation of 4-estrene-17β-ol-3-one and DDBO and the reduction of 4-estrene-3,17-dione and PQ (Fig. 2) were followed spectrophotometrically in the presence of the coenzymes $NADP^+$ and NADPH and wild-type 17β-HSDcl and 3HNR and their mutant proteins, using a Perkin Elmer, Lambda 45, UV/VIS spectrophotometer. The differences in NADPH absorbance were measured from 10 s to 130 s at 340 nm and 25 °C. The assays were carried out in 1 ml 100 mM phosphate buffer, pH 8.0 and the final dimethyl sulfoxide concentration of 1% was used. The substrate concentration of 100 μM was used, with 200 μM coenzyme and 0.5 μM enzyme. The measurements were performed in triplicate.

2.3.2. Thin-layer chromatography

Recombinant 17β-HSDcl and 3HNR wild-type and mutant proteins (0.33 μM) were incubated with 200 μM 4-estrene-17β-ol-3-one, 4-estrene-3,17-dione or DDBO in the presence of 400 μM of the appropriate coenzyme ($NADP^+$ or NADPH) in 1 ml 100 mM phosphate buffer, pH 8.0, for 15 min at room temperature. The final dimethyl sulfoxide concentrations of 1% to 2% were used to improve the substrate solubilities. The products of the enzymatic reactions were extracted by ethyl acetate (steroids) and dichloromethane and ethyl acetate (DDBO), and analyzed by thin-layer chromatography (TLC) on silica gel plates in the solvent systems of chloroform:methanol:water (94:6:0.5) for steroids and dichloromethane:methanol:acetic acid (90:10:0.2) for DDBO. The substrates and products were visualized under UV light.

2.4. Docking simulations

Three-dimensional homology modeling of 3HNR from the fungus *C. lunata* was carried out as previously reported [6]. Briefly, the sequence of 3HNR was aligned with the sequence of 3HNR from *Magnaporthe grisea* (PDB code 1G0N) and 17β-HSDcl (PDB code 3IS3). Multiple alignment gave 74% and 58% sequence identities, respectively. Both of these resolved crystal structures were thus used as templates for homology building using the BLDPIR command in the WHATIF molecular modeling package. As 3IS3 represents an apo form of 17β-HSDcl, we used the binding conformation of the coenzyme and the surrounding residues from the 1G0N structure to obtain its holo form. For the two compensatory mutants, we substituted the coordinates of residues Ala231 from 17β-HSDcl and Trp227 from 3HNR accordingly. The docking of the substrates 4-estrene-3,17-dione, DDBO and PQ into the active sites of the proteins was carried out by putting the appropriate carbon atoms of the ligands above the C-4 of the nicotinamide moiety of the $NADP^+$ and NADPH, respectively. The hydroxyl oxygen of DDBO and the carbonyl oxygens at C-17 of

Table 1
Substrate specificities of the wild-type and mutant 17β-HSDcl and 3HNR proteins.

Substrate	Initial velocity ± SD (μmol/l s)			
	17β-HSDcl, wild-type	17β-HSDcl, Ala231Trp	3HNR, wild-type	3HNR, Trp227Ala
4-estrene-17β-ol-3-one	0.156 ± 0.012	NA	NA	0.172 ± 0.009
4-estrene-3,17-dione	0.219 ± 0.010	0.015 ± 0.007	0.032 ± 0.007	0.145 ± 0.007
DDBO	NA	NA	4.67 ± 0.42	1.13 ± 0.01
PQ	0.037 ± 0.002	0.208 ± 0.014	0.482 ± 0.011	0.704 ± 0.015

Initial velocities (means ± standard deviation) for the conversion of the four substrates tested (100 μM) in the presence of the appropriate coenzyme (200 μM) at pH 8.0 and 25 °C with wild-type 17β-HSDcl and its Ala231Trp mutant protein and wild-type 3HNR and its Trp227Ala mutant protein (all at 0.5 μM). DDBO, 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one; PQ, 9,10-phenanthrenequinone; NA, no activity observed.

4-estrene-3,17-dione and C-9 of PQ were set at the H-bond distance to the OH-1 atom of the active-site tyrosine for each enzyme. Furthermore, two subsequent 100-step quantum mechanics and molecular mechanics (QMMM) optimization runs were performed: the first with fixed coenzyme and the two above-mentioned atoms of each ligand; and the second without any constraints. In each complex, we treated the ligands (4-estrene-3,17-dione, DDBO or PQ), the nicotinamide ring with ribose, the active-site tyrosine, and the serine and lysine terminal protonated amino groups quantum mechanically, while the rest of the system was treated molecular mechanically.

3. Results and discussion

In the present study, we focused our attention on a comparison of the active sites of 17β-HSDcl and 3HNR, and consequently on a comparison of their substrate specificities. The aim was twofold: to test the hypotheses that (i) the side-chain residue of Ala231 in 17β-HSDcl, which is oriented toward the A ring of the steroid substrate, allows the correct accommodation of steroids within the active site, and (ii) the corresponding larger Trp227 side-chain residue in 3HNR blocks the binding of large substrates, although it provides additional interactions for the binding of smaller substrates. We prepared Ala231Trp 17β-HSDcl and Trp227Ala 3HNR mutant proteins in *E. coli* and purified them to homogeneity (Fig. 3). We then followed the oxidoreductions of four substrates, 4-estrene-17β-ol-3-one, 4-estrene-3,17-dione, DDBO and PQ, both spectrophotometrically and by TLC. Finally, docking simulations of these substrates into the active sites of these wild-type and mutant proteins were performed to demonstrate their accommodation and determine the potentiality for their conversion.

3.1. Ala231 allows the correct accommodation and conversion of steroids in 17β-HSDcl

As previously known, wild-type 17β-HSDcl preferentially catalyses oxidoreductions of the steroid substrates 4-estrene-17β-ol-3-one and 4-estrene-3,17-dione. When we tested oxidation of DDBO and reduction of PQ by wild-type 17β-HSDcl spectrophotometrically, it showed no activity toward DDBO, while conversion of PQ was detected (Table 1 and Fig. 4). On the other hand, the Ala231Trp 17β-HSDcl mutant protein did not oxidize the steroid substrate 4-estrene-17β-ol-3-one and showed only minor reduction of 4-estrene-3,17-dione (less than 10% of the initial rate in comparison to wild-type 17β-HSDcl). This substitution of Ala231 with tryptophan in 17β-HSDcl did not improve its activity toward DDBO. However, this substitution resulted in a 5.6-fold increase in the initial rate for the reduction of PQ. TLC analysis of the steroid conversions by wild-type 17β-HSDcl and its Ala231Trp mutant confirmed these findings. In the case of the wild-type enzyme, the reactions proceeded to equilibrium in a 15-min incubation (K_{eq} = 0.42; [22]), while the Ala231Trp 17β-HSDcl mutant protein did not oxidize 4-estrene-17β-ol-3-one, although some reduction of 4-estrene-3,17-dione did occur (Fig. 5). Additionally, TLC analysis demonstrated that oxidation of DDBO was not catalyzed by either 17β-HSDcl or its Ala231Trp mutant protein (Fig. 5).

We can therefore conclude that the substitution of Ala231 with Trp in 17β-HSDcl prevents the oxidoreduction of these steroids. The bulkier tryptophan side-chain residue still does not allow the correct accommodation and stabilization of two-ring substrates (e.g. DDBO) for any reaction to occur; however, it provides the appropriate binding interactions for three-ring substrates, as shown by the increased initial velocity for PQ reduction.

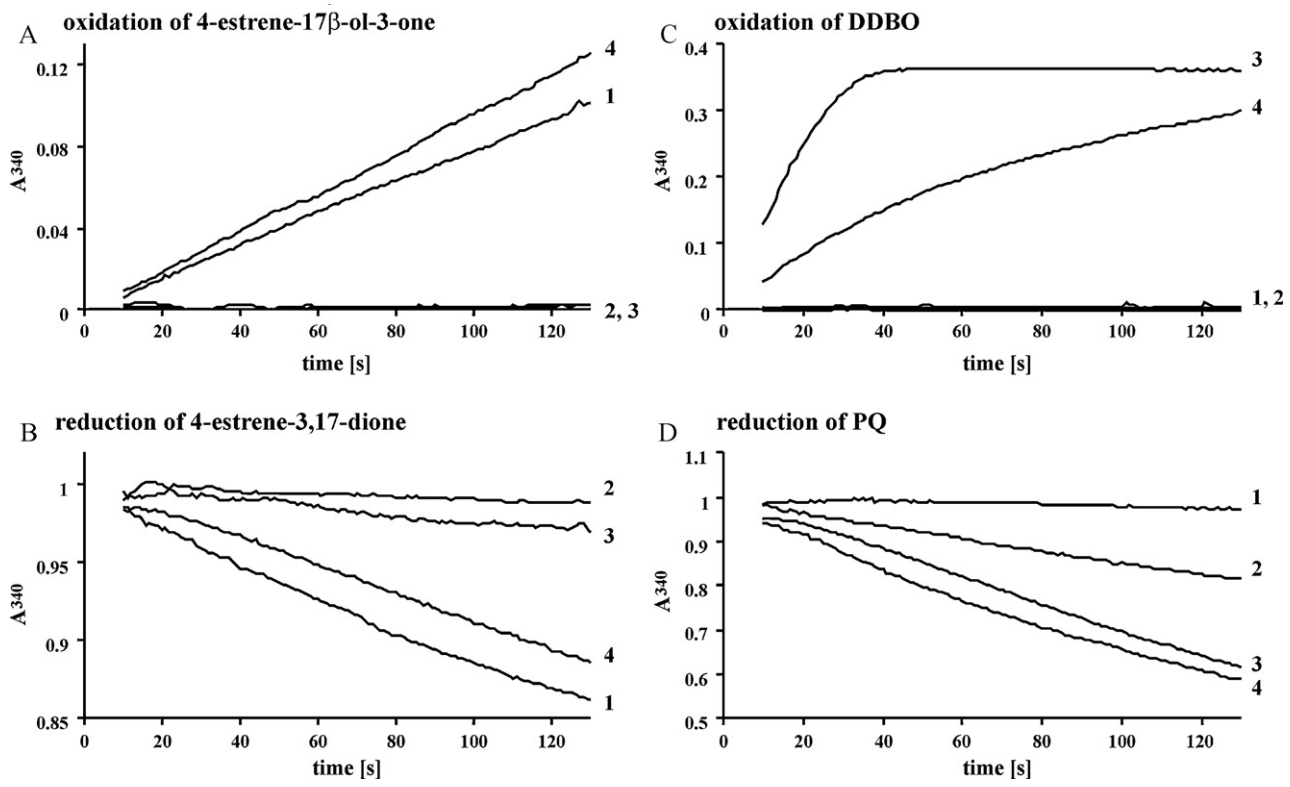

Fig. 4. Spectrophotometric analyses of the substrate specificities. Progress curves for the oxidation of 4-estrene-17β-ol-3-one (A) and DDBO (C) and the reduction of 4-estrene-3,17-dione (B) and PQ (D) (all at 100 μM) by wild-type 17β-HSDcl (1), the Ala231Trp 17β-HSDcl mutant protein (2), wild-type 3HNR (3) and the Trp227Ala 3HNR mutant protein (4) (all at 0.5 μM) in the presence of the respective coenzymes (200 μM) at pH 8.0 and 25 °C. DDBO, 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one; PQ, 9,10-phenanthrenequinone.

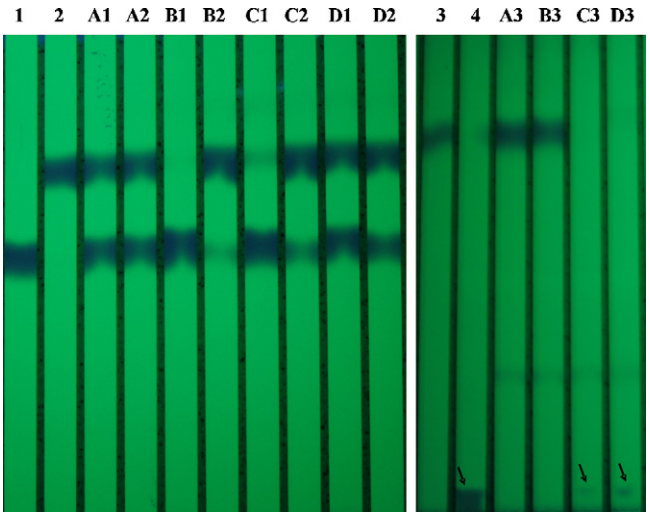

Fig. 5. TLC analysis of the substrate specificities. Lane labels: (1), 4-estrene-17β-ol-3-one; (2), 4-estrene-3,17-dione; (3), DDBO; (4), DBO; (A1), oxidation of 4-estrene-17β-ol-3-one by 17β-HSDcl; (A2), reduction of 4-estrene-3,17-dione by 17β-HSDcl; (B1), oxidation of 4-estrene-17β-ol-3-one by Ala231Trp; (B2), reduction of 4-estrene-3,17-dione by Ala231Trp; (C1), oxidation of 4-estrene-17β-ol-3-one by 3HNR; (C2), reduction of 4-estrene-3,17-dione by 3HNR; (D1), oxidation of 4-estrene-17β-ol-3-one by Trp227Ala; (D2), reduction of 4-estrene-3,17-dione by Trp227Ala; (A3) oxidation of DDBO by 17β-HSDcl; (B3) oxidation of DDBO by Ala231Trp; (C3) oxidation of DDBO by 3HNR; (D3) oxidation of DDBO by Trp227Ala. The substrate specificities of the enzymes (0.33 μM) were tested with 200 μM substrate and 400 μM coenzyme, in 100 mM phosphate buffer, pH 8.0, for 15 min at room temperature. Samples were extracted by ethyl acetate (steroids) and dichloromethane and ethyl acetate (DDBO), analyzed by TLC in the solvent system chloroform:methanol:water (94:6:0.5) for steroids and dichloromethane:methanol:acetic acid (90:10:0.2) for DDBO, and visualized under UV light. The position of DBO in the TLC plate is shown by the arrows. DDBO, 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one; DBO, 4,5-dihydroxy-2*H*-benzopyran-2-one.

3.2. Trp227 is responsible for stabilization of two-ring and three-ring substrates in 3HNR

Our spectrophotometric analysis showed that the wild-type 3HNR enzyme does not oxidize 4-estrene-17β-ol-3-one, with only a minor reduction of 4-estrene-3,17-dione observed (Table 1 and Fig. 4). On the other hand, a nonphysiological substrate of 3HNR, DDBO, was rapidly oxidized, and PQ was also reduced with about a 10-fold lower initial velocity, compared to that of DDBO. In the case of the Trp227Ala 3HNR mutant protein, there was rapid oxidation of the steroid substrate 4-estrene-17β-ol-3-one, with a 10% higher initial rate compared to that of its conversion by wild-type 17β-HSDcl. This substitution also resulted in a 4.5-fold increased reduction of 4-estrene-3,17-dione by the Trp227Ala 3HNR mutant protein, in comparison to wild-type 3HNR. In addition to a better conversion of steroid substrates with this Trp227Ala 3HNR mutant protein, there was a 1.5-fold higher initial rate for its reduction of PQ, in comparison to wild-type 3HNR. As expected, the Trp227Ala 3HNR mutant protein oxidized DDBO 4.1-fold slower compared to wild-type 3HNR. TLC analysis showed that the steroid substrates 4-estrene-17β-ol-3-one and 4-estrene-3,17-dione are converted by wild-type 3HNR and its Trp227Ala mutant protein (Fig. 5); here, with the mutant protein equilibrium was reached, while with wild-type 3HNR steroid substrates were only converted to a small extent. When DDBO oxidation was followed by TLC analysis, with wild-type 3HNR and its Trp227Ala mutant protein, no DDBO was detected on the silica gel plates; only its oxidized product 4,5-dihydroxy-2*H*-benzopyran-2-one (DBO) was seen (Fig. 5). Thus we can conclude that DDBO was completely oxidized by both wild-type 3HNR and its Trp227Ala mutant protein in the 15-min incubation. This result is in agreement with our previous findings, where we showed that the equilibrium of this reaction at pH 8 is largely ($K_{eq} = 96 \pm 25$) shifted toward the oxidized substrate form (DBO) [16].

Our data thus show that Trp227 has a very important role in substrate specificity of 3HNR. In the absence of this bulky amino acid, steroids and substrates with three rings are better converted by 3HNR, while the initial rates for the conversion of the two-ring substrate (DDBO) were notably lower.

3.3. Docking simulations of 4-estrene-3,17-dione, DDBO and PQ within the active sites of the 17β-HSDcl and 3HNR proteins

To further clarify the substrate recognition by these various proteins, the substrates 4-estrene-3,17-dione, DDBO and PQ were docked into the active sites of the wild-type and mutant 17β-HSDcl and 3HNR proteins. The docking simulations revealed that all three of these substrates are sandwiched between the nicotinamide ring of the coenzyme ($NADP^+$ or NADPH) and the Pro197-Gly198-Gly199 (for 17β-HSDcl) or Pro193-Gly194-Gly195 (for 3HNR) on one side, and the phenol ring of Tyr212 or Tyr208 (for 17β-HSDcl and 3HNR, respectively) on their other side (Fig. 6). All three of the substrates were positioned for potential hydride transfer from/to the nicotinamide of NADPH or $NADP^+$, although they differed in the distances between the C-4 of the nicotinamide moiety and the C-17 of 4-estrene-3,17-dione, the C-9 of PQ and the C-2 of DDBO. All of these three substrates also formed H-bonds with the catalytic amino acids of Tyr167 and Tyr163 (17β-HSDcl, 3HNR, respectively), although again, they differed in the additional stabilization through H-bonding to Ser153 and Ser149 (17β-HSDcl, 3HNR, respectively), as well as in their hydrophobic interactions.

In the Ala231Trp 17β-HSDcl mutant protein, the Trp231 forced 4-estrene-3,17-dione into a bent position, where this steroid cannot be optimally stabilized (Fig. 6A), thus lowering its catalytic activity. In wild-type 17β-HSDcl and its Ala231Trp mutant protein, the distance between DDBO and the catalytic Tyr167 was greater, as compared to that for the steroid substrates and PQ (Fig. 6B). In the Ala231Trp 17β-HSDcl mutant protein, DDBO interacted with the Trp231, but did not form an H-bond with the Ser153, and thus DDBO was still not appropriately stabilized for any reaction to occur. PQ was in contact distance to Trp231 and formed an H-bond with Ser153 as well as a bifurcated H-bond with Tyr167. PQ was thus better stabilized in the Ala231Trp 17β-HSDcl mutant protein, as compared to the wild-type 17β-HSDcl enzyme, where only one H-bond was formed, with Tyr167 (Fig. 6C). These data thus correlate well with the increased initial rate for PQ reduction by the Ala231Trp 17β-HSDcl mutant protein.

With the Trp227Ala 3HNR mutant protein, 4-estrene-3,17-dione formed H-bonds with Tyr163 and Ser149, as it also did in the wild-type 3HNR enzyme (Fig. 6D). The 4.5-fold difference in the activity here can be explained by the forced bent conformation of the steroid substrate in the wild-type 3HNR enzyme, as also seen in the Ala231Trp 17β-HSDcl mutant protein. In wild-type 3HNR and its Trp227Ala mutant protein, DDBO H-bonded to Tyr163 (Fig. 6E). In spite of the lack of an H-bond with Ser149 in the wild-type 3HNR enzyme, DDBO was here still better stabilized by the hydrophobic interactions, which included Trp227; this can explain the higher initial rate with the wild-type 3HNR protein for DDBO oxidation, as compared to its Trp227Ala mutant protein. In both the wild-type 3HNR and its Trp227Ala mutant protein, PQ formed an H-bond with Ser149 and a bifurcated H-bond with Tyr163 (Fig. 6F). As the Trp227Ala 3HNR mutant protein showed only a 1.5-fold higher initial rate for PQ reduction than the wild-type 3HNR protein, the docking simulation here did not provide any reasonable explanation of this observation.

These docking simulation studies of 4-estrene-3,17-dione, DDBO and PQ in the active sites of the wild-type and the mutant

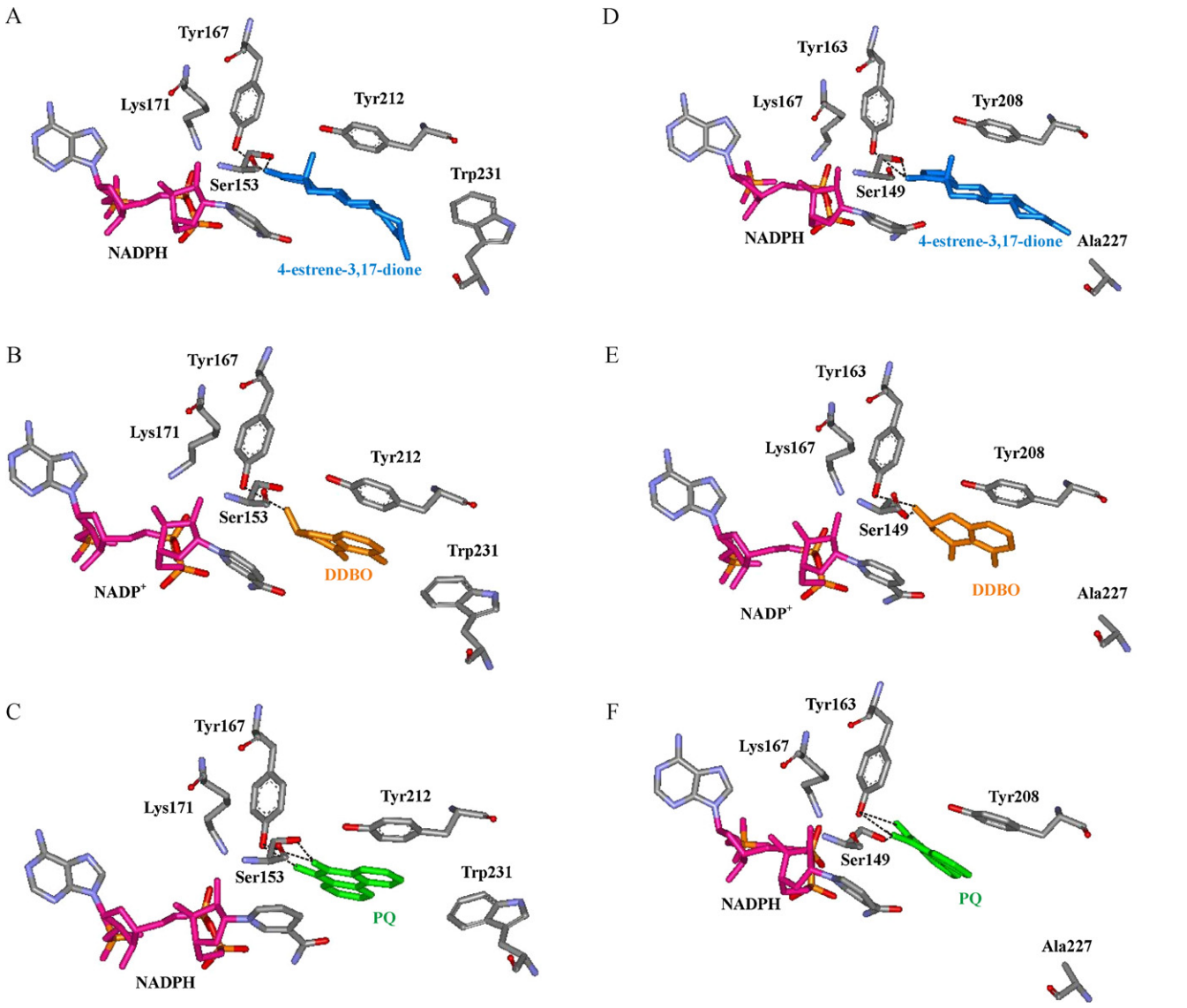

Fig. 6. The docking positions of 4-estrene-3,17-dione, DDBO and PQ in the mutant protein active sites. (A–C) Within the Ala231Trp 17β-HSDcl mutant protein active site, for 4-estrene-3,17-dione and coenzyme NADPH (A), DDBO and coenzyme $NADP^+$ (B), and PQ and coenzyme NADPH (C). (D–F) Within the Trp227Ala 3HNR mutant protein active site, for 4-estrene-3,17-dione and coenzyme NADPH (D), DDBO and coenzyme $NADP^+$ (E), and PQ and coenzyme NADPH (F). The catalytic amino acids Ser153/149, Tyr167/163 and Lys171/167, and the amino acids Tyr212/208 and Trp231/Ala227 are shown for the Ala231Trp 17β-HSDcl and Trp227Ala 3HNR mutant proteins, respectively. DDBO, 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one; PQ, 9,10-phenanthrenequinone.

17β-HSDcl and 3HNR proteins mainly support our spectrophotometric and TLC data.

4. Conclusions

In the present study, we have shown that the amino-acid residues of Ala231 in 17β-HSDcl and the corresponding Trp227 in 3HNR have important roles in the substrate specificities of these enzymes. Substitution of the small Ala231 side-chain residue by this larger tryptophan in 17β-HSDcl resulted in its decreased activity toward steroid substrates and increased initial rate of reduction of PQ. On the other hand, substitution of the bulky Trp227 side-chain residue in 3HNR by alanine increased the conversion of steroid substrates and PQ, and decreased the oxidation of the smaller DDBO. Our docking simulation studies mainly confirmed these experimental data. We can thus conclude that Ala231 in 17β-HSDcl and Trp227 in 3HNR are the key amino acids for the determination of substrate recognition based on substrate size.

Acknowledgments

This study was supported by a P1-170 grant and a young researcher grant to M.B.Š, both from the Slovenian Research Agency. The authors thank Dr. Chris Berrie for critical reading of the manuscript.

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