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Factorizing the role of a critical leucine residue in the binding of substrate to human 20α-hydroxysteroid dehydrogenase (AKR1C1): Molecular modeling and kinetic studies of the Leu308Val mutant enzyme

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ABSTRACT

A comparison of the structures and kinetic properties of human 20α-hydroxysteroid dehydrogenase (AKR1C1) and its mutant enzymes (Leu308Val and Leu308Ala) indicates that Leu308 is a selectivity determinant for substrate binding. While the Leu308Val mutation improved the catalytic efficiency (k_{cat}/K_m) of AKR1C1 towards the two substrates 5α-pregnane-3α,20α-diol (PregA) and 5β-pregnan-3α-ol-20-one (PregB), the Leu308Ala mutation rendered the enzyme inactive. In the docked model of PregA the conformation of the steroid molecule was similar to that of 20α-hydroxyprogesterone in the crystal structure of the AKR1C1 complex where the steroid did not interact with the catalytic residues Tyr55 and His117. In the case of PregB the steroid interacted with the catalytic residue His117 and formed close contacts with Leu308, suggesting that the binding mechanism of 3α-hydroxysteroids in the active site of AKR1C1 is different from that of 20α-hydroxysteroids.

Hydroxysteroid dehydrogenases (HSDs) are broadly classified into two protein superfamilies, the short-chain dehydrogenase/reductase family and the aldo–keto reductases (AKR) family.[1,2] The four human NADP(H)-dependent HSDs belonging to the AKR1C subfamily, namely AKR1C1 (20α-HSD), AKR1C2 (type 3 3α-HSD), AKR1C3 (type 2 3α-HSD, and type 5 17β-HSD) and AKR1C4 (type 1 3α-HSD), share over 86% amino acid sequence homology.[3–6] The AKR1C isoforms have different expression patterns in human tissues, where they regulate ligand occupancy and trans-activation of nuclear receptors by controlling the concentrations of active steroid hormones such as androgens, estrogens and progestins in target tissues.[4,6–8] Although the AKR1C isoforms catalyze the oxidation and reduction reactions with comparable efficiencies, they thermodynamically favor the ketosteroid reduction in vitro.[9] The direction of catalysis in vivo is also governed by the cellular ratio of NADPH/$NADP^+$. In metabolically active cells, reduction is favored over oxidation since NADPH is the predominant form of the cofactor and the dehydrogenase activities of the four isoforms are potently inhibited by NADPH.[10,11] The four AKR1C isoforms have overlapping catalytic properties,[8] but display distinct positional and stereochemical preferences for steroid substrates. Differences in affinity for the 3-, 17- or 20-ketosteroid substrates and mode of steroid binding in the active site among the four AKR1C isoforms is dictated by residues lining their substrate binding pockets.[4–6]

Although AKR1C1 exhibits both 3α/β- and 20α-HSD activities, it preferentially acts as a 20-ketosteroid reductase converting progesterone and its metabolites (5α-pregnane-3,20-dione and 5α-pregnan-3α-ol-20-one) into the corresponding 20α-hydroxysteroids.[10–15] Since the metabolite 20α-hydroxyprogesterone (OH-Prog) has a low affinity for the progesterone receptors, AKR1C1 plays an important role in controlling the cellular concentration of progesterone, which is an essential hormone required for endometrial development and maintenance of pregnancy.[16] Progesterone is a natural hormone, synthesized in both males and females, and also affects neuronal function by modulating gene expression and cellular receptors abundant in the central nervous system.[17] Progesterone and its metabolites exert neuroprotective effects in traumatic and ischemic brain injury,[17] nerve crush injury,[18] peripheral neuropathy induced by an antineoplastic agent docetaxel,[19] and diabetic neuropathy.[20] In addition, 5α-pregnan-3α-ol-20-one and its 5β-isomer are potent positive modulators of the $GABA_A$ receptors in the brain.[21]

We have recently reported a selectivity pocket lined by the hydrophobic side-chain of Leu308 in the active site of AKR1C1 that is involved in inhibitor binding.[22,23] In an effort to investigate the role of Leu308 in substrate binding, we have examined the effects of shortening the side-chain of Leu308 by site-directed mutagenesis, to Val308 and Ala308, on the positional and stereochemical preferences of the enzyme. Furthermore, the role of Leu308 in

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determining substrate selectivity was investigated by molecular modeling of 20α- and 3α-hydroxysteroid substrates in the crystal structures of the wild-type (WT) and Leu308Val mutant (MT) AKR1C1s.

Site-directed mutagenesis and purification of recombinant enzymes: Mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) and pGEX-2T expression plasmid harboring cDNA for AKR1C1[24] as the template according to the protocol described by the manufacturer. The primer pair used for the mutagenesis was composed of sense and antisense oligonucleotides to alter a single codon of the AKR1C1 cDNA. The 30-mer primers were synthesized to give the Leu308Val and Leu308Ala mutations. The coding regions of the cDNAs in the expression plasmids were sequenced by using a Beckman CEQ2000XL DNA sequencer in order to confirm the presence of the desired mutation and ensure that no other mutation had occurred. The recombinant WT and MT enzymes were expressed in *Escherichia coli* JM109, and purified to homogeneity as previously described.[24] The final protein concentration was determined by a bicinchoninic acid protein assay reagent kit (Pierce) using bovine serum albumin as the standard.

Assay of enzyme activity: The dehydrogenase activities of WT and MT AKR1C1s were determined at 25 °C by measuring the rate of change in NADPH fluorescence (at 455 nm with an excitation wavelength of 340 nm).[14] The K_m and k_{cat} values for the substrates were determined in the reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4, a saturated concentration of $NADP^+$ (0.25 mM), a range of five substrate concentrations ($0.2 - 5 \times K_m$) and enzyme, in a total volume of 2.0 mL. The kinetic constants are expressed as the means ± standard errors of at least three determinations. The hydroxysteroid substrate (20 μM) was incubated at 37 °C for 15 min in a reaction mixture (4.0 mL) as described above. The substrate and products were extracted twice by ethyl acetate, and analyzed by thin-layer chromatography as described previously.[14,15]

Molecular docking: Atomic coordinates for the WT and Leu308Val MT AKR1C1s were obtained from the RCSB Protein Data Bank (ID codes 3C3U and 3GUG, respectively). For ligand docking the structure of the WT was prepared using the Maestro (Shrödinger, LLC, Portland, OR) software package Version 8.5 where hydrogen atoms in the structure were generated. The Protein Preparation module in Maestro was used to perform a brief relaxation on the starting structures with the 'Refinement Only' option, which optimizes the hydroxyl and thiol torsions as well as performing an all-atom constrained minimization to relieve any clashes. The formal charges and appropriate bond orders of the ligands were manually adjusted in Maestro. In order to eliminate any potential bond length and bond angle biases in the structure, the ligands were subjected to a full minimization prior to the docking. The docking calculations were performed using GLIDE 5.0[25] on a Linux workstation. A grid box was generated and the center of the grid box was defined by the catalytic residues Tyr55 and His117. The calculations were run in the 'Extra Precision' (XP) mode of GLIDE where the ligands were docked flexibly, and to soften the potential for the nonpolar parts of the ligands, the van der Waals radius was scaled by 0.8. In order to ensure that the poses generated were conformationally distinct and to increase the diversity of the retained poses, poses with a RMSD less than 0.5 Å and a maximum atomic displacement less than 1.3 Å were discarded as duplicates. The figure showing the superimposition of the structural models was prepared by PyMOL (DeLano Scientific, San Carlos, CA, USA).

The role of Leu308 in substrate binding: Although AKR1C1 predominantly functions as a 20α-HSD reducing active steroid hormones such as progesterone to OHProg and shows moderate 3α-HSD activity for 5β-dihydrosteroids,[14] the structural basis for its stereochemical preference remains to be elucidated. The steroid binding cavity is composed mainly of residues belonging to three loops (loop A, residues 117–134; loop B, 217–238; and loop C, 299–323).[26] Since the interactions between the residues belonging to these loops and the substrate are critical for catalysis to occur, we investigated the effect resulting from the mutations of Leu308 into valine (with a side-chain smaller by one methyl group) and alanine (with only one methyl group as the side-chain) on the enzyme's catalytic efficiency for both 3α- and 20α-hydroxysteroids. The results from the kinetic analyses of the two steroid substrates, 5α-pregnane-3α,20α-diol (PregA) and 5β-pregnan-3α-ol-20-one (PregB), and a non-steroidal substrate *S*-tetralol are presented in Table 1.

The 3α-HSD activity of the Leu308Val MT enzyme for PregB was 60-fold higher than the 20α-HSD activity for PregA, whereas the Leu308Ala MT enzyme did not show any significant activity towards the two substrates. However, both MT enzymes exhibited dehydrogenase activity towards the small non-steroidal substrate *S*-tetralol. In order to investigate the role of Leu308 in dictating the enzyme's catalytic efficiency we determined the K_m and k_{cat} values of the WT and two MT enzymes. The kinetic representation of the catalytic efficiency by the k_{cat}/K_m value is shown in Table 1. It should be noted that the oxidized products of PregA and PregB by the MT Leu308Val enzyme were identified to be 5α-pregnan-3α-ol-20-one and 5β-pregnane-3,20-dione, respectively. Thus, like WT AKR1C1[14] the Leu308Val MT enzyme predominantly oxidizes the 20α-hydroxyl group of PregA.

The three steroid substrates OHProg, PregA and PregB were docked in the active site of AKR1C1 using the extra-precision mode of the program GLIDE and the resulting structural models of the complexes were analyzed. The docking of OHProg in AKR1C1 produced a structural model similar to the published crystal structure of AKR1C1 with OHProg bound.[26] In the crystal structure the reactive hydroxyl group at the C20 position is present within hydrogen bonding distance of His222 but far away from the catalytic residues (Tyr55 and His117) and the C4 atom of the nicotinamide ring, an orientation which does not favor catalysis (Fig. 1). This observation led to the speculation that progesterone binds in the active site with an alternate conformation in order to favor catalysis of the C20-ketone. Following catalysis, the product OHProg moves away from the catalytic residues due to a conformational change in the enzyme. Thus, the crystal structure of AKR1C1 with OHProg bound is thought to be a snapshot of product release.[26] Based on the crystal structure, modeling studies on progesterone had identified Leu308 as the key residue involved in the conformational change due to steric clashes between the side-chain of Leu308 and the incoming substrates.[26] Thus, the results from the modeling suggested that Leu308 which belongs to a highly variable and flexible part of loop C initially adopts a conformation that can easily accommodate the substrate for catalysis following which the enzyme undergoes a conformational change whereby the side-chain

Table 1
Kinetic alterations in the oxidation of 20α- and 3α-hydroxysteroids and *S*-tetralol resulting from the Leu308Val and Leu308Ala mutations

Substrate	WT[a]	Leu308Val	Leu308Ala
PregA			
K_m (μM^{-1})	0.9	0.4 ± 0.01 (0.4)	—
k_{cat}/K_m ($min^{-1}\ \mu M^{-1}$)	2.4	16 ± 1 (7)	na[b]
PregB			
K_m (μM^{-1})	4.5	6.2 ± 0.1 (1.4)	—
k_{cat}/K_m ($min^{-1}\ \mu M^{-1}$)	0.04	1.6 ± 0.2 (40)	na[b]
S-Tetralol			
K_m (μM^{-1})	7.0	16 ± 1 (2)	470 ± 50 (67)
k_{cat}/K_m ($min^{-1}\ \mu M^{-1}$)	3.7	0.8 ± 0.1 (0.2)	0.05 ± 0.01 (0.01)

Values for MT/WT ratios are given in parentheses.
[a] Values taken from Ref. 14.
[b] No activity was detected.

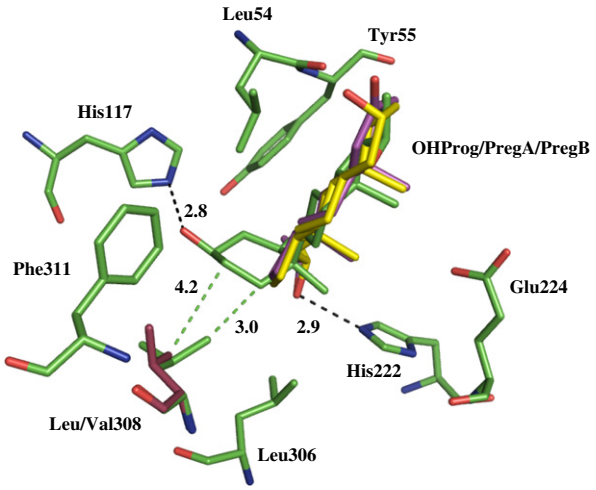

Figure 1. Superimposition of the docked substrates 20α-hydroxyprogesterone (OHProg; purple), 5α-pregnane-3α,20α-diol (PregA; yellow) and 5β-pregnan-3α-ol-20-one (PregB; green) in the crystal structure of WT AKR1C1. Distances in angstroms for hydrogen bonds (black dotted lines) with His117 and His222, and shortest contacts (green dotted lines) with Leu308 (green)/Val308 (magenta) are shown.

of Leu308 adopts a conformation that no longer supports product binding in the catalytic position for reduction/oxidation.[26] Similarly, a catalytically favorable orientation for PregA (20α-hydroxysteroid) was not produced by our docking studies (Fig. 1), consistent with the orientation of OHProg in the crystal structure of AKR1C1 and the proposed conformational change of Leu308 during catalysis.

In order to obtain a model of the binding mode for 3α-hydroxysteroids in AKR1C1, PregB was docked in the active site of the enzyme. The 3-hydroxyl group of the substrate was within 4 Å of the C4 atom of the nicotinamide ring and pointing towards the catalytic residues Tyr55 (5.2 Å) and His117 (2.8 Å). Since the position of the C3-hydroxyl group of the steroid is too far away to allow proton transfer to Tyr55, this orientation may represent a snapshot for substrate binding where the substrate molecule is stabilized by His117. Additionally, in this orientation the C3-hydroxyl group of the steroid molecule formed close interactions with the side-chain of Leu308 (Fig. 1), and as such Leu308 may play an important role in the binding of 3α-hydroxysteroids as well as 20α-hydroxysteroids through conformational changes that allow the binding of substrate and release of product. Our results (Table 1) indicated that the WT enzyme catalyzed the oxidation of PregA more efficiently than PregB. On the other hand, the mutation of Leu308Val increased the k_{cat}/K_m values for PregA and PregB (7- and 40-fold, respectively), mainly due to elevations in k_{cat} values. The noticeable improvement in the catalytic efficiency (k_{cat}/K_m) of the Leu308Val MT for PregB may be attributed to more favorable interactions due to the shortening of the side-chain of residue 308, which then becomes closer to an optimal van der Waals distance from the substrate (Fig. 1). The mutation of Leu308Ala significantly impaired both affinity (K_m) and catalytic efficiency for the small substrate *S*-tetralol, and rendered the enzyme inactive towards both hydroxysteroid substrates, suggesting that Ala308 did not form favorable interactions that resulted in product release.

In conclusion, the docked models of PregA and PregB predict that binding mechanism of 3α-hydroxysteroids in the active site of AKR1C1 is different from that of 20α-hydroxysteroids. The docked model of PregA suggests that the 20α-hydroxyl group of the steroid molecule would not interact with the catalytic residues Tyr55 and His117 due to the steric hindrance by the side-chain of Leu308. In case of 3α-hydroxysteroids, the docked model of PregB suggests that the 3α-hydroxy group of the substrate would interact with the catalytic residue His117 and form close contacts with Leu308. Therefore, the movement of the Leu308 side-chain is essential in forming a mature binding site allowing the steroidal substrates to form hydrogen bonds with the catalytic residues Tyr55 and His117 for catalysis to occur. The important role of the bulky side-chain of Leu308 in the binding of both 3α- and 20α-hydroxysteroid substrates is further emphasized by the improved catalytic efficiency of the Leu308Val mutant towards both substrates, whereas the replacement of Leu308 with alanine abolished the dehydrogenase activity towards the substrates. Our molecular modeling and site-directed mutagenesis results on the binding of 3α- and 20α-hydroxysteroid substrates to the enzyme identify structural features that provide novel insights into the positional and stereochemical preferences of AKR1C1. Furthermore, since AKR1C1 plays an important role in controlling the cellular concentration of progesterone that is required for endometrial development, and also affects neuronal function, these structural features may be useful in the design of novel ligands for the treatment for endometriosis and mental illness.

References and notes

1. Persson, B.; Kallberg, Y.; Bray, J. E.; Bruford, E.; Dellaporta, S. L.; Favia, A. D.; Duarte, R. G.; Jörnvall, H.; Kavanagh, K. L.; Kedishvili, N.; Kisiela, M.; Maser, E.; Mindnich, R.; Orchard, S.; Penning, T. M.; Thornton, J. M.; Adamski, J.; Oppermann, U. *Chem. Biol. Interact.* **2009**, *178*, 94.
2. Jez, J. M.; Penning, T. M. *Chem. Biol. Interact.* **2001**, *130–132*, 499.
3. Penning, T. M. *Endocr. Rev.* **1997**, *18*, 281.
4. Penning, T. M. *Hum. Reprod. Update* **2003**, *9*, 193.
5. Penning, T. M.; Jin, Y.; Heredia, V. V.; Lewis, M. *J. Steroid Biochem. Mol. Biol.* **2003**, *85*, 247.
6. Penning, T. M.; Burczynski, M. E.; Jez, J. M.; Hung, C. F.; Lin, H. K.; Ma, H.; Moore, M.; Palackal, N.; Ratnam, K. *Biochem. J.* **2000**, *351*, 67.
7. Bauman, D. R.; Steckelbroeck, S.; Penning, T. M. *Drug News Perspect.* **2004**, *17*, 563.
8. Penning, T. M.; Jin, Y.; Rizner, T. L.; Bauman, D. R. *Mol. Cell. Endocrinol.* **2008**, *281*, 1.
9. Cooper, W. C.; Jin, Y.; Penning, T. M. *J. Biol. Chem.* **2007**, *282*, 33484.
10. Steckelbroeck, S.; Jin, Y.; Gopishetty, S.; Oyesanmi, B.; Penning, T. M. *J. Biol. Chem.* **2004**, *279*, 10784.
11. Jin, Y.; Penning, T. M. *Biochemistry* **2006**, *45*, 13054.
12. Ishikura, S.; Nakajima, S.; Kaneko, T.; Shintani, S.; Usami, N.; Yamamoto, I.; Carbone, V.; El-Kabbani, O.; Hara, A. Comparison of Stereoselective Reduction of 3- and 20-Oxosteroids among Mouse and Primate 20α-Hydroxysteroid Dehydrogenases. In *Enzymology and Molecular Biology of Carbonyl Metabolism*; Weiner, H., Plapp, B., Lindahl, R., Maser, E., Eds.; Purdue University Press: West Lafayette, 2005; pp 341–351.
13. Zhang, Y.; Dufort, I.; Rheault, P.; Luu-The, V. *J. Mol. Endocrinol.* **2000**, *25*, 221.
14. Higaki, Y.; Usami, N.; Shintani, S.; Ishikura, S.; El-Kabbani, O.; Hara, A. *Chem. Biol. Interact.* **2003**, *143–144*, 503.
15. Usami, N.; Yamamoto, T.; Shintani, S.; Ishikura, S.; Higaki, Y.; Katagiri, Y.; Hara, A. *Biol. Pharm. Bull.* **2002**, *25*, 441.
16. Piekorz, R. P.; Gingras, S.; Hoffmeyer, A.; Ihle, J. N.; Weinstein, Y. *Mol. Endocrinol.* **2005**, *19*, 431.
17. Sayeed, I.; Stein, D. G. *Prog. Brain Res.* **2009**, *175*, 219.
18. Roglio, I.; Bianchi, R.; Gotti, S.; Scurati, S.; Giatti, S.; Pesaresi, M.; Caruso, D.; Panzica, G. C.; Melcangi, R. C. *Neuroscience* **2008**, *155*, 673.
19. Roglio, I.; Bianchi, R.; Camozzi, F.; Carozzi, V.; Cervellini, I.; Crippa, D.; Lauria, G.; Cavaletti, G.; Melcangi, R. C. *J. Peripher. Nerv. Syst.* **2009**, *14*, 36.
20. Leonelli, E.; Bianchi, R.; Cavaletti, G.; Caruso, D.; Crippa, D.; Garcia-Segura, L. M.; Lauria, G.; Magnaghi, V.; Roglio, I.; Melcangi, R. C. *Neuroscience* **2007**, *144*, 1293.
21. Lambert, J. J.; Belelli, D.; Shepherd, S. E.; Pistis, M.; Peters, J. A. The Selective Interaction of Neurosteroids with the $GABA_A$ Receptor. In *Neurosteroids: A New Regulatory Function in the Nervous System*; Baulieu, E.-E., Robel, P., Schumacher, M., Eds.; Humana Press: Totowa, 1999; pp 125–142.
22. El-Kabbani, O.; Scammells, P. J.; Gosling, J.; Dhagat, U.; Endo, S.; Matsunaga, T.; Soda, M.; Hara, A. *J. Med. Chem.* **2009**, *52*, 3259.
23. Dhagat, U.; Endo, S.; Sumii, R.; Hara, A.; El-Kabbani, O. *J. Med. Chem.* **2008**, *51*, 4844.
24. Matsuura, K.; Hara, A.; Deyashiki, Y.; Iwasa, H.; Kume, T.; Ishikura, S.; Shiraishi, H.; Katagiri, Y. *Biochem. J.* **1998**, *336*, 429.
25. Friesner, R. A.; Banks, J. L.; Murphy, R. B.; Halgren, T. A.; Klicic, J. J.; Mainz, D. T.; Repasky, M. P.; Knoll, E. H.; Shelley, M.; Perry, J. K.; Shaw, D. E.; Francis, P.; Shenkin, P. S. *J. Med. Chem.* **2004**, *47*, 1739.
26. Couture, J. F.; Legrand, P.; Cantin, L.; Luu-The, V.; Labrie, F.; Breton, R. *J. Mol. Biol.* **2003**, *331*, 593.