Available online at www.sciencedirect.com
SCIENCE DIRECT®
Chemico-Biological Interactions
Chemico-Biological Interactions 143–144 (2003) 493–501
www.elsevier.com/locate/chembioint

Significance of individual amino acid residues for coenzyme and substrate specificity of 17β-hydroxysteroid dehydrogenase from the fungus *Cochliobolus lunatus*

Katja Kristan [a], Tea Lanišnik Rižner [a,*], Jure Stojan [a], Josef K. Gerber [b], Elisabeth Kremmer [c], Jerzy Adamski [b]

[a] *Medical Faculty, Institute of Biochemistry, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia*
[b] *Institute of Experimental Genetics, GSF National Research Centre for Environment and Health, Neuherberg, Germany*
[c] *Institute of Molecular Immunology, GSF National Research Centre for Environment and Health, Neuherberg, Germany*

Abstract

17β-Hydroxysteroid dehydrogenase from the fungus *Cochliobolus lunatus* (17β-HSDcl) is a NADPH dependent member of the short-chain dehydrogenase reductase (SDR) superfamily. Recently, we prepared a homology-built structural model of 17β-HSDcl using the known three-dimensional structure of homologous 1,3,8-trihydroxynaphthalene reductase from the fungus *Magnaporthe grisea*. This model structure directed our studies of structure–function relationship of the fungal 17β-HSD, as one of the model enzymes of the SDR superfamily. In this work, we investigated the significance of individual amino acid residues for coenzyme and substrate specificity. We performed site directed mutagenesis of R28, a basic residue conserved in most NADPH dependent SDR structures; T200, found only in *Streptomyces hydrogenans* 3α,20β-HSD and *Drosophila* alcohol dehydrogenases; and H230, a residue corresponding to the substrate specificity important H221 in human 17β-HSD type 1. All recombinant proteins were expressed in *Escherichia coli* and purified to homogeneity. Kinetic evaluation of individual mutations was performed by analysis of progress curves of interconversions between 4-estrene-3,17-dione and 4-estrene-17β-ol-3-one, in the presence of NADPH and $NADP^+$; according to the Theorell–Chance reaction mechanism. The results demonstrate the role of the selected amino acid residues; R28 seems to interact with the NADPH 2′-phosphate group; T200 may be involved in binding and dissociation of NADPH/$NADP^+$; while H230 and the neighboring A231 appears not to be responsible for substrate specificity of 17β-HSDcl.

Keywords: 17β-Hydroxysteroid dehydrogenase; SDR; *Cochliobolus lunatus*; Fungi

1. Introduction

Short-chain dehydrogenases/reductases (SDR) constitute one of the largest protein superfamilies

* Corresponding author. Tel.: +386-1-543-7657; fax: +386-1-543-7641.
E-mail address: tea.lanisnik-rizner@mf.uni-lj.se (T.L. Rižner).

0009-2797/02/$ - see front matter © 2002 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0009-2797(02)00205-3

known to date, represented in all kingdoms of life [1–3]. SDR proteins are non-metalloenzymes, mostly 25–35 kDa, that usually function as dimers or tetramers [4]. They share ≈15–30% of identical amino acids, including the typical SDR motifs, that are conserved among all members, e.g. the coenzyme binding motif G-X-X-X-G-X-G and the catalytic site Y-S-X-X-K [5]. Site directed mutagenesis of the conserved amino acid residues have been performed in several members of the SDR superfamily. These studies have delimited Y, K and S as a catalytically important 'triad' of residues [4,6]. Tyrosine acts as a basic catalyst, lysine binds NAD(P)H and lowers the pK_a value of tyrosine, and serine stabilizes the substrate, reaction intermediate and product in catalysis [7]. Crystal structures of several SDR members have shown that despite low amino acid sequence similarity protein fold is highly conserved [4].

The SDR members catalyze diverse biochemical reactions, such as isomerization, epimerization and oxidoreduction [2]. They are involved in hormone metabolism (retinol dehydrogenases, prostaglandin dehydrogenases, hydroxysteroid dehydrogenases), fatty acid oxidation (hydroxyacyl CoA dehydrogenases) or biotransformation of xenobiotics (carbonyl reductases, quinone reductases) [8]. Hydroxysteroid dehydrogenases (HSDs) from the SDR super-family are implicated in the development of steroid dependent cancer forms [9], polycystic kidney disease [10], regulation of blood pressure [11], Alzheimer's disease [12] and obesity [13].

Although there are about 2000 members of the SDR superfamily [1] 17β-hydroxysteroid dehydrogenase from the filamentous fungus *Cochliobolus lunatus* (17β-HSDcl) is currently the only fungal HSD member. The NADPH dependent enzyme was purified, cloned and expressed in *Escherichia coli* [14,15]. The enzyme is homologous to fungal reductases involved in aflatoxin and melanin biosynthesis as well as to bacterial 7α-HSD and even human 17β-HSD types 4 and 8 [16]. Recently, we prepared a homology-built structural model of 17β-HSDcl using the known three-dimensional structure of 1,3,8-trihydroxy-naphthalene reductase (3HNR) from the fungus *Magnaporthe grisea* as a template [17]. The model structure directed our studies of structure–function relationship of the fungal 17β-HSD, as one of the model enzymes of the SDR family.

In this study, we investigated the significance of individual amino acid residues for coenzyme and substrate specificity. We performed site directed mutagenesis of R28, a residue that is conserved in most NADPH dependent SDR structures; T200 found only in *Streptomyces hydrogenans* 3α,20β-HSD and *Drosophila* alcohol dehydrogenases; and H230, a residue corresponding to the substrate specificity important H221 in human 17β-HSD type 1. All recombinant proteins were expressed in *E. coli* and purified to homogeneity. Kinetic evaluation of individual mutations was performed by analysis of progress curves of interconversions between the best substrates of 17β-HSDcl known so far, 4-estrene-3,17-dione and 4-estrene-17β-ol-3-one, according to the Theorell–Chance reaction mechanism.

2. Materials and methods

2.1. Site directed mutagenesis

The mutant proteins were prepared using Quick Change Site Directed Mutagenesis kit (Stratagene), pGex-17β-HSDcl expression vector and the following primers (only forward primers are shown):

- R28E for: 5′ – GTCACTGGCTCTGGTGAG GGCATCGGTGCAGCC – 3′
- R28A for: 5′ – GTCACTGGCTCTGGTGCC GGCATCGGTGCAGCC – 3′
- T200V for: 5′ – CCGTTGCCCCAGGCGGT GTCGTGACAGATATGTTCC – 3′
- H230A for: 5′ – CAGATGGCTGCGGCC GCATCCCCACTGC – 3′
- A231S for: 5′ – CAGATGGCTGCGCACTCC TCCCCACTGCACCG – 3′

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

2.2. Expression and purification of mutant proteins

Recombinant 17β-HSD (wild type and mutant proteins) were expressed in *E. coli* JM 107 cells and purified as described [15]. Protein concentrations were determined according to the Bradford method with BSA as a standard [18].

2.3. Preparation of 17β-HSDcl specific monoclonal antibodies

Recombinant 17β-HSDcl was expressed and purified to homogeneity as a GST fusion protein [15]. GST-17β-HSDcl ($\approx$ 50 μg) was injected both intraperitoneally and subcutaneously into Lou/C rats. A final boost of 50 μg of protein was given after 4 weeks. Fusion of the myeloma cell line P3X63-Ag8.653 with rat immune spleen cells was performed as described [19]. Hybridoma supernatants were tested in a solid phase immunoassay using GST-17HSDcl adsorbed to microtiter plates. Western blot analysis was performed as described under SDS-PAGE and Western blot analysis. Monoclonal antibody mAb 7F2 (rat IgG1) gave the best results.

2.4. SDS-PAGE and Western blot analysis

Homogeneity of the proteins was checked by SDS-PAGE and Coomassie blue staining. A total of 4 μg of the wild type and purified mutants were denatured in a Laemmli buffer and applied to a 12% acrylamide gel [20]. Electrophoresis and transfer were performed as described elsewhere. Rat monoclonal antibodies against 17β-HSDcl diluted 1:1 and incubated overnight, were used as primary antibodies. The secondary were mouse and goat anti-rat IgG+IgM (H+L) conjugated with horseradish peroxidase (Dianova, Hamburg), diluted 1:1000 and incubated for 2 h. Color was developed using diaminobenzidine and H_2O_2.

2.5. Activity staining

Screen for activity of the expressed proteins was carried out by activity staining. PAGE under non-denaturing conditions was performed on a 9% acrylamide gel using 8 μg of purified wild type and mutants. Following electrophoresis, gels were equilibrated in 50 mM Tris buffer pH 9.0 to which 0.5 mM $NADP^+$, 0.5 mM substrate 4-estrene-17β-ol-3-one dissolved in DMF and 1.5 mM β-cyclodextrine, all final concentrations were added. The activity staining was started by the addition of 0.1 mM phenazine methosulfate and 0.3 mM nitroblue tetrazolium salt, both final concentrations. The gels were incubated in the dark at 4 °C until the color developed.

2.6. Determination of coenzyme and substrate specificity

Homogenous 17β-HSDcl (wild type and T200V, R28E and R28A mutants, 0.5 μM) were incubated with either 100 μM 4-estrene-3,17-dione or 4-estrene-17β-ol-3-one in the presence of 500 μM coenzymes (NADPH, NADH, $NADP^+$ and NAD^+) in 100 mM phosphate buffer pH 8.0 for 1 h at RT. Mutants H230A and A231S (0.5 μM) were incubated with 100 μM steroids (4-estrene-3,17-dione, 5α-androstane-3,17-dione, 5α-androstane-3α-ol-17-one or 5α-androstane-3β-ol-17-one) in the presence of 500 μM NADPH, as described above. The products were extracted with chloroform and analyzed by TLC. Substrates and products were visualized by spraying the plates with 30% H_2SO_4 in EtOH.

2.7. Kinetic characterization of mutants

Interconversions between 4-estrene-3,17-dione and 4-estrene-17β-ol-3-one (20–200 μM) by the wild type enzyme or mutants (0.5–5 μM) in the presence of various coenzymes (100–500 μM) were also followed photometrically at 340 nm on the stopped-flow apparatus (SF-PQ-53, Hi-Tech, UK) and on a spectrophotometer Beckman DU7500. Progress curves from rapid and conventional experiments were analyzed according to the procedure described previously [17].

We used the Theorell–Chance reaction mechanism:

$$E + A \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} EA + B \underset{k_{-2}}{\overset{k_{+2}}{\leftrightarrow}} EQ + P \underset{k_{-3}}{\overset{k_{+3}}{\leftrightarrow}} E + Q$$

E represents enzyme, A, Q, B and P are NADPH,

$NADP^+$, 4-estrene-3,17-dione and 4-estrene-17β-ol-3-one, respectively. The kinetic parameters are second or first order rate constants (see Table 1).

3. Results and discussion

3.1. Expression, purification and kinetic characterization of 17β-HSDcl mutants

All recombinant proteins were expressed in *E. coli* and purified in one step to homogeneity. They all appeared on SDS-PAGE as single 28 kDa bands corresponding to the molecular mass of the wild type enzyme (Fig. 1A). Mutations did not change the recognition of the mutant proteins by anti-17β-HSDcl monoclonal antibodies prepared against the wild type enzyme (Fig. 1B). Screening with activity staining revealed that all mutants, except R28E, possess some 17β-HSD activity (Fig. 2). The kinetic evaluation of individual mutations was performed according to the Theorell–Chance reaction mechanism, as described before [17].

3.2. Amino acid residues important for coenzyme specificity of 17β-HSDcl

3.2.1. Role of arginine 28

In most structures of NADPH preferring enzymes, two negative charges of the NADPH 2′-phosphate group are compensated by one or two positively charged residues [21]. These residues are evident in NADPH dependent SDR enzymes, as in

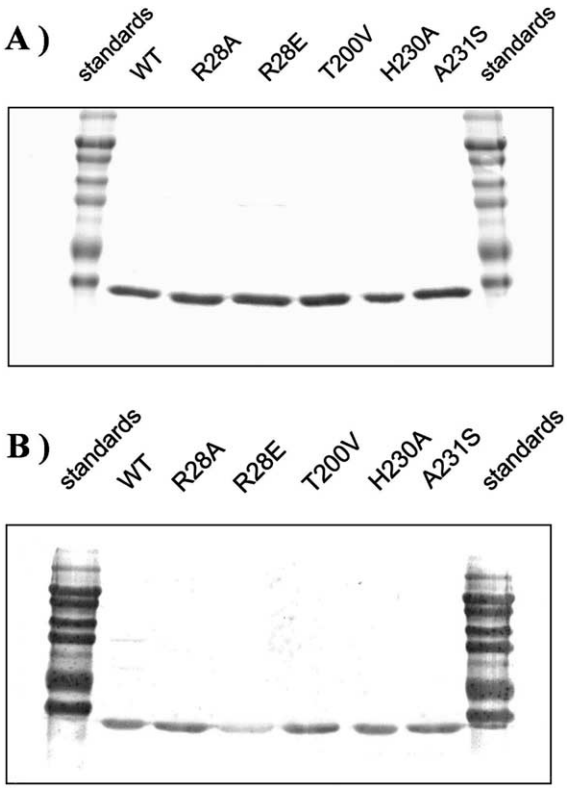

Fig. 1. SDS-PAGE and Western analysis of purified mutants. (A) A total of 4 μg of 17β-HSDcl (wild type and of each mutant) was applied to SDS-PAGE and stained with Coomassie blue. (B) Western analysis was performed using monoclonal rat antibodies against recombinant 17β-HSDcl and visualized with goat anti-rat IgG-horseradish peroxidase conjugate. Prestained molecular mass markers were 26.6, 36.5, 48, 58, 84, 116 and 180 kDa, respectively.

Table 1
Characteristic rate constants for reduction of 4-estrene-3,17-dione and oxidation of 4-estrene-17β-ol-3-one in the presence of NADPH and $NADP^+$

	Wild type	R28A	T200V	H230A	A231S
k_{+1} (M^{-1} s^{-1})	$1.77 \pm 0.20 \times 10^6$	551313 ± 253724	616810 ± 216614	$1.17 \pm 0.16 \times 10^6$	$1.04 \pm 0.15 \times 10^6$
k_{-1} (s^{-1})	278.9 ± 162.6	98.6 ± 19.6	3.62 ± 0.46	66.9 ± 18.99	193 ± 152
k_{+2} (M^{-1} s^{-1})	241703 ± 128052	123035 ± 25790	19384 ± 2309	105950 ± 29753	209766 ± 164956
k_{-2} (M^{-1} s^{-1})	5162 ± 24	359*	1944*	4335*	3392*
k_{+3} (s^{-1})	1.04 ± 0.02	0.0196 ± 0.00015	0.83 ± 0.05	0.66 ± 0.01	0.76 ± 0.02
k_{-3} (M^{-1} s^{-1})	$1.13 \pm 0.13 \times 10^6$	124840 ± 40269	$5.2 \pm 1.6 \times 10^6$	$1.04 \pm 0.11 \times 10^6$	$0.93 \pm 0.09 \times 10^6$

* Set fixed by using the overall equilibrium constant $((k_{-1} \times k_{-2} \times k_{-3})/(k_{+1} \times k_{+2} \times k_{+3}))$ of 0.273 as determined with wild type enzyme.

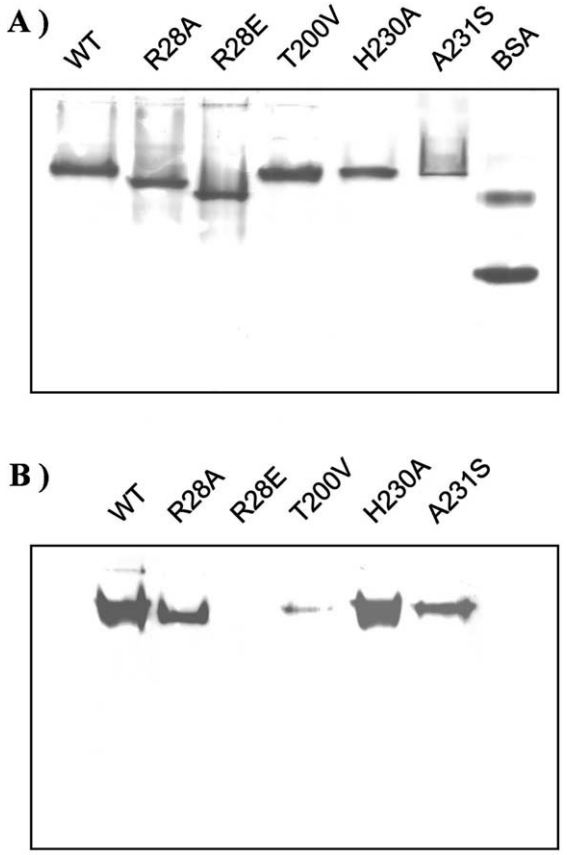

Fig. 2. Native PAGE and activity staining of mutants. (A) A total of 8 μg of each mutant was applied to native PAGE and stained with Coomassie blue. (B) Activity staining was performed with nitroblue tetrazolium and phenazine methasulfate in the presence of 4-estrene-17β-ol-3-one and $NADP^+$.

mouse lung carbonyl reductase (MLCR-1CYD, residues K17 and R39) [22]; in tropinone reductases (TR-I and TR-II) from *Datura stramonium* (TR-I-1AE1, residues K31 and R53, TR-II-2AE1, residues R19 and R41) [23] and in human 17β-HSD type 1 (1EQU, residues R37 and K195) [21]. There are, however, some NADPH dependent enzymes like 3HNR of *M. grisea* (1YBV, residue R39) and protochlorophyllide reductase of *Arabidopsis thaliana* (residue R122) that lack one of the two basic residues [22].

Interestingly, the atomic model of 17β-HSDcl also reveals only one basic residue (R28) that appears to interact with the 2′-phosphate. The presence of only one basic residue was suggested as insufficient to determine NADPH specificity [24]. Since to our knowledge this residue has not yet been changed in SDR proteins that possess only one basic amino acid residue, we decided to study the effect of R28A and R28E mutations on kinetic properties of the recombinant enzyme.

Introduction of a negative charge at the position 28 (R28E) completely abolished enzyme activity using NADPH/$NADP^+$ (Fig. 3). The mutant, however, retained a very low activity for the oxidation of 4-estrene-17β-ol-3-one with NAD^+, as observed by TLC analysis after 1 h incubation (Fig. 4), but photometric data did not allow any relevant kinetic analysis. On the other hand, the R28A mutant remained active, although significantly less than the wild type. The mutant revealed lower enzyme activity in the reductive and the oxidative direction with all four coenzymes tested (Figs. 3 and 4 and Table 1). Very low activity with NADH and NAD^+ made kinetic analysis of these reactions impossible. The reduction of 4-estrene-3,17-dione with NADH and the oxidation of 4-estrene-17β-ol-3-one with NAD^+ were detected photometrically only when ten times higher concentration of R28A was used (Fig. 3), while no products were seen on TLC plates with the usual concentration of the enzyme and NADH or NAD^+ (Fig. 4). It seems that in the R28E mutant NADPH could be repelled by the negatively charged glutamate residue, but the substitution R28E seems not to be sufficient for appropriate binding of NAD^+/NADH (Fig. 4). For the R28A mutant one would expect that NADPH is not stabilized by the presence of the basic residue, but also the binding of NAD^+/NADH is not enabled because of the absence of an aspartate residue that is conserved in NAD^+ dependent enzymes. However, kinetic analysis of this mutant revealed decrease in the dissociation rate constant for $NADP^+$. It seems that the loss of positive charge (R to A) enhance the affinity for the positively charged oxidized coenzyme.

3.2.2. Role of threonine 200

In addition to the basic residues that electrostatically interact with the 2′-phosphate group of

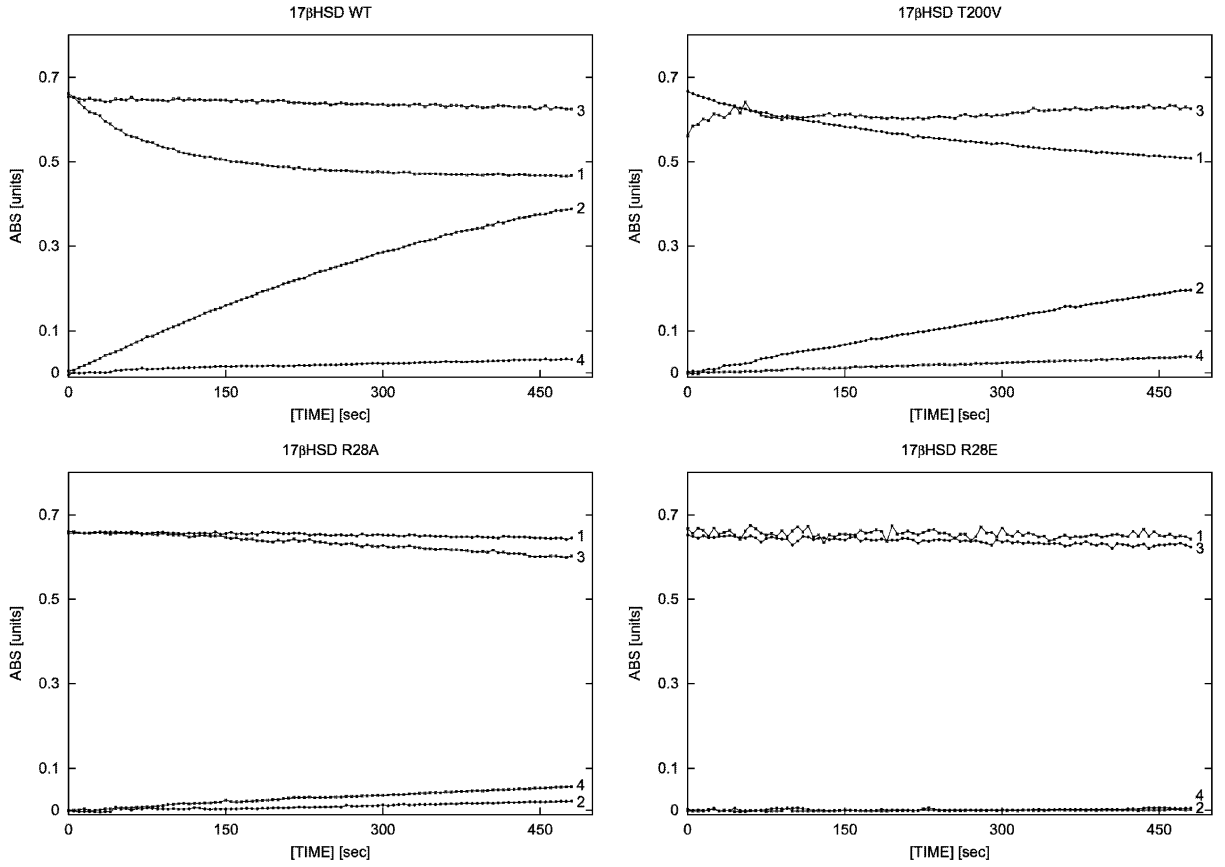

Fig. 3. Progress curves for reduction of 4-estrene-3,17-dione and oxidation of 4-estrene-17β-ol-3-one in the presence of NADPH, NADH, $NADP^+$ and NAD^+ by different mutants. Progress curves of reduction of 4-estrene-3,17-dione and oxidation of 4-estrene-17β-ol-3-one (100 μM) by the wild type and different mutants in the presence of 100 μM coenzymes (1) NADPH, (2) $NADP^+$, (3) NADH and (4) NAD^+. The concentrations of the wild type enzyme, T200V and R28A were 0.5 μM. In the presence of NAD^+ or NADH T200V and R28A were 5 μM. The concentration of R28E was always 1.5 μM. Reactions proceeded in 0.1 M phosphate buffer pH 8.0.

NADPH, residues that anchor the carboxyamide group of the nicotinamide ring are also important [23]. Tight binding of the carboxyamide group to the protein directs the B-face of the nicotinamide ring toward void of the cleft, consistent with specificity for the pro-*S* hydride transfer [23]. In crystal structures of the SDR members, the side-chain oxygen of threonine was found to form a H-bond with the amino group of the nicotinamide moiety: T206 in *D. stramonium* TR [23]; T213 in *M. grisea* 3HNR [25]; T194 in 7α-HSD of *E. coli* [26]; T190 in human 17β-HSD type 1 [27] and others. In the model structure of 17β-HSDcl T202, equivalent to the mentioned threonines, is within H-bond distance to the amide moiety of NADPH [17]. However, another threonine (T200) appears to be in a close contact with the carbonyl part of the nicotinamide. Interestingly, this threonine is conserved in NAD^+ dependent *S. hydrogenans* 3α/20β-HSD (2HSD, T185) and *Drosophila* alcohol dehydrogenases (1B14, T184) [28], while it is replaced by a hydrophobic amino acid, valine or isoleucine, in most other SDR enzymes. Since the threonine at this position has not yet been replaced in SDR proteins we changed it for a hydrophobic valine and investigated the effect of this mutation.

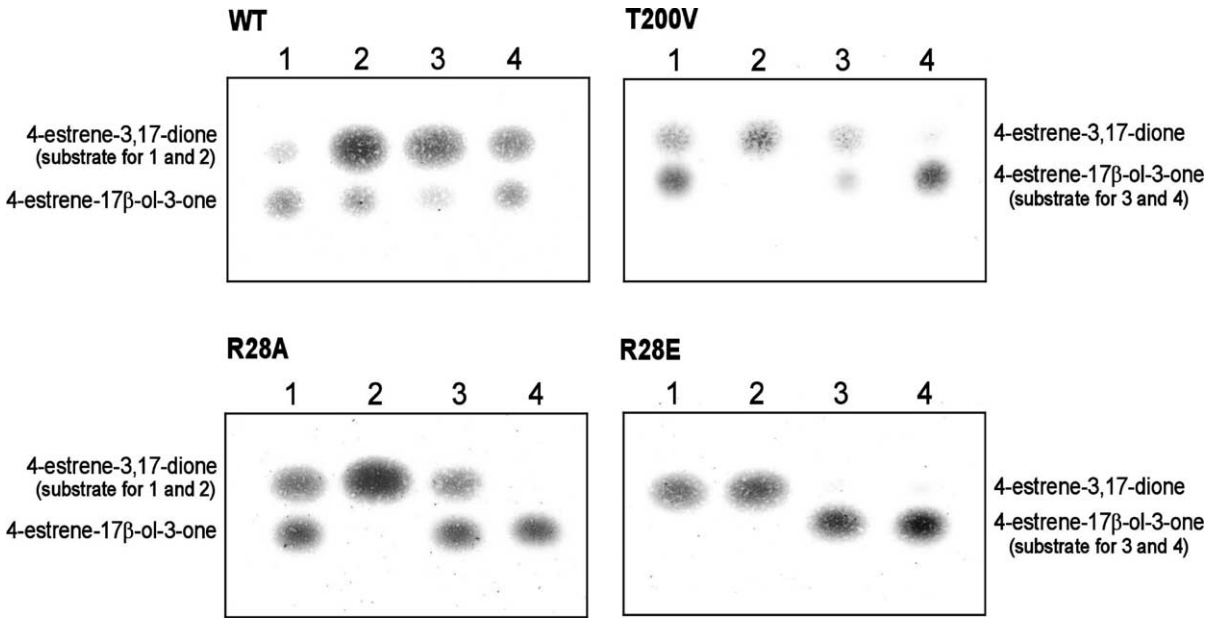

Fig. 4. TLC analysis for reduction of 4-estrene-3,17-dione and oxidation of 4-estrene-17β-ol-3-one in the presence of NADPH, NADH, $NADP^+$ and NAD^+ by different mutants. Coenzyme specificity of the wild type, R28A, R28E and T200V mutants was tested with 100 μM substrates (4-estrene-3,17-dione or 4-estrene-17β-ol-3-one) in the presence of 500 μM coenzymes: (1) NADPH, (2) NADH, (3) $NADP^+$ and (4) NAD^+. Reactions proceeded in 0.1 M phosphate buffer pH 8.0 for 1 h. Samples were extracted and analyzed by TLC in the solvent system Chex:EtAc = 1:2.

The T200V mutant was essentially less active than the wild type in the oxidative and the reductive direction with all four coenzymes tested (Figs. 3 and 4). Low enzyme activities with NADH/NAD^+ did not allow kinetic analysis of the reactions catalyzed in the presence of these two coenzymes. When we analyzed progress curves for the reduction and the oxidation in the presence of NADPH/$NADP^+$, we saw that reduction of the substrate in the presence of NADPH was very little affected, while the rate for substrate oxidation considerably decreased (Fig. 3). While k_{+3}, the dissociation rate constant for $NADP^+$ responsible for the rate of substrate reduction, was almost unchanged; k_{-1} dissociation rate constant for NADPH that determines the rate of substrate oxidation, was 77-fold lower (Table 1). This indicates that valine that was introduced at position 200 may stabilize NADPH, but it seems that threonine at the same position may also be involved in interaction with NADPH/$NADP^+$.

3.3. Amino acid residues potentially important for substrate specificity of 17β-HSDcl: roles of histidine 230 and alanine 231

According to site directed mutagenesis and the resolved crystal structure of human 17β-HSD type 1 substrate specificity of this human enzyme is appointed by the presence of H221 that forms a hydrogen-bond with the O3 of the steroid substrate. This H-bond determines specificity of the enzyme for steroids with a hydroxyl group at C3 and aromatic A ring [21,29]. The hydrogen bond between H221 and the C3 OH group helps to establish the catalytically relevant steroid/protein interaction of O17, T155 Oη and S142 Oγ [21].

Although, in the 17β-HSDcl atomic model H230 that corresponds to H221 is not close enough to the steroid O3 atom to form a hydrogen bond, we wanted to clear up the position of H230 and the neighboring A231 that might also be involved. To test their putative importance, we changed H230 and A231 to A and S, respectively.

Both mutants (H230A and A231S) showed slightly lower enzyme activity for the reduction of 4-estrene-3,17-dione and the oxidation of 4-estrene-17β-ol-3-one in the presence of NADPH and $NADP^+$ than the wild type enzyme (results not shown). No significant changes in the rate constants were determined (Table 1). Both were also tested with the substrates bearing a keto or hydroxyl group at C3 (4-estrene-3,17-dione, 5α-androstane-3,17-dione, 5α-androstane-3α-ol-17-one or 5α-androstane-3β-ol-17-one), but we could not observe a significant difference in comparison with the wild type enzyme (Fig. 5). Thus, H230 appears not to be involved in H-bonding with the keto or hydroxyl group at the C3 position of the steroids tested. These results are in accordance with the position of H230 in the model structure of 17β-HSDcl and demonstrate that H230, that corresponds H221 in 17β-HSD type 1 and the neighboring A231 most probably do not interact with the substrate. Such a finding is not unexpected, since importance of the H221 residue could only be observed in human 17β-HSD type 1; in rat 17β-HSD type 1 that equally uses both estrone and androstenedione, E282 is found at the equivalent position [30].

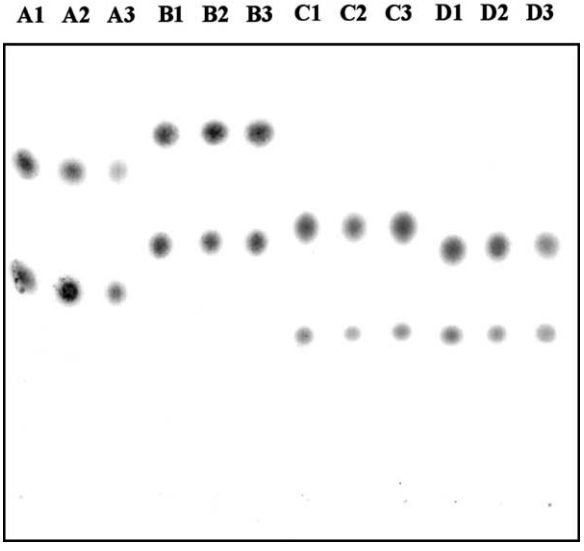

Fig. 5. Effect of H230A and A231S mutations on substrate specificity of 17β-HSDcl. Reduction of 17-keto steroids with keto or hydroxyl group at C3 position is shown. Wild type (1), H230A (2) and A231S (3) mutants (0.5 μM) were incubated with 100 μM substrates: (A) 4-estrene-3,17-dione; (B) 5α-androstane-3,17-dione; (C) 5α-androstane-3α-ol-17-one; and (D) 5α-androstane-3β-ol-17-one in the presence of 500 μM NADPH. Reactions proceeded in 0.1 M phosphate buffer pH 8.0 for 1 h. Samples were extracted and analyzed by TLC in the solvent system Chf:MetOH:H_2O = 94:6:0.5.

Acknowledgements

This work was supported by a NATO grant to J.A. and T.L.R. by the Ministry of Education, Science and Sport of Slovenia and partly by Deutsche Forschungsgemeinschaft grant to J.A.

References

[1] Y. Kallberg, U. Oppermann, H. Joernvall, B. Persson, Short-chain dehydrogenase/reductase (SDR) relationships: a large family with eight clusters common to human, animal, and plant genomes, Prot. Sci. 11 (2002) 636–641.
[2] H. Joernvall, B. Persson, M. Krook, S. Atrian, R. Gonzales-Duarte, J. Jeffery, D. Ghosh, Short-chain dehydrogenases/reductases (SDR), Biochemistry 34 (1995) 6003–6013.
[3] H. Joernvall, J.O. Hoog, B. Persson, SDR and MDR: completed genome sequences show these protein families to be large, of old origin and of complex nature, FEBS Lett. 445 (1999) 254–261.
[4] T.M. Penning, Molecular endocrinology of hydroxysteroid dehydrogenases, Endocr. Rev. 18 (1997) 281–305.
[5] Z. Krozowski, The short-chain alcohol dehydrogenase superfamily: variations on a common theme, J. Steroid Biochem. Mol. Biol. 51 (1994) 125–130.
[6] U.C. Oppermann, C. Filling, K.D. Berndt, B. Persson, J. Benach, R. Ladenstein, H. Jornvall, Active site directed mutagenesis of 3β/17β-hydroxysteroid dehydrogenase establishes differential effects on short-chain dehydrogenase/reductase reaction, Biochemistry 36 (1997) 34–40.
[7] T. Tanabe, N. Tanaka, K. Uchikawa, T. Kabashima, K. Ito, T. Nonaka, Y. Mitsui, M. Tsuru, T. Yoshimoto, Roles of the Ser146, Tyr159, and Lys163 residues in the catalytic action of 7α-hydroxysteroid dehydrogenase from *Escherichia coli*, J. Biochem. 124 (1998) 634–641.

[8] U.C.T. Oppermann, C. Filling, H. Joernvall, Forms and functions of human SDR enzymes, Chem.-Biol. Interact. 130–132 (2001) 699–705.
[9] S.X. Lin, Q. Han, A. Azzi, D. Zhu, A. Gangloff, R.L. Campbell, 3D-structure of human estrogenic 17β-HSD1: binding with various steroids, J. Steroid Biochem. Mol. Biol. 69 (1999) 425–429.
[10] J. Fomitcheva, M.E. Baker, E. Anderson, G.Y. Lee, N. Aziz, Characterization of Ke6, a new 17β-hydroxysteroid dehydrogenase, and its expression in gonadal tissues, J. Biol. Chem. 273 (1998) 22664–22671.
[11] A. Li, R. Tedde, Z.S. Krozowski, A. Pala, K.X. Li, C.H. Shackleton, F. Mantero, M. Palermo, P.M. Stewart, Molecular basis for hypertension in the 'type II variant' of apparent mineralocorticoid excess, Am. J. Hum. Genet. 63 (1998) 370–379.
[12] X.Z. He, G. Merz, P. Mehta, H. Schultz, S.-Y. Yang, Human brain short-chain L-3-hydroxyacyl coenzyme A dehydrogenase is a single domain multifunctional enzyme, J. Biol. Chem. 274 (1999) 15014–15019.
[13] H. Masuzaki, J. Peterson, H. Shinyama, N.M. Morton, J.J. Mullins, J.R. Seckl, J.S. Flier, A transgenic model of visceral obesity and the metabolic syndrome, Science 294 (2001) 2071–2072.
[14] T. Lanišnik Rižner, M. Žakelj-Mavrič, A. Plemenitaš, M. Zorko, Purification and characterization of 17β-hydroxysteroid dehydrogenase from the filamentous fungus *Cochliobolus lunatus*, J. Steroid Biochem. Mol. Biol. 59 (1996) 205–214.
[15] T. Lanišnik Rižner, G. Moeller, H.H. Thole, M. Žakelj-Mavrič, J. Adamski, A novel 17β-hydroxysteroid dehydrogenase in the fungus *Cochliobolus lunatus*: new insights into the evolution of steroid-hormone signalling, Biochem. J. 337 (1999) 425–431.
[16] T. Lanišnik Rižner, J. Stojan, J. Adamski, 17β-Hydroxysteroid dehydrogenase from the fungus *Cochliobolus lunatus*: structural and functional aspects, Chem.-Biol. Interact. 130-132 (2001) 793–803.
[17] T. Lanišnik Rižner, J. Adamski, J. Stojan, 17β-Hydroxysteroid dehydrogenase from *Cochliobolus lunatus*: model structure and substrate specificity, Arch. Biochem. Biophys. 384 (2000) 255–262.
[18] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[19] J.K. Gerber, T. Richter, E. Kremmer, J. Adamski, H. Hofler, R. Balling, H. Peters, Progressive loss of PAX9 expression correlates with increasing malignancy of dysplastic and cancerous epithelium of the human oesophagus, J. Pathol. 197 (2002) 293–297.
[20] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[21] C. Mazza, R. Breton, D. Housset, J.C. Fontecilla-Camps, Unusual charge stabilization of $NADP^+$ in 17β-hydroxysteroid dehydrogenase, J. Biol. Chem. 273 (1998) 8145–8152.
[22] M. Nakanishi, M. Kakumoto, K. Matsuura, Y. Deyashiki, N. Tanaka, T. Nonaka, Y. Mitsui, A. Hara, Involvement of two basic residues (Lys17 and Arg39) of mouse lung carbonyl reductase in NADP(H)-binding and fatty acid activation: site directed mutagenesis and kinetic analysis, J. Biochem. 120 (1996) 257–263.
[23] K. Nakajima, A. Yamashita, H. Akama, T. Nakatsu, H. Kato, T. Hashimoto, J. Oda, Y. Yamada, Crystal structures of two tropinone reductases: different reaction stereospecificities in the same protein fold, Proc. Natl. Acad. Sci. USA 95 (1998) 4876–4881.
[24] N. Tanaka, T. Nonaka, M. Nakanishi, Y. Deyashiki, A. Hara, Y. Mitsui, Crystal structure of the ternary complex of mouse lung carbonyl reductase at 1.8 Å resolution: the structure origin of the coenzyme specificity in the short-chain dehydrogenase/reductase family, Structure 4 (1995) 33–45.
[25] S. Andersson, D. Jordan, G. Schneider, Y. Lundqvist, Crystal structure of the ternary complex of 1,3,8-trihydroxy-naphthalene reductase from *Magnaporthe grisea* with NADPH and an active-site inhibitor, Structure 4 (1996) 1161–1170.
[26] N. Tanaka, T. Nonaka, T. Tanabe, T. Yoshimoto, D. Tsuru, Y. Mitsui, Crystal structures of the binary and ternary complexes of 7α-hydroxysteroid dehydrogenases from *Escherichia coli*, Biochemistry 35 (1996) 7715–7730.
[27] R. Breton, D. Housset, C. Mazza, J.C. Fontecilla-Camps, The structure of a complex of human 17β-hydroxysteroid dehydrogenase with estradiol and $NADP^+$ identifies two principal targets for the design of inhibitors, Structure 4 (1996) 905–915.
[28] J. Benach, S. Atrian, R. Gonzales-Duarte, R. Ladenstein, The refined crystal structure of *Drosophila lebanonensis* alcohol dehydrogenase at 1.9 Å resolution, J. Mol. Biol. 282 (1998) 383–399.
[29] Y.-W. Huang, I. Pineau, H.-J. Chang, A. Azzi, V. Bellemare, S. Laberge, S.-X. Lin, Critical residues for the specificity of cofactors and substrates in human estrogenic 17β-hydroxysteroid dehydrogenase 1: variants designed from the three-dimensional structure of the enzyme, Mol. Endocrinol. 15 (2001) 2010–2020.
[30] T. Puranen, M. Poutanen, D. Ghosh, R. Vihko, P. Vihko, Origin of substrate specificity of human and rat 17β-hydroxysteroid dehydrogenase type 1, using chimeric enzymes and site-directed substitutions, Endocrinology 138 (1997) 3532–3539.