Articles

The Crystal Structure of Human Δ4-3-Ketosteroid 5β-Reductase Defines the Functional Role of the Residues of the Catalytic Tetrad in the Steroid Double Bond Reduction Mechanism†

Frédérick Faucher,‡ Line Cantin, Van Luu-The, Fernand Labrie, and Rock Breton*

Oncology and Molecular Endocrinology Research Center, Laval University Medical Center, Québec (QC), G1V 4G2 Canada

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ABSTRACT: The 5β-reductases (AKR1D1−3) are unique enzymes able to catalyze efficiently and in a stereospecific manner the 5β-reduction of the C4−C5 double bond found into Δ4-3-ketosteroids, including steroid hormones and bile acids. Multiple-sequence alignments and mutagenic studies have already identified one of the residues presumably located at their active site, Glu_{120}, as the major molecular determinant for the unique activity displayed by 5β-reductases. To define the exact role played by this glutamate in the catalytic activity of these enzymes, biochemical and structural studies on human 5β-reductase (h5β-red) have been undertaken. The crystal structure of h5β-red in a ternary complex with $NADP^+$ and 5β-dihydroprogesterone (5β-DHP), the product of the 5β-reduction of progesterone (Prog), revealed that Glu_{120} does not interact directly with the other catalytic residues, as previously hypothesized, thus suggesting that this residue is not directly involved in catalysis but could instead be important for the proper positioning of the steroid substrate in the catalytic site. On the basis of our structural results, we thus propose a realistic scheme for the catalytic mechanism of the C4−C5 double bond reduction. We also propose that bile acid precursors such as 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one, when bound to the active site of h5β-red, can establish supplementary contacts with Tyr_{26} and Tyr_{132}, two residues delineating the steroid-binding cavity. These additional contacts very likely account for the higher activity of h5β-red toward the bile acid intermediates versus steroid hormones. Finally, in light of the structural data now available, we attempt to interpret the likely consequences of mutations already identified in the gene encoding the h5β-red enzyme which lead to a reduction of its enzymatic activity and which can progress to severe liver function failure.

The 5β-reductase enzyme (5β-red) catalyzes the reduction of the Δ4 double bond of Δ4-3-ketosteroids, converting those steroids into 5β-dihydro-3-ketosteroids. The best known activity of 5β-red is the formation of biliary acids. It transforms the bile acid precursors, 7α-hydroxycholest-4-en-3-one and 7α,12α-dihydroxycholest-4-en-3-one, into their corresponding 5β-reduced derivatives (*1–3*). Human 5β-reductase (h5β-red)[1] belongs to the aldo-keto reductase (AKR) superfamily and is the first member of the 1D subfamily (AKR1D1), which also encompasses the 5β-reductases from rat (AKR1D2) (*4*) and rabbit (AKR1D3). As observed for the rat enzyme, h5β-red is strongly expressed in the liver (*5*) and, at lower levels, in testes and placenta (*1, 6*). Defects in the AKR1D1 gene result in high urinary and plasma levels of Δ4-3-oxo steroids which lead to liver failures and hemochromatosis, conditions which are potentially lethal in newborn infants (*7–9*).

Human 5β-red is also able to efficiently catalyze the reduction of all steroid hormones carrying a 4-ene-3-keto group. It thus transforms Prog into 5β-pregnan-3,20-dione (5β-DHP), an endogenous ligand for the pregnane X receptor and for the constitutive androstane receptor, both implicated in the clearance of xenobiotics (*10*). This Prog metabolite is also a potent tocolytic steroid (*11*) for which circulating concentrations have been found to decrease significantly in association with the active phase of the first stage of human labor (*6*). Because the relative abundance of 5β-red mRNA in placenta and myometrium also decreases significantly in association with labor (*6*), it has been hypothesized that this enzyme, through the formation of 5β-reduced Prog metabo-

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* To whom correspondence should be addressed: Centre de recherche en endocrinologie moléculaire et oncologique, Centre hospitalier de l'Université Laval (CHUL), 2705, boul. Laurier, Québec (Qc), Canada G1V 4G2. Telephone: (418) 654-2296. Fax: (418) 654-2761. E-mail: rock.breton@crchul.ulaval.ca.

‡ Present address: Department of Microbiology and Molecular Genetics, University of Vermont, Burlington, VT 05405.

[1] Abbreviations: h5β-red, human 5β-reductase; h3α-HSD3, human type 3 3α-hydroxysteroid dehydrogenase; 5β-DHP, 5β-pregnan-3,20-dione; Prog, progesterone; HSD, hydroxysteroid dehydrogenase; SDR, short-chain dehydrogenase/reductase; AKR, aldo-keto reductase; rmsd, root-mean-square deviation.

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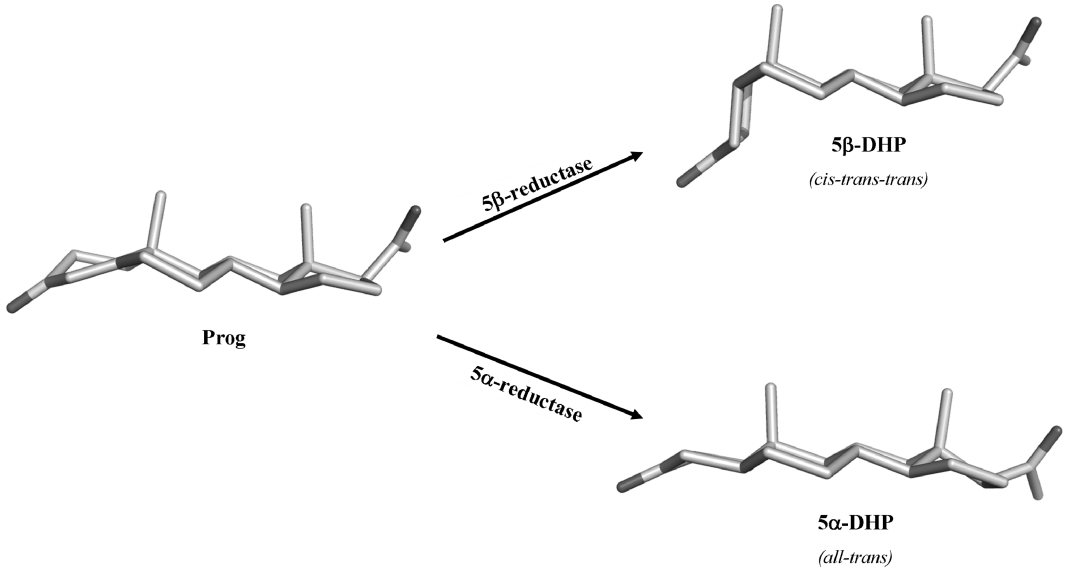


FIGURE 1: Representation of the steroid nucleus conformational changes caused by the reduction of the C4−C5 double bond by 5β-reductase (addition of a proton at C5 on the β-face of the steroid) and by 5α-reductase (addition of a proton at C5 on the α-face of the steroid).

lites, could play a role in regulating myometrial activity and in the onset of spontaneous labor in humans.

Like the other AKR members, h5β-red is thought to adopt an (α/β)-barrel fold with a cylindrical core of eight parallel β-strands surrounded by eight α-helices running antiparallel to the strands (*12*). The carboxy ends of the β-strands are connected to amino ends of the α-helices by loops of varying lengths. Residues forming the active site or participating in substrate binding mainly belong to the loops located in the C-terminal portion of the β-sheet (*13*). The NADPH cofactor binds AKR enzymes in an extended conformation with the nicotinamide in the center of the barrel. This cofactor binding mode contrasts with that found in the 5β-red isolated from leaves of *Digitalis* plants, the only other 5β-red enzyme whose structure has been determined so far (*14*). Indeed, this 5β-red contains a dinucleotide-binding Rossmann fold (*15*) typical of members of the short-chain dehydrogenase/reductase (SDR) superfamily. Although they catalyze the same reaction, it thus appears that vertebrate and plant 5β-reductases have evolved independently after divergence from the last universal common ancestor (*16*).

The reduction reaction catalyzed by 5β-red is considered to be irreversible. Indeed, in contrast to AKR1C subfamily members, which are reversible enzymes capable of performing reduction or oxidization reactions depending on the added cofactor (NADPH or $NADP^+$), there is no indication that 5β-reduction is reversible since it is believed that steroid C5 β-face reduction and the formation of the double bond between C4 and C5 are thermodynamically unfavorable. It is important to note that the reduction of a C4−C5 double bond involves the transfer of a proton to the β-face of the steroid at C5. This transfer produces a major structural change, resulting in a transition of the steroid nucleus from an *all-trans* and planar conformation to a *cis*−*trans*−*trans* conformation with the A-ring bent 90° relative to the steroid nucleus (Figure 1).

Enzymes of the AKR1D subfamily also differ from members of the AKR1C subfamily in the number of amino acids composing their primary structures and in the identity of residues forming their catalytic tetrad. AKR1C subfamily members are all composed of 323 residues, while AKR1D subfamily members have three additional residues. Multiple-sequence alignments show that these additional residues are all located in the N-terminal portion of the protein, around positions 3, 11, and 28. However, the most important difference distinguishing the two subfamilies is the substitution of the His_{117} residue found in the catalytic tetrad of all AKR1C members (Asp_{50}, Tyr_{55}, Lys_{84}, and His_{117}) with a negatively charged amino acid residue (Glu_{120}) in the AKR1D sequences (Asp_{53}, Tyr_{58}, Lys_{87}, and Glu_{120}). The importance of this glutamate residue in the specificity of the 5β-reductase activity has been tentatively studied by Jez and Penning (*17*), who have succeeded in introducing a stereospecific 5β-reductase activity into rat 3α-HSD simply by mutating the conserved His_{117} residue within the catalytic tetrad of this enzyme to a glutamate. These authors have proposed a hypothetical mechanism which implies a direct interaction between two residues of the catalytic tetrad: the Tyr and Glu residues. According to this mechanism, the protonated glutamate (Glu_{117}) residue establishes a hydrogen bond with the catalytic tyrosine (Tyr_{55}), thus lowering the pK_b of the tyrosine and making it a stronger acid able to promote acid-catalyzed enolysation of Δ4-3-ketosteroids. The presence of a glutamate residue in the catalytic tetrad of the AKR and its proximity to the catalytic tyrosine thus seem of primary importance for enzymes displaying 5β-reductase activity. However, since no three-dimensional structure for an enzyme containing a glutamate in its active site has been determined so far, the exact position of this residue within the catalytic tetrad as well as its position relative to the steroid nucleus and to the C4−C5 double bond to be reduced is currently unknown.

To further refine the catalytic role played by the Glu_{120} residues conserved in all 5β-reductase sequences characterized to date, we performed crystallographic studies on the human enzyme (AKR1D1) in complex with the NADPH cofactor as well as with a few of its steroid substrates. We report here the crystallization and the structure analysis of

Table 1: Summary of the Crystallization Conditions

	h5β-reductase in complex with $NADP^+$ and 5β-pregnandione
protein concentration (mg/mL)	12
crystallization temperature (K)	277
composition of mother liquor	20% PEG-4K, 0.1 M sodium cacodylate (pH 6.5), 0.2 M sodium citrate, 0.5 M $(NH_4)_2SO_4$
ratio of protein to mother liquor in the drop	2:1
largest crystal size (mm)	0.3 × 0.3 × 0.1
time to reach the maximum size (days)	30
cryoprotecting agent and final concentration used (%)	20% ethylene glycol and 1 mM progesterone

the h5β-red enzyme in a ternary complex with $NADP^+$ and 5β-DHP. Analysis of the geometry of residues within the catalytic tetrad (Asp_{53}, Tyr_{58}, Lys_{87}, and Glu_{120}) of the active site has allowed us to further refine their role in the catalysis and to understand the ability of h5β-red to bind and transform Δ4-3-ketosteroids, including steroid hormones and bile acid precursors. A realistic mechanism for the C4−C5 double bond reduction reaction catalyzed by h5β-red is thus proposed.

MATERIALS AND METHODS

Recombinant Human 5β-Reductase Expression and Purification. Escherichia coli cells, transformed with a plasmid containing the h5β-red cDNA coding region in fusion with glutathione *S*-transferase (GST), were used to produce at a high level a protein of 65 kDa, a molecular mass corresponding to the recombinant GST−h5β-reductase fusion protein. This protein remained in the soluble fraction after centrifugation at 100000*g* for 30 min. Soluble proteins were then applied directly to a glutathione-Sepharose column. After elution, the fusion protein was incubated overnight at 4 °C in the presence of the Precision protease, and the cleaved fragments were purified by anion-exchange chromatography. The fraction containing the purified h5β-red protein was then concentrated (to approximately 8.0 mg/mL), and a volume of approximately 500 μL was applied to a Sephacryl-S100 gel filtration column. The highly purified protein was finally concentrated to 10−12 mg/mL in a solution containing 10 mM Tris (pH 8.0) and 1 mM β-mercaptoethanol and stored at 4 °C.

Crystallization and X-ray Analysis of the Recombinant h5β-red. Prior to crystallization, a 4-fold molar ratio of NADPH and Prog was added to the concentrated and highly purified h5β-red protein. Crystals were obtained at 4 °C using the hanging-drop vapor diffusion technique. h5β-red crystals were generally obtained in drops of a 2:1 (v:v) ratio of protein and well solution (see Table 1 for crystallization conditions). Crystals appeared rapidly and grew to a suitable size for X-ray diffraction in approximately 5 days. Prior to diffraction, h5β-red−$NADP^+$−5β-DHP crystals were soaked for 5 min in a cryopreservative solution containing 1 mM Prog to achieve a full occupancy of the steroid-binding site. X-ray diffraction images were recorded at 100 K using our laboratory R-AXIS IIc image plate detector mounted on a RU-200 copper rotating anode generator equipped with focusing mirrors. Data were integrated and scaled using XDS (*18*). The h5β-red structure was obtained by the molecular replacement method performed with MOLREP from the CCP4 suite (*19*) using coordinates of the m17α-HSD−NADPH binary complex (Protein Data Bank entry 2HEJ) (*20*) as a search model. The refinement procedure was performed using Refmac (*21*) and CNS (*22*). The initial model issued from rigid body refinement was manually mutated to match the h5β-reductase sequence and was submitted to a cycle of simulated annealing at 3000 K followed by energy minimization cycles. After electronic density map calculations and manual rebuilding using O (*23*), NADPH cofactor and steroid were added to the model. Models were refined by simple energy minimization followed by isotropic *B*-factor refinement (restrained, individual *B*-factor refinement) and corrected by manual rebuilding. Missing parts of the models, β-mercaptoethanol, ethylene glycol, and water molecules were progressively added during the refinement procedure. Finally, the quality of the model was verified with PROCHECK (*24*).

Protein Data Bank Entries. Atomic coordinates for the h5β-reductase−$NADP^+$−5β-DHP ternary complex have been deposited with the Protein Data Bank and are available as entry 3CAV.

RESULTS AND DISCUSSION

Crystallization of h5β-Reductase in the Presence of NADPH and Progesterone and Overall Description of the Ternary Complex Structure. Ternary complex crystals of h5β-red (see Table 1 for crystallization conditions) belong to orthorhombic space group $P2_12_12_1$. A near-complete data set obtained at a resolution of 1.9 Å (Table 2) allowed us to calculate well-defined electron density maps and to build a molecular model that encompasses two protein molecules per asymmetric unit. The good quality of the density maps also allowed us to easily identify 5β-pregnan-3,20-dione (5β-DHP) in the ligand binding site instead of Prog, which was added to the protein solution before crystallization and to the cryoprotective solution. The structure was refined to a crystallographic *R*-factor of 20.9% (R_{free} = 23.7%). The final model lacks only the first methionine of each monomer; two β-mercaptoethanol molecules, five ethylene glycol molecules, and 644 water molecules were unambiguously fitted into the $2F_o - F_c$ map.

It is noteworthy that, in both monomers, a water molecule was found in the bottom of the steroid-binding cavity, stabilized well between the two residues essential for the catalytic activity of h5β-red, Tyr_{58} and Glu_{120}. This water molecule very likely occupies the position of the O3 atom found in the structure of all Δ4-3-ketosteroid substrates of this enzyme (see below). Above all, it should also be noted that the Glu_{120} residue does not interact directly with the Tyr_{55} in our h5β-red model, the reactive oxygen atoms of their side chains being separated by more than 4.2 Å. This observation thus forces us to question the tentative mechanism based on a sequence alignment and site-directed mutagenesis, which had previously been proposed by Penning et al. (*25*) concerning the reduction of the double bond

Table 2: Summary of Data Collection and Refinement Statistics for h5β-red in Complex with NADP$^+$ and 5β-Pregnandione (data of the last shell of resolution)

Data Collection	
resolution (Å)	19.7−1.9 (1.95−1.9)
unit cell parameters *a*, *b*, *c* (Å)	50.65, 111.27, 130.89
space group	$P2_12_12_1$
total no. of reflections	247882 (17845)
no. of unique reflections	55083 (4072)
completeness (%)	93 (92.4)
$I/\sigma(I)$	23.6 (6.3)
R_{merge} (%)	6.8 (25.3)
redundancy	4.5 (4.4)
Refinement	
no. of reflections used in the R_{work} set, in the R_{free} set	52975, 2831
R_{cryst} (%)	20.9
R_{free} (%)	23.7
rmsd from ideal	
bond lengths (Å)	0.007
bond angles (deg)	1.2
no. of non-hydrogen atoms	
all atoms	6136
proteins	5322
NADPHs	96
steroids	46
waters	644
heterogeneous atoms	28
overall *B*-factor (Å^2)	14.2
cofactor *B*-factor (Å^2)	8.1
steroid *B*-factor (Å^2)	24.2
Verification with PROCHECK (%)	
most favored regions	88.6
allowed regions	11.4
disallowed regions	0

of Δ4-3-ketosteroids performed by the h5β-red enzyme and the role played by the Glu_{120} residue in this activity (see below).

During the refinement of the h5β-red−NADP$^+$−5β-DHP ternary complex structure, we were astonished to discover that the steroid molecule present in monomers A and B adopted two different orientations. Indeed, in monomer A, the 5β-DHP molecule was oriented with its A-ring toward the catalytic site in a position that is very probably the one adopted by the steroid just after the enzyme has performed its 5β-reduction reaction. However, the steroid nucleus is slightly set back from the catalytic site with the oxygen atom of its ketone group on the C3 being hydrogen bound to the water molecule now occupying the catalytic site of the enzyme (Figure 2A). In monomer B, the position of the steroid nucleus in the binding cavity was similar to that observed in monomer A. However, the 5β-DHP molecule was in the opposite direction, its C17 acetyl side chain pointing toward the active site of the enzyme with its C20 ketone group also bound to the water molecule at the catalytic site (Figure 2B). The difference in the orientation of the steroid molecule between the two monomers was clearly visible when the shape of the electron density on the β-face of the steroid where the C18 and C19 angular methyl groups are located was observed. To eliminate any doubt, the positioning of the steroid was finally confirmed by omit map calculation. It thus appears that, contrary to the situation observed in monomer A, where the 5β-DHP formed from the 5β-reduction of Prog stays confined inside the steroid-binding site of the enzyme, in monomer B the newly formed

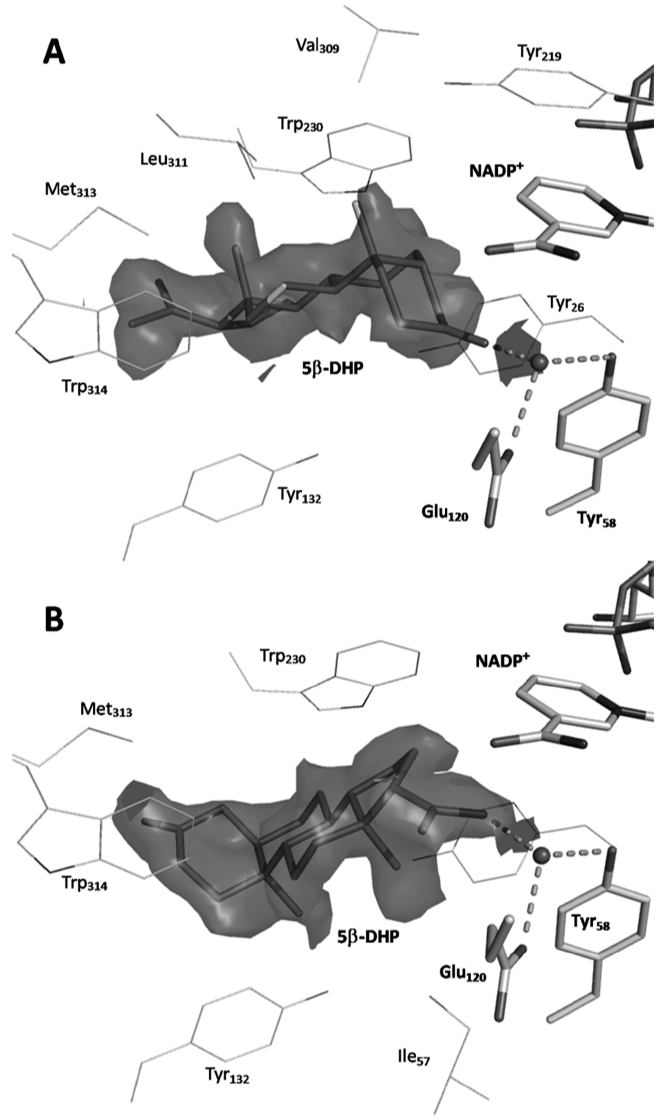


FIGURE 2: Difference in the orientation of the 5β-dihydroprogesterone molecule, the product of the C4−C5 double bond 5β-reduction of Prog, bound to monomer A (A) and monomer B (B) of the h5β-reductase ternary complex structure. Only residues (side chains) delineating the steroid binding cavity are depicted. Putative H-bonds are represented by dashed lines. Both omit maps corresponding to the steroid molecule are contoured at the 1σ level.

5β-DHP leaves the catalytic site and re-enters in the binding site with its O20 atom forward. The fact that the change in the steroid orientation occurs only in monomer B can be explained by the differences in crystal packing contacts around the entry of the steroid-binding cavity of the two monomers. Indeed, the large conical entry of the steroid-binding cavity of monomer B opens onto a solvent channel, the dimensions of which are large enough to allow the passage and/or reversal of a steroid molecule.

Spatial Organization of the Catalytic Tetrad Residues at the Active Site of h5β-Reductase. Members of the AKR enzyme superfamily retain a conserved catalytic tetrad of residues (Asp, Tyr, Lys, and His) at their active sites. The spatial organization of these residues (Figure 3A) shows that the tyrosine is flanked and hydrogen-bonded to the histidine on one side and to the lysine on the other side. The aspartate is also located in the proximity, being salt-linked to the lysine. It has been found that the tyrosine acts as a catalytic

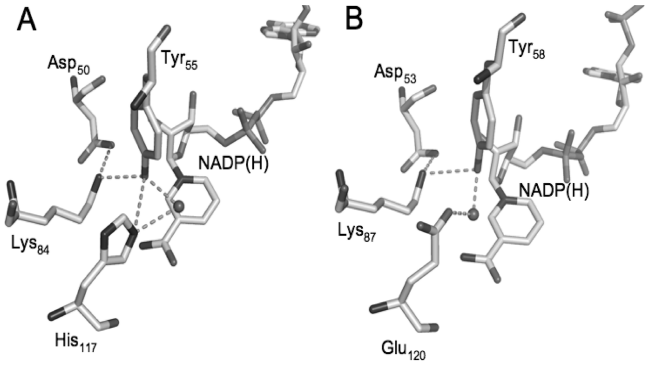


FIGURE 3: Comparison of the spatial organization between the catalytic tetrad residues of (A) an enzyme of the AKR1C subfamily (AKR1C1; PDB entry 1MRQ) and (B) h5β-red, a member of the AKR1D subfamily (AKR1D1; the structure reported here). Only residues of the catalytic tetrad (Asp_{50}, Tyr_{55}, Lys_{84}, and His_{117} of AKR1C1 and Asp_{53}, Tyr_{58}, Lys_{87}, and Glu_{120} of AKR1D1) are depicted. The water molecules and the putative H-bonds are represented by dark gray spheres and dashed lines, respectively.

general acid/base in both the reduction and oxidation reactions. In this way, the remaining residues of the catalytic tetrad, notably, the histidine and lysine, affect the ionization state of the tyrosine. Thus, in the reduction direction, the histidine facilitates donation of a proton from the catalytic tyrosine to the acceptor carbonyl group, whereas in the oxidation direction, the lysine facilitates removal of a proton from the donor hydroxyl group to the tyrosine.

h5β-red, like the other members of the AKR1D subfamily, is distinguished by the presence of a glutamic acid residue instead of a histidine in its catalytic tetrad (Asp_{53}, Tyr_{58}, Lys_{87}, and Glu_{120}). The substitution of the histidine with a glutamate in the structure of h5β-red has an impact on the shape of the steroid-binding cavity of this enzyme. A glutamate, which possesses a smaller and more flexible side chain than a histidine, contributes to making the cavity of h5β-red deeper than that of the other AKR enzymes, in particular those which exert their activity at the extremities of the steroid nucleus (Figure 4A,B). This seems logical since, to stereospecifically reduce the C4−C5 double bond on a steroid nucleus, a steroidal substrate must penetrate more profoundly toward the end of the cavity and the active site of the enzyme to perfectly position the β-face of its C5 atom over the C4 atom of the pyridine head of the cofactor, and thus make possible the *pro-R* hydride transfer from NADPH.

The importance of the Glu-for-His substitution with respect to the activity of AKR enzymes has been elegantly demonstrated by Jez and Penning (*17*), who have successfully introduced a 5β-reductase activity into rat 3α-HSD by substituting the wild-type His_{117} residue of this enzyme with a glutamate. After characterizing the new activity acquired by the His_{117}Glu mutant rat 3α-HSD, they then proposed a mechanism for C−C double bond reduction as well as a possible role which could be played by the glutamate residue. The central element of this tentative mechanism was that the protonated glutamate should be hydrogen-bonded to the catalytic tyrosine to make the tyrosine able to promote acid-catalyzed enolysation of the Δ4-3-ketosteroid substrate. However, our 5β-red structure clearly shows that these two residues do not interact together directly but are instead involved in the stabilization of a water molecule which may be at the position normally occupied by the O3 atom of the Δ4-3-ketosteroid substrate (Figure 3B). Four buried water molecules were also observed in the neighborhood of the Glu_{120} residue, connecting the active site with the protein surface via a very well-defined channel. These water molecules very likely contribute to the maintenance of Glu_{120} in a protonated state. In addition, considering the extremely apolar nature of the h5β-red steroid-binding site, this channel could be the passage taken by water molecules to reach the active site of the enzyme to stabilize side chains of Glu_{120} and Tyr_{58} residues. It thus appears that some of the elements of this previously proposed mechanism are incorrect or, at least, remain to be defined. We thus decided to use this work, taking advantage of the fact that the enzyme's spatial organization is now completely defined, to clarify the role of all the implicated residues.

Because 5β-DHP, the product of the reaction, was moved slightly away from the catalytic site in the ternary complex structure, we first wanted to determine the approximate position of the steroid substrate in the steroid-binding site during catalysis. A Prog molecule was thus manually modeled in the binding cavity of the h5β-red structure and initially positioned in the same orientation as 5β-DHP in the ternary complex (monomer A), with its A-ring pointing toward the end of the cavity. The Prog molecule was then moved by approximately 2 Å directly toward the end of the cavity and the catalytic site until its O3 atom was superimposed on the water molecule found between the Glu_{120} and Tyr_{58} residues (Figure 4C). Interestingly, modeled in this position, the A-ring (β-face) of the Prog was perfectly parallel to the nicotinamide ring (A side) of the cofactor (Figure 4D). The distance between the nicotinamide C4 atom (hydride donor) and the Prog C5 atom is between 2.8 and 3.0 Å, while the angle made by these two atoms with the pyridine head of the cofactor is approximately 100°. The relative position of these two atoms thus appears to be optimal for the promotion of the *pro-R* hydride transfer according to the orbital steering mechanism proposed by Heredia et al. (*26*). The rest of the Prog molecule, including its C20, C21, and O20 atoms, seems easily contained inside the steroid-binding site of h5β-red, since no clashes with any of the amino acids delineating the cavity were detected. In addition to the interactions made by its O3 atom with the Glu_{120} and Tyr_{58} residues, Prog could be stabilized by contacts with hydrophobic residues, namely, Ile_{229}, Trp_{230}, and Met_{313}, all of which are situated in the proximity of its steroid nucleus. For all these reasons, it is very likely that the hypothetical position in which we modeled the Prog molecule inside the 5β-red steroid binding cavity is that adopted by Δ4-3-ketosteroid substrates to form the productive ternary complex with this enzyme.

Putative Roles Played by the Tyr_{58} and Glu_{120} Residues in the Catalytic Mechanism of 5β-red. On the basis of this model and the structural findings concerning the relative position of the Tyr_{58} and Glu_{120} residues at its catalytic site, a tentative description of the mechanism of 5β-reduction performed by h5β-red can now be formulated (Scheme 1). Briefly, the C4−C5 double bond reduction mechanism proposed here occurs in two successive and concerted reaction steps. The first step involves the transfer of a hydride radical from NADPH to the C5 atom followed by, in the second step, the protonation of the C4 atom. In the first concerted reaction step, the transfer of the hydride from

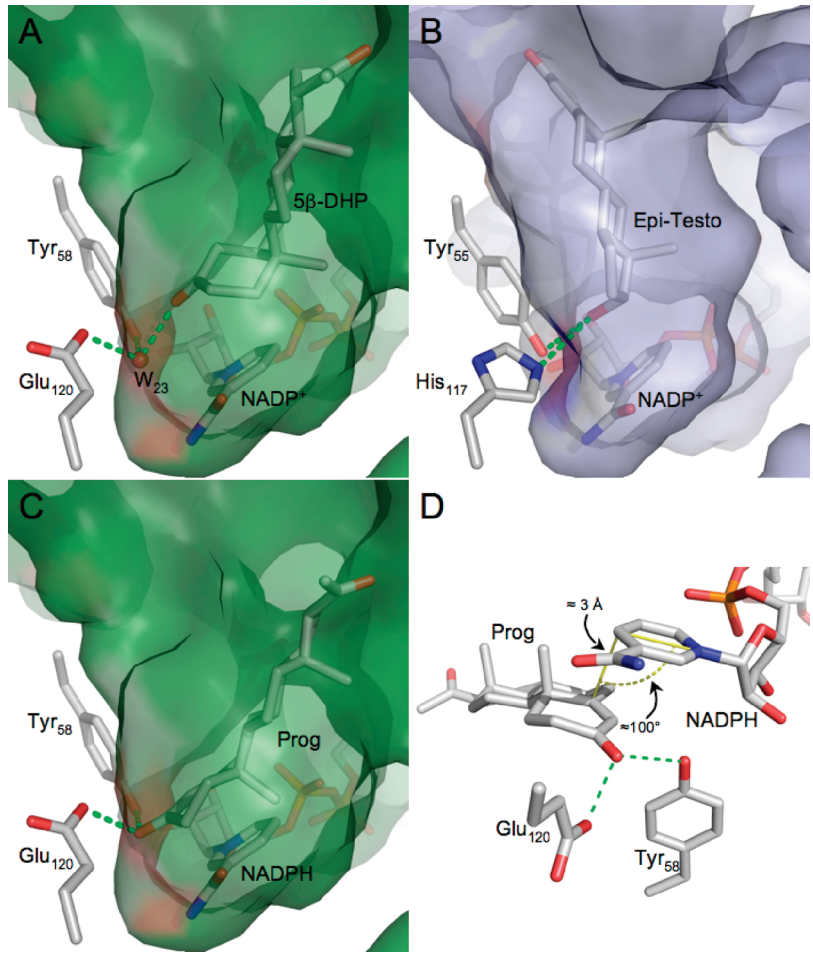


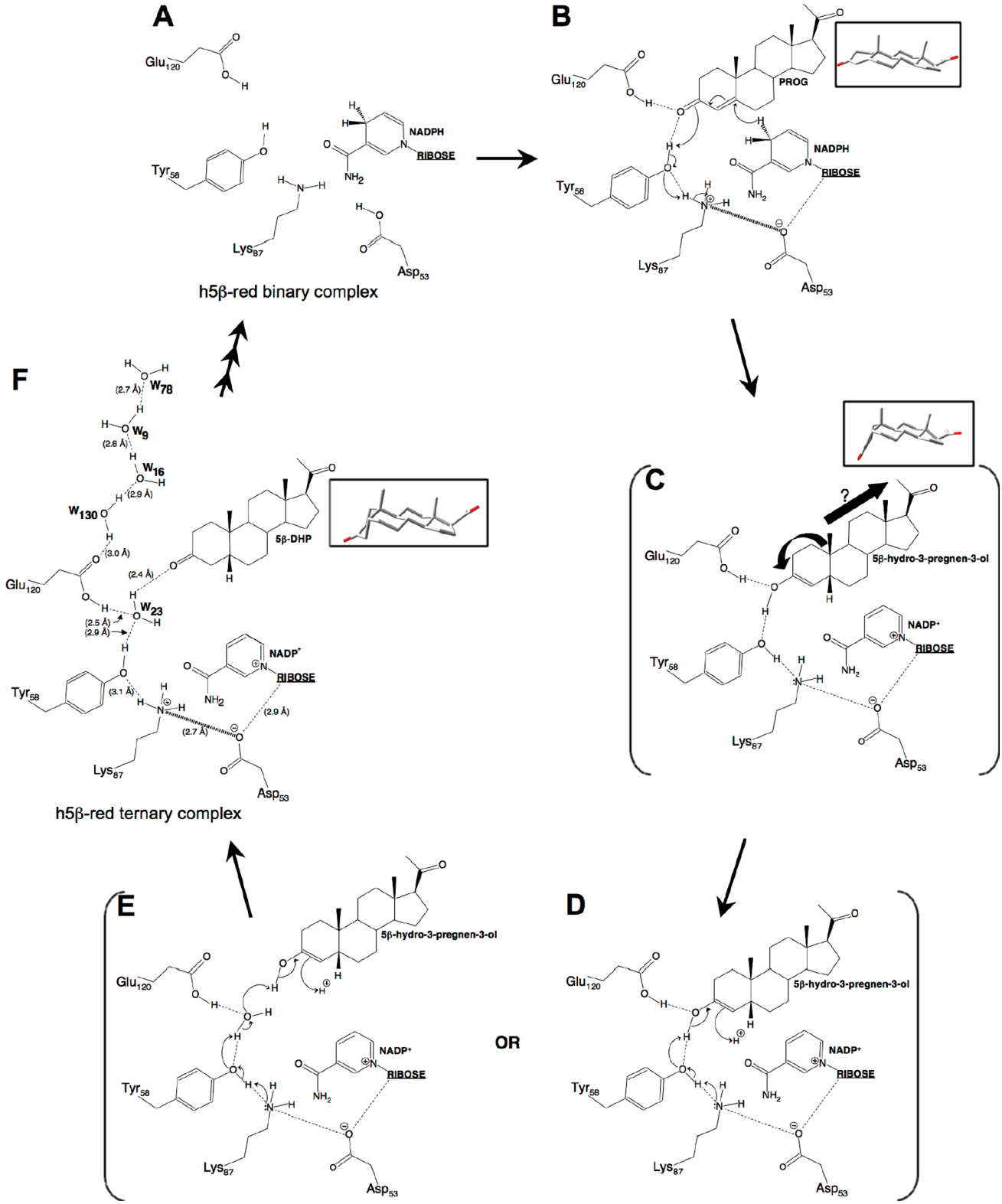
FIGURE 4: Comparison of the steroid-binding cavities of (A) h5β-red, in a ternary complex with $NADP^+$ and 5β-DHP, and (B) m17α-HSD, in a ternary complex with $NADP^+$ and Epi-Testo (AKR1C21; PDB entry 2IPF), showing how the substitution of a histidine with a glutamate contributes to making the cavity of h5β-red deeper than that of m17α-HSD, allowing (C) the steroid substrate (here a Prog molecule manually docked) to penetrate more profoundly into the steroid-binding cavity and (D) to adopt a productive binding orientation (*26*). The angle of the hydride with the target double bond and the distance from C4 of the pyridine head of the cofactor are indicated.

NADPH to the β-face of the steroid C5 atom occurs simultaneously with donation of a proton to the steroid O3 atom (Scheme 1, step B). In fact, the interactions made by Tyr_{58} and Glu_{120} with the O3 atom of the steroid strongly polarize the C3 carbonyl and contribute to a cationic character of C5 through the conjugated double bonds, facilitating the hydride transfer from NADPH. Both Tyr_{58} and Glu_{120} can donate a proton to O3, but the candidacy of Tyr_{58} as a putative proton donor is strongly supported by mutagenesis studies performed on the rat 3α-HSD, which have shown that the newly acquired 5β-reductase activity, resulting from the substitution of its His_{117} residue with a glutamate, was lost in the double mutant (Y55F/H117E) (*17*). By interacting with its hydroxyl group, Lys_{87} can influence the p*K* of Tyr_{58}, making it a stronger acid. Indeed, a positively charged lysine can stabilize the unprotonated state of the tyrosine residue, facilitating donation of a proton by Tyr_{58}. On the basis of our h5β-red crystal structures, we cannot, however, exclude the possibility that Glu_{120} can also be the proton donor, especially when it is in its protonated state, which seems to be the case here.

The first step of our proposed mechanism leads to the formation of a 5β-hydro-3,4-enol, a reaction intermediate whose structure already adopts the *cis*−*trans*−*trans* conformation with the A-ring bent 90° relative to the rest of the steroid nucleus (Scheme 1, step C). In fact, this structural change of the steroid nucleus is so important that it has been very difficult to model a *cis*−*trans*−*trans* steroid molecule in a position still allowing an interaction between its O3 atom and the catalytic residues (Tyr_{58} and/or Glu_{120}) without making an important repositioning of the steroid nucleus to prevent clashes with residues delineating the binding cavity (especially side chains of Tyr_{26}, Tyr_{132}, and Trp_{230} residues) or with the pyrimidine head of the cofactor. Interestingly, in the very few positions which satisfy these conditions, the orientation of the 5β-hydro-3,4-enol intermediate is similar to that of 5β-DHP in our h5β-red ternary complex structure (Figure 5A). In the case where, in spite of the drastic structural change in its steroid nucleus, the first step reaction intermediate (5β-hydro-3,4-enol) remains bound to the catalytic residues (Scheme 1, step D), it is likely that the tyrosine acts as a general base to abstract the hydrogen from the hydroxyl group at C3, initiating tautomerization and protonation at C4 to complete the reaction. This is supported by the fact that the side chain of Tyr_{58} can exist as a phenolate anion following the donation of the proton of its hydroxyl group in the first step of the reaction.

As mentioned above, during the first catalytic step, the steroid nucleus of the reaction product undergoes major structural changes which certainly affect its ability to remain bound to the catalytic site of the enzyme. In the event that the reaction intermediate is effectively released from the

Scheme 1: Proposed Mechanism for the C4−C5 Double Bond Reduction Catalyzed by h5β-red (see the text for details)[a]



[a] Thin curved arrows indicate electron transfers, while the dashed lines and the number in parentheses represent the putative hydrogen bonds and the H-bond distances, respectively. The structures of the steroid nucleus of the substrate (Prog), reaction intermediate (5β-hydro-3-pregnen-3-ol), and product (5β-DHP) are shown in boxes.

active site because of its bent conformation (Scheme 1, step E), we can imagine many mechanisms which would allow the completion of the reaction. First, assuming that the proximal binding site identified in our h5β-red−NADP$^+$−5β-DHP ternary complex structure is important for the catalytic activity of this enzyme, specifically for the second step of the reaction, the reaction intermediate could be transferred from the active site to the proximal binding site, which seems better adapted to bind steroids in a *cis*−*trans*−*trans* conformation. In this case, the abstraction of the hydrogen from

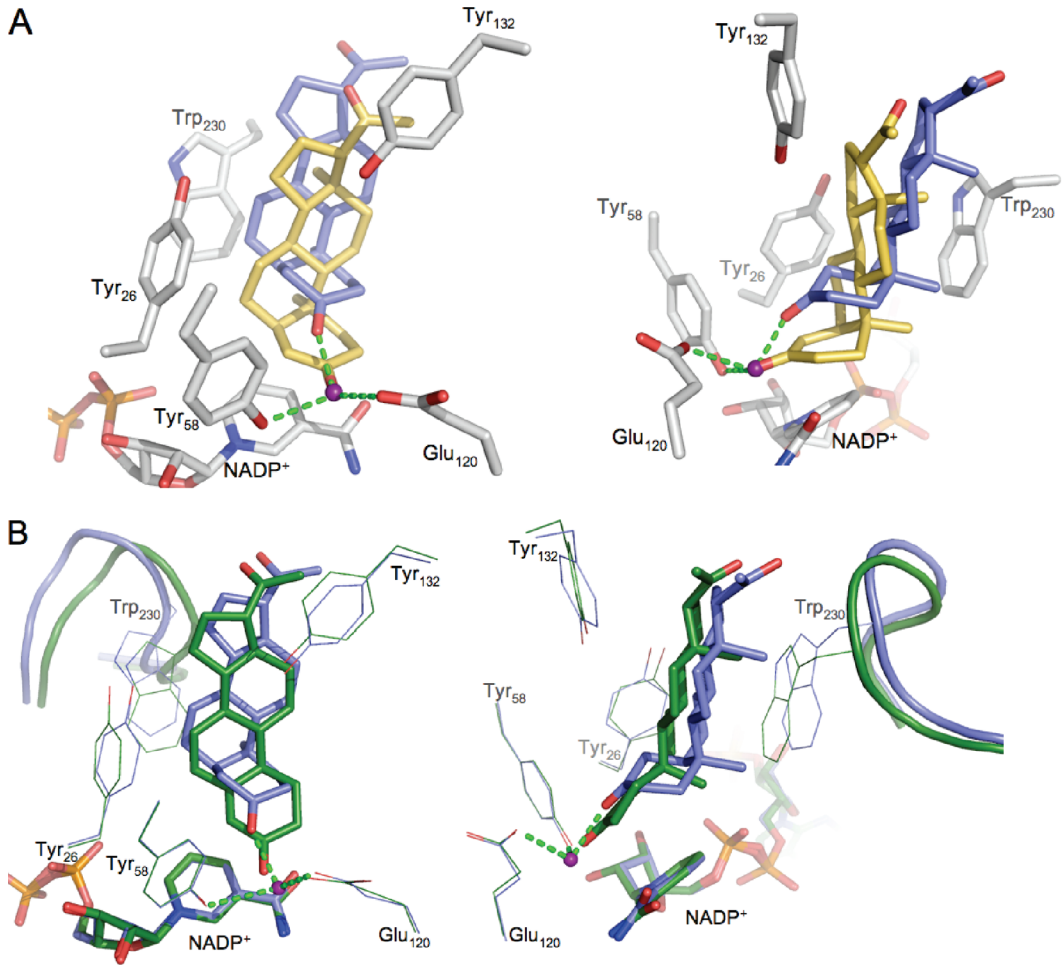


FIGURE 5: Orthogonal views showing (A) how the 5β-hydro-3,4-enol (yellow), the reaction intermediate produced from Prog, can be modeled in the binding cavity of the h5β-red structure in a ternary complex with 5β-DHP (blue) in a position still allowing its O3 atom to interact with the catalytic residues (Tyr_{58} and/or Glu_{120}) without clashing with residues delineating the binding cavity and (B) a comparison of the structure of some key residues forming the steroid-binding cavity of h5β-red in a ternary complex with 5β-DHP (blue) and Prog (green) [PDB entry 3COT (*31*)]. The water molecule occupying the catalytic site of the enzyme is depicted as a purple sphere.

the O3 hydroxyl group of the 5β-hydro-3,4-enol intermediate could be done by Tyr_{58} via the water molecule found between this residue and the O3 atom. Moreover, it would be interesting to determine the origin of this water molecule, considering the extremely apolar nature of the steroid-binding site of the h5β-red enzyme, and the fact that the narrow active site is largely filled by the steroid molecule. In this regard, Glu_{120} could play a major role, not only by contributing to the stabilization of the water molecule at this position but also by directly participating in its transfer and positioning in the catalytic site, immediately after the reaction intermediate is released from this site before being bound again by the enzyme at its alternative site. The presence of four buried water molecules in the neighborhood of the glutamate residue that connects the active site with the protein surface supports the idea that Glu_{120} is involved in a water transfer chain.

Second, because the 5β-hydro-3,4-enol intermediate is a highly unstable compound, the presence of a water molecule in the vicinity of the OH group at C3 may be sufficient to induce its final conversion into the 5β-dihydro-3-keto product. In this case, the water molecule would grab the hydrogen at O3 while liberating, in the neighborhood of C4, the proton required for completion of the reaction. Finally, the 5β-hydro-3,4-enol intermediate could simply dissociate from the enzyme, leaving the steroid-binding site before being spontaneously and nonenzymatically transformed into 5β-dihydro-3-keto.

Metabolism of Biliary Acids by 5β-red. We have determined the crystallographic structure of human 5β-reductase (AKR1D1), the only known human enzyme capable of performing the stereospecific 5β-reduction of Δ4-3-ketosteroids, an essential reaction for the production of bile acid precursors and some neuroactive steroids. In spite of many attempts, we were unable to obtain a structure of h5β-red in complex with one of its bile acid precursor substrates, either 7α-hydroxy-4-cholesten-3-one or 7α,12α-dihydroxy-4-cholesten-3-one; these compounds are not sufficiently soluble to be added in the crystallization solution. Nevertheless, it has been easy to model such an enzyme−bile acid precursor complex by manually positioning these molecules in our h5β-red structure (Figure 6). Placed in a productive position, that is to say with the O3 atom of the substrate placed in the proximity of the Tyr_{58} and Glu_{120} residues and with its C5 atom near C4 of the pyridine head of the cofactor, both 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one molecules are shown to be easily contained in the h5β-red-binding cavity. It is also interesting to note that, compared to steroid molecules, bile acid intermediates can establish supplementary contacts with residues delineating the steroid-binding cavity. Indeed, the 7α-hydroxyl group

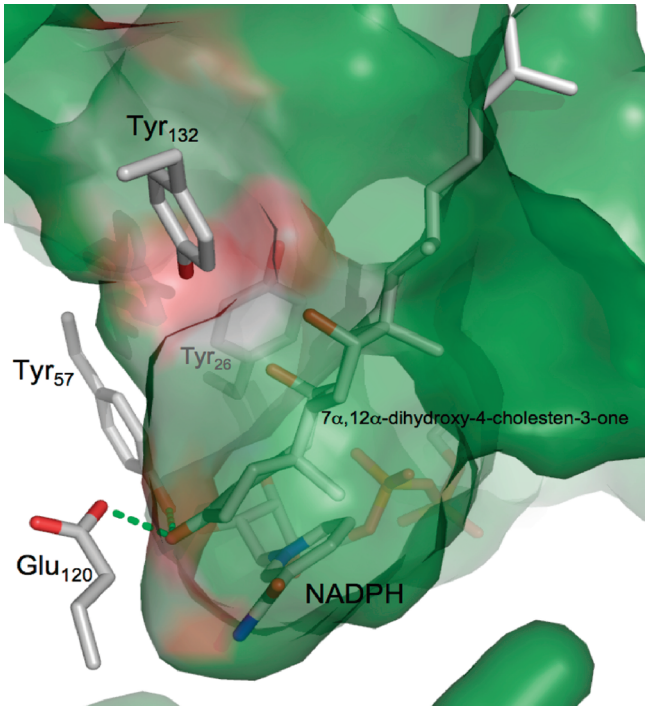


FIGURE 6: Hypothetical orientation of a bile acid precursor substrate (7α,12α-dihydroxy-4-cholesten-3-one) placed in a productive position in the h5β-red steroid-binding site.

found in the structure of these molecules is located <3 Å from the side chain of Tyr_{26}, while the hydroxyl at C12α may establish a hydrogen bond with the side chain of Tyr_{132}. Together with those provided by the long aliphatic chain at C17β, these additional contacts may account for the higher activity of this enzyme toward the bile acid intermediates.

To date, at least five different mutations leading to reduced activity of the hepatic enzyme have been identified in the gene encoding the 5β-red enzyme (*7, 27*). All patients with these mutations excreted 3-oxo-Δ4 bile acids as the major urinary bile acid and presented in the neonatal period with cholestatic jaundice which can progress rapidly to liver failure. Even though treatment with bile acid replacement therapy can lead to normalization of liver function, in at least two cases liver transplantation was eventually required. Four of these mutations are missense mutations resulting in a single amino acid change (L106F, P133R, P198L, or R260C). The other mutation was a 1 bp deletion leading to a frame shift and a premature stop codon. Even when using the high-resolution structure, we have determined for this enzyme, the real impact of these mutations on the activity of h5β-red is very difficult to explain, since the modified residues, especially Leu_{106} and R_{260}, are located some distance from the catalytic site and are not directly involved in the steroid or cofactor stabilization. We can, however, hypothesize that a replacement of the proline residue might have a major impact on the structure of the enzyme. Indeed, when located inside or near flexible structures which is the case here, proline residues can play a role in preventing conformational changes from propagating to the rest of the protein (*28–30*). In the h5β-red structure, the mutated prolines (Pro_{133} and Pro_{198}) belong to flexible loops, both located at the C-terminal end of the β-barrel. The flexibility of these loops has functional relevance, since it enables the enzymes to accommodate substrates of varying shapes and sizes and to control many molecular events such as catalytic action. Considering that these two proline residues are highly conserved among enzymes of the AKR1 family, it is very likely that their replacement could have a direct impact on the mobility of these loop motifs, thus affecting the capacity of the enzyme to recognize and bind its substrates and to coordinate their binding (or release) of cofactor NADPH. Further experiments, including expression, crystallization, and structure determination of these mutated h5β-red enzymes, will be necessary to understand how these mutations can affect the normal functioning of this enzyme.

Concluding Remarks. During the review of this work, crystal structures of the human 5β-red in complex with various substrates have been published by another group (*31*). A close comparison of our model with these structures, particularly the h5β-red−NADPH−Prog complex structure (PDB entry 3COT), revealed no significant differences, with the exception of the positioning of the steroid inside the binding cavity and that of some residues belonging to the mobile loop B (Asn_{227}−Val_{230}) which are slightly pushed away, apparently to allow the 5β-DHP molecule to bind to the proximal site (Figure 5B). The other residues delineating the steroid-binding site, including those forming the active site, occupy nearly identical positions in both structures. Interestingly, the position of the Prog molecule in the h5β-red−NADPH−Prog complex structure is very similar to the hypothetical one predicted by our modeling studies (Figure 4D). As anticipated, the steroid O3 atom is located between the two catalytic residues (Tyr_{58} and Glu_{120}) in a position allowing the establishment of two hydrogen bonds with the reactive oxygen atoms of their side chains. However, the O3 atom does not occupy the exact position of the water molecule found in the active site of our ternary complex structure, the two positions being separated by approximately 1.0 Å (Figure 5B). Consequently, in the crystal structure model determined by Di Costanzo et al. (*31*), the C5 atom of the Prog molecule is farther from the C4 atom of the pyridine head of the cofactor (3.8 and 4.0 Å for monomers A and B, respectively). These distances appear to be largely suboptimal for the *pro-R* hydride transfer from NADPH according to the orbital steering mechanism proposed by Heredia et al. (*26*) and shown in Figure 4D. In that light, it is very likely that the h5β-red−NADPH−Prog ternary complex structure does not correspond to the catalytically productive complex for this enzyme. In fact, to be 5β-reduced, a Δ4-3-ketosteroid substrate must enter more profoundly toward the enzyme active site to perfectly position its C5 atom with respect to the C4 hydride donor atom of the cofactor. This clearly explains why the Prog was found in the active site rather than the reaction product, the 5β-DHP. It is also interesting to note that the site occupied by the Prog is not the same site as the proximal binding site where the 5β-DHP was found in our ternary complex structure (Figure 5B). Besides, because of its shape, the proximal binding site seems to be much more adapted for a steroid with a *cis*−*trans*−*trans* conformation. Finally and more importantly, the h5β-red−NADPH−Prog ternary complex structure demonstrates that, even with a steroid substrate molecule bound to its active site, Glu_{120} does not directly interact with the other catalytic residues. This is in perfect agreement with the catalytic mechanism we propose for the C4−C5 double bond reduction (Scheme 1, step B) and

strongly supports our hypothesis according to which the Glu_{120} residue is not directly involved in catalysis but could instead be important for the proper positioning of the steroid substrate in the catalytic site.

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