

Crystal Structures of Human Δ 4-3-Ketosteroid 5β -Reductase (AKR1D1) Reveal the Presence of an Alternative Binding Site Responsible for Substrate Inhibition^{†,‡}

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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 in Δ 4-3-ketosteroids, including steroid hormones and bile acids precursors such as 7α -hydroxy-4-cholesten-3-one and $7\alpha,12\alpha$ -dihydroxy-4-cholesten-3-one. In order to elucidate the binding mode and substrate specificity in detail, biochemical and structural studies on human 5β -reductase (h 5β -red; AKR1D1) have been recently undertaken. The crystal structure of a h 5β -red binary complex provides a complete picture of the NADPH–enzyme interactions involving the flexible loop B, which contributes to the maintenance of the cofactor in its binding site by acting as a “safety belt”. Structural comparison with binary complexes of AKR1C enzymes, specifically the human type 3 3α -hydroxysteroid dehydrogenase (AKR1C2) and the mouse 17α -hydroxysteroid dehydrogenase (AKR1C21), also revealed particularities in loop B positioning that make the steroid-binding cavity of h 5β -red substantially larger than those of the two other enzymes. Kinetic characterization of the purified recombinant h 5β -red has shown that this enzyme exerts a strong activity toward progesterone (Prog) and androstenedione (Δ 4) but is rapidly inhibited by these substrates once their concentrations reach 2-times their K_m value. A crystal structure of the h 5β -red in ternary complex with NADPH and Δ 4 has revealed that the large steroid-binding site of this enzyme also contains a subsite in which the Δ 4 molecule is found. When bound in this subsite, Δ 4 completely impedes the passage of another substrate molecule toward the catalytic site. The importance of this alternative binding site for the inhibition of h 5β -red was finally proven by site-directed mutagenesis, which demonstrated that the replacement of one of the residues delineating this site (Val₃₀₉) by a phenylalanine completely abolishes the substrate inhibition. The results of this report provide structural insights into the substrate inhibition of h 5β -red by C19- and C21-steroids.

The 5β -reductase (h 5β -red)¹ is known to play a major role in the formation of the cholic and chenodeoxycholic biliary acids (1–3). More precisely, this enzyme catalyzes the reduction of the bile acid precursors 7α -hydroxycholest-4-en-3-one and $7\alpha,12\alpha$ -dihydroxycholest-4-en-3-one, to their corresponding 5β -reduced derivatives. Defects in the 5β -red gene result in high urinary and plasma levels of Δ 4-3-oxo steroids, which lead to liver failure and hemochroma-

toxis, conditions that are potentially lethal in newborn infants (4–6).

This enzyme is also important for metabolism of steroid hormones since it efficiently catalyzes the transformation of C21- and C19-steroids and, to a lesser extent, C27-steroids (7, 8). Because certain 5β -reduced steroids are active molecules and are possibly involved in many physiological functions (9–13), the activity of this enzyme is of primary importance. For example, 5β -pregnan-3,20-dione (5β -DHP), the product of the 5β -reduction of progesterone (Prog), is an endogenous ligand for the pregnane X receptor and for the constitutive androstane receptor, both implicated in clearance of xenobiotics (14). This Prog metabolite is also a potent tocolytic steroid (15) circulating concentrations of which have been found to decrease significantly in the active phase of the first stage of human labor (16). Because the relative abundance of 5β -red mRNA in placenta and myometrium also decreases significantly in association with labor (16), it has been hypothesized that this enzyme, through the formation of 5β -reduced Prog metabolites, could play a role in regulating myometrial activity and in onset of spontaneous labor in humans.

Contradictory results have however been obtained by different investigators concerning the substrate specificity of

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[‡] Atomic coordinates have been deposited with the RCSB Protein Data Bank and are available under the following accession codes: 3CAQ for h 5β -reductase/NADPH binary complex and 3CAS h 5β -reductase/NADPH/D4 substrate inhibition complex.

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¹ Abbreviations: Δ 4, androstenedione; h 5β -red, human 5β -reductase; h 3α -HSD3, human type 3 3α -hydroxysteroid dehydrogenase; 5β -DHP, 5β -pregnan-3,20-dione; AKR, aldo–keto reductases; Prog, progesterone; HSD, hydroxysteroid dehydrogenase; MPD, 2-methyl-2,4-pentandiol; SDR, short-chain dehydrogenase/reductase; Testo, testosterone.

Table 1: Dissociation Constant (K_d) of NADPH for Some AKR Enzymes

enzyme	K_d (nM)
h3 α -HSD3	2702 \pm 140
h5 β -reductase	528 \pm 28
m17 α -HSD	459 \pm 24

human 5 β -red. Indeed, it was first demonstrated that h5 β -red expressed in transiently transfected COS cells showed a small but significant 5 β -reduction activity toward cortisol and testosterone (Testo), whereas no activity was detected toward Prog or androstenedione (Δ 4) (17). It was later found that the same enzyme stably expressed in HEK293 cells catalyzed with similar efficiency the 5 β -reduction of Prog, Δ 4, and Testo (7). These authors also found that cortisol was a poorer substrate for h5 β -red, being transformed into its 5 β -reduced metabolite at an approximately 20-fold lower rate than Prog. The difference in the experimental conditions used by the two groups (assay of the 5 β -red activity in cell homogenates vs intact cells) was then used to explain these contradictory results (7). Complementary experiments, especially enzymatic characterization of the purified enzyme, thus seem necessary to better define the steroid substrate specificity of h5 β -red.

Human 5 β -red belongs to the aldo-keto reductase (AKR) superfamily and is the first member of the 1D subfamily (AKR1D1), which also encompasses the 5 β -reductases from rat (AKR1D2) (18) and rabbit (AKR1D3). Currently, AKR1D1 encodes the only known human enzyme capable of efficiently catalyzing the 5 β -reduction of Δ 4-3-ketosteroids. It is highly expressed in the liver (17) and, at lower levels, in testes and placenta (16, 19). Enzymes of the AKR1D subfamily differ from members of the AKR1C subfamily by the identity of residues forming their catalytic tetrad. Indeed, the His₁₁₇ residue found in the catalytic tetrad of all AKR1C members (Asp₅₀, Tyr₅₅, Lys₈₄, and His₁₁₇) is substituted by a negatively charged amino acid residue (Glu₁₂₀) in the AKR1D sequences (Asp₅₃, Tyr₅₈, Lys₈₇, and Glu₁₂₀). To further refine the catalytic role played by the Glu₁₂₀ residue in the specificity of the 5 β -reductase, we have initiated crystallographic studies on the human enzyme (AKR1D1). Very recently, we have determined the first structure for h5 β -red in ternary complex with NADP⁺ and 5 β -dihydroprogesterone (20) and found that Glu₁₂₀ is not directly involved in catalysis, as previously hypothesized (21), but may instead be important for the proper positioning of the steroid substrate in the catalytic site.

Using our homogeneous and highly active enzyme preparation, we decided to more completely characterize the enzymatic activity of h5 β -red toward steroid substrates. We thus noted that this enzyme is strongly inhibited by the steroids Δ 4 and Prog at concentrations that do not exceed 2-times their K_m value. It has been previously reported that h5 β -red can be strongly reduced or inhibited by mineralocorticoids, especially cortisol (22). However, the mechanism by which cortisol affects h5 β -red activity is currently unknown, as is the capacity of the other steroid hormones to inhibit this enzyme. To better understand, at a molecular level, the mechanism underlying the substrate inhibition, we also performed structural characterization of this enzyme, in two different complexes: a binary complex with NADPH and a ternary complex with cofactor and Δ 4. The position

and orientation of Δ 4 bound inside the steroid-binding site of the h5 β -red gave us crucial information on the mechanism by which steroids can influence the activity of this enzyme.

MATERIAL AND METHODS

Recombinant Human 5 β -Reductase Expression and Purification. h5 β -Reductase was expressed and purified as described previously (20). Briefly, a GST-h5 β -red fusion protein was expressed in *E. coli*. The expressed fusion protein was then purified and cleaved using successively a glutathione-sepharose column, anion exchange, and gel filtration. 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.

Steady-State Enzyme Kinetics. Determination of kinetic constants for Δ 4 reduction was performed as described previously (23, 24). Briefly, the reactions were performed for 15–60 min at 37 °C using a homogeneous preparation of h5 β -red enzyme (0.03–0.1 μ M/assay) in a final volume of 800 μ L in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) containing 0.5 mM NADPH, 0.1 μ M ¹⁴C-labeled steroid, and various concentrations of unlabeled steroid. Each velocity measurement was performed using a five-point curve in duplicate. After incubation, the steroids were extracted twice with 1 mL of ether, then resuspended in 40 μ L of CH₂Cl₂ and subjected to silica gel 60 thin layer chromatography (MERCK), before separation by migration in the CHCl₃/ether (10:3 v/v) solvent system. Substrate and metabolites were revealed by autoradiography and identified by comparison with reference steroids. Kinetic parameters were calculated using the GraphPad Prism 4 program using the Michaelis-Menten equation. When substrate inhibition was detected, an equation for substrate inhibition ($Y = (V_{max}S + V_i(S^2/K_i))/(K_m + S + S^2/K_i)$) was instead used.

Determination of Binding Constant for NADPH by Fluorescence Titration. Fluorescence titration assays were conducted essentially as previously described (23). Briefly, the binding constant (K_d) of recombinant h5 β -red for NADPH was determined by measurement of protein fluorescence on a SLM 8000 fluorometer. Measurements were made at 22 °C in a 2 mL cell containing a magnetic stirrer, the purified enzyme (20 mg/mL) and PBS buffer in a final volume of 1 mL. Protein fluorescence was measured following the incremental addition of NADPH (0–5 μ M). The total volume change was less than 0.5%. Excitation of samples was at 290 nm with fluorescence emission scanned from 300 to 500 nm at 120 nm/min. Emission and excitation bandpasses were both set to 4 nm. Data were plotted as the percentage change in fluorescence at emission λ_{max} (% Δ fluorescence) versus NADPH concentration. K_d values were estimated by fitting these data to a saturation absorption isotherm using the GraphPad Prism program.

Crystallization and X-Ray Analysis of the Recombinant h5 β -Reductase. Prior to crystallization, a 4-fold molar ratio of NADPH was added to the concentrated and highly purified h5 β -red protein. For h5 β -red/NADPH/ Δ 4 complex, Δ 4 was added at a 4-fold molar ratio. Crystals were obtained at 4 °C using the hanging-drop vapor diffusion technique. h5 β -Reductase crystals were generally obtained in drops of a 2:1 (v/v) ratio of protein and well solution (see Table 2 for

Table 2: Summary of the Crystallization Conditions

	h5 β -reductase in complex with NADPH	h5 β -reductase in complex with NADP ⁺ and androst-4-ene-3,20-dione
protein concentration (mg/mL)	12.8	9.55
crystallization temperature (K)	277	277
composition of mother liquor	PEG-4K 20%, 0.1 M Tris, pH 7.5, MPD 5%	PEG-4K 20%, 0.1 M Tris, pH 7.7, MPD 5%, 0.05 M CaCl ₂
ratio of protein to mother liquor in the drop	2/1	2/1
largest crystal size (mm ³)	0.6 × 0.2 × 0.2	0.6 × 0.2 × 0.2
time to reach the maximum size (days)	5	5
cryoprotecting agent and final concentration used (%)	ethylene glycol 20%	ethylene glycol 20% + 1 mM androst-4-ene-3,20-dione

crystallization conditions). Crystals appeared rapidly and grew to a suitable size for X-ray diffraction in about 5 days. Prior to diffraction, crystals of h5 β -red/NADPH/ Δ 4 complex were soaked for 5 min in a cryopreservative solution containing 1 mM Δ 4 in order 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 the XDS program (25). The h5 β -red structure was obtained by the molecular replacement method with the MOLREP software from CCP4 suite (26) using coordinates of the m17 α -HSD/NADPH binary complex (2HEJ) (24) as a search model. The refinement procedure was performed using Refmac (27) and CNS (28) programs. The initial model issued from rigid body refinement was manually mutated to match the h5 β -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 (29), 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 (30).

Site-Directed Mutagenesis. Plasmid pGEX-6P1-5 β -red Y132F and V309F mutations were performed using the QuickChange site-directed mutagenesis kit (Stratagene) with 5'-GCC-AGG-AGA-TGA-AAT-ATT-CCC-TAG-AGA-TGA-GAA-TGG-3' and 5'-GAA-TAA-AAA-TGT-CCG-CTT-TTT-TGA-ATT-GCT-CAT-GTG-GCG-CG-3' (mutated nucleotides are bold) as forward primers, respectively. Positive clones were sequenced to confirm the presence of the desired mutation.

RESULTS AND DISCUSSION

Characterization of Enzymatic Activity. Since certain steroid metabolizing enzymes of the AKR gene superfamily are labile and lose their activity upon cell homogenization (31, 32), we first determine whether our purified h5 β -red enzyme preparation had retained its activity toward steroid substrates. Kinetic studies were conducted immediately (or a few days later to test the stability of the recombinant enzyme) after the purification of the enzyme by varying initial concentrations of Δ 4 and Prog in the presence of saturating concentrations of NADPH. We found that our highly purified

recombinant h5 β -red preparation efficiently transforms Δ 4 and Prog into 5 β -androstenedione and 5 β -dihydroprogesterone, respectively, and remains active several days after its purification without marked loss of activity.

We noticed that the h5 β -red displays a strong substrate inhibition at relatively low steroid concentrations. Indeed, as seen in Figure 1A, at lower concentrations (up to 0.6 μ M of Δ 4), the kinetic data fit very well with the Michaelis–Menten equation ($K_m = 0.366 \pm 0.094 \mu$ M and a turnover of 0.78 min⁻¹). However, at higher Δ 4 concentrations, the velocity of the enzyme tends to decrease, indicating a substrate inhibition (Figure 1B). The passage from linear Michaelis–Menten behavior to substrate inhibition occurs in a transition zone of Δ 4 concentrations ranging from 0.6 to 1.0 μ M. Similar substrate inhibition of the enzyme was also observed with Prog (results not shown). Interestingly, it has been previously reported that h5 β -red activity can also be reduced or inhibited by an excess of mineralocorticoid (22). Thus, patients with mutations affecting the activity of 11 β -hydroxysteroid dehydrogenase type 2, the enzyme converting cortisol to cortisone, also have reduced serum and urine levels of 5 β -cortisol metabolites, suggesting that cortisol or cortisol metabolites are responsible for inhibition of h5 β -red. Human 5 β -red is not the only AKR enzyme for which substrate inhibition phenomena have been reported. Indeed, the AKR1C2 enzyme has been found to be substrate-inhibited by the synthetic compound tibolone, while the AKR1C1 enzyme is inhibited by two structurally similar xenobiotic substrates, *E*- and *Z*-10-oxonortriptyline (33, 34). However, h5 β -red is, to our knowledge, the only AKR enzyme for which substrate inhibition occurs with endogenous ligand. A low level of circulating steroids is probably not sufficient to inhibit h5 β -red, but under certain circumstance, like the one described by intracrinology (35), the steroid concentrations can potentially be high enough to inhibit the enzyme.

The fact that h5 β -red is inhibited by its steroid substrates Δ 4 and Prog explains the previously reported contradictory results concerning the activity of this enzyme toward C19- and C21-steroids. Using transiently transfected COS cell homogenates, Kondo et al. (17) first reported that h5 β -red could not metabolize Δ 4 and Prog. Subsequently, Charbonneau and Luu-The (7) found that the same enzyme expressed in stably transfected embryonic kidney (HEK-293) cells and assayed in intact cells efficiently catalyzes the 5 β -reduction of progestins and androgen hormones, including Prog, 17 α -hydroxyprogesterone, Δ 4, and Testo. At that time, it was hypothesized that the difference in the experimental conditions used by the two groups (assay of the 5 β -red activity in cell homogenates vs intact cells) probably explained the

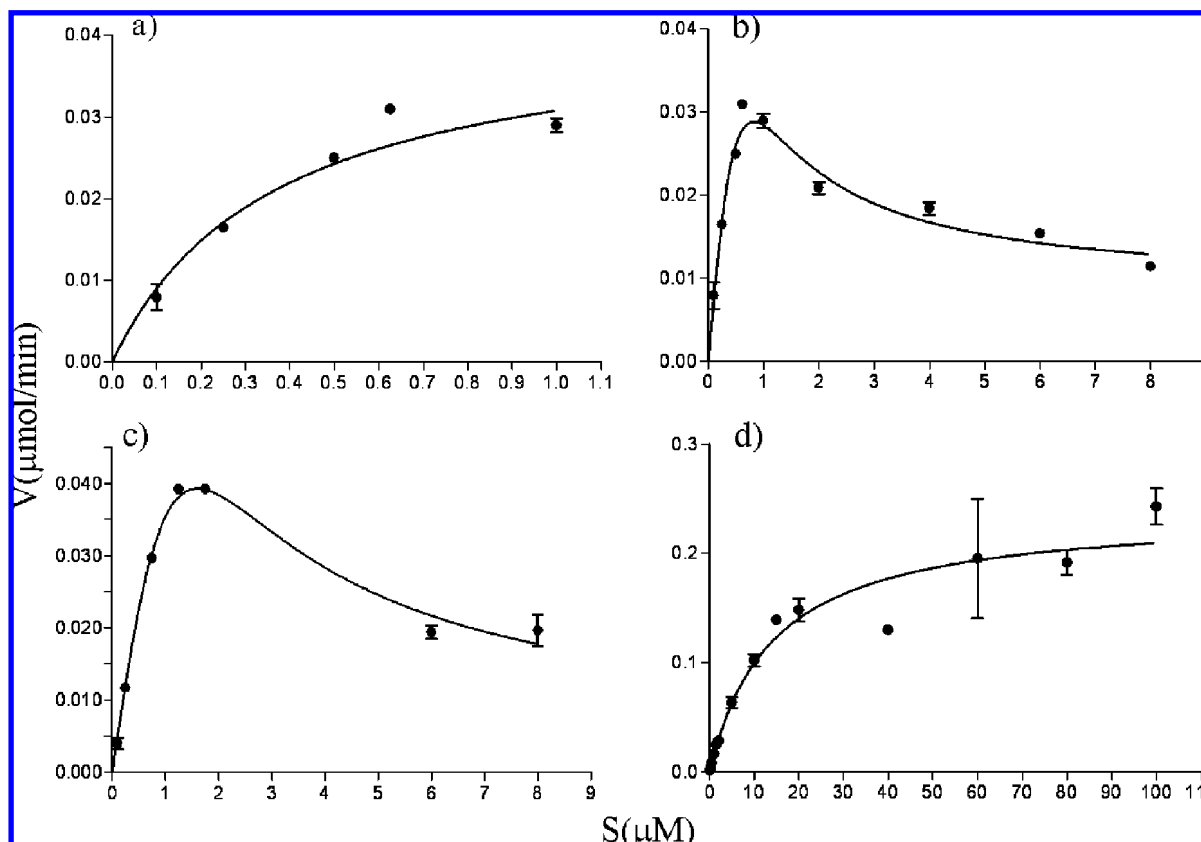


FIGURE 1: Michaelis–Menten kinetics for the reduction of androstenedione by (a) wild-type 5β -red at low substrate concentrations, (b) wild-type 5β -red at higher substrate concentrations, (c) 5β -red(Y132F) mutant, and (d) 5β -red(V309F) mutant. Each point represents the mean of duplicates. Curves in panels a and d are fitted using the Michaelis–Menten equation, while curves in panels b and c are fitted to a substrate inhibition equation.

discrepancy between these two results (7). This hypothesis cannot be ruled out; however, our findings about the stability of the recombinant $h5\beta$ -red upon cell homogenization and, more importantly, the fact that the activity of this enzyme is strongly reduced in the presence of relatively low concentrations of its steroid substrates urged us to re-evaluate the question. Considering that in their activity assays Kondo et al. (17) used a steroid concentration ($50\ \mu\text{M}$) that is sufficient to inhibit $h5\beta$ -red (see above), it is very likely that the amount of 5β -reduced steroids produced in this condition was too low to be easily detected using HPLC techniques. On the other hand, because they used very low steroid substrate concentrations (less than $0.1\ \mu\text{M}$) coupled with a more sensitive method for the detection of the steroidal products, Charbonneau and Luu-The (7) have successfully observed the reductive activity of the $h5\beta$ -red toward the sexual steroids that have a C4–C5 double bond in their structure.

Finally, we used a protein fluorescence quenching assay to determine the dissociation constant (K_d) for the $h5\beta$ -red-NADPH complex in order to compare it with those previously measured for two other AKR enzymes, $h3\alpha$ -HSD3 (AKR1C2) and $m17\alpha$ -HSD (AKR1C21) (Table 1). As previously demonstrated (23), the number of hydrophilic interactions forming the well-known “safety belt” of AKR enzymes directly affects the binding of the cofactor. We have shown that higher the K_d , tighter is the safety belt, because of the increased number of H-bonds maintaining the two loops forming the safety belt in a closed conformation. On the other hand, low K_d means fewer interactions favoring the two loops to be in an open conformation. In the open

conformation, AKR enzymes are more prone to bind NADPH but also are more prone to expel the oxidized NADP^+ . The high K_d value directly indicates a better stabilization of the safety belt in the closed conformation, and the enzyme needs a higher cofactor concentration to bind. Once bound to a high K_d AKR, the cofactor is trapped in the active site, and the tighter safety belt is less prone to open again and to release the cofactor. We thus found that the K_d for binding of NADPH by $h5\beta$ -red ($528 \pm 28\ \text{nM}$) is very similar to that of $m17\alpha$ -HSD ($459 \pm 24\ \text{nM}$) but more than 5-fold lower than that determined for $h3\alpha$ -HSD3 ($2702 \pm 140\ \text{nM}$) by the same method. This indicates that NADPH binding is easier in $h5\beta$ -red than in $h3\alpha$ -HSD3 and at a comparable level to that in $m17\alpha$ -HSD. This is in perfect agreement with the fact that binary complex crystals have been easily obtained for $h3\alpha$ -HSD3 without the need to add cofactor during the purification and crystallization processes, whereas addition of NADPH is absolutely required to obtain such binary complex crystals for $h5\beta$ -red and $m17\alpha$ -HSD enzymes (24, 36, 37).

Crystallization of $h5\beta$ -Reductase in Complex with NADPH. In order to better understand the peculiarities of $h5\beta$ -red in terms of cofactor binding and stabilization, we crystallized this enzyme in the presence of NADPH and compared it to other AKR1C enzyme binary complexes. Human 5β -red crystals obtained in the presence of NADPH were used for X-ray diffraction experiments, and a near complete data set was collected to $2.2\ \text{\AA}$ resolution (see Table 2 for crystallization conditions and Table 3 for data collection and refinement statistics). The $h5\beta$ -red structure adopts the

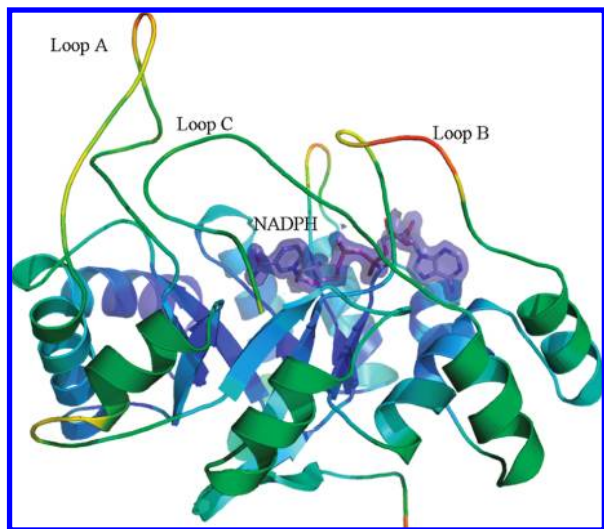


FIGURE 2: Overall view of h5 β -reductase in binary complex with NADPH. The ribbon representation shows the three mobile loops (loops A, B, and C), all of which are located at the C-terminal end of the β -barrel and which contribute to forming the cofactor and steroid binding sites. $2F_o - F_c$ electronic density map of NADPH is contoured at 1σ level. Protein structure is colored according to B-factor (from dark blue for low B-factor to red for high B-factor). The figure was generated with Pymol (Delano Scientific).

conserved TIM barrel fold and a very clear electronic density was observed for the NADPH molecule in each monomer (see Figure 2). Our h5 β -red binary complex model was refined to an R -factor of 21.6% ($R_{\text{free}} = 25.1\%$). The three additional residues of h5 β -red (Leu₃, Pro₁₁, and Lys₃₀) were unambiguously fit in the $F_o - F_c$ electronic map. However, for Lys₃₀ electron density was only present for the backbone atoms. In addition, the first residue of each monomer (Met₁) was not modeled, since no electron density was observed. Finally, a water molecule was found in the catalytic site of both monomers, in a position that could correspond to that of the O3 atom found in all steroid and bile acid substrates of this enzyme. This water molecule is indeed hydrogen-bonded to Tyr₅₈ and Glu₁₂₀, two residues of the catalytic tetrad. Finally, a MPD molecule originating from the crystallization was found in the ligand binding pocket of monomer A.

Binding of the Cofactor by h5 β -Reductase and Identification of the Safety Belt Interactions. Binding of the cofactor molecule constitutes the first step of the sequential ordered Bi-Bi mechanism characterizing enzymes of the AKR superfamily (38, 39). The NADPH molecule binds at the C-terminal end of the β -barrel (see Figure 2) and interacts with many hydrophilic residues distributed all along the cofactor molecule. A close examination of the hydrogen bond network established by h5 β -red with the NADPH molecule in our binary complex structure has allowed us to identify 18 putative H-bonds involving 16 amino acid residues. This hydrogen bond network is essentially the same as the one observed in the h3 α -HSD3 binary complex (24), in which we have identified a total of 19 hydrogen bonds formed by 16 residues. Moreover, there is no major difference in the nature of the amino acid residues involved in cofactor binding between these two enzymes nor in the distance separating the atoms making these interactions. It is thus very likely that other structural factors exist that can explain the

difference in cofactor binding affinity measured for h5 β -red and h3 α -HSD3.

Binding of the cofactor induces a large structural rearrangement of loop B over the NADPH molecule, providing additional interactions, especially with the central phosphate groups (24). Furthermore, in this new conformation, loop B residues are able to establish other strong hydrophilic interactions with residues of a small loop $\beta 1-\alpha 1$ located on the opposite side of the cofactor binding cleft. In this particular position over the cofactor, loop B acts as a “safety belt” (40) and contributes to the maintenance of the cofactor tightly bound to the enzyme. By comparing the structure of different AKR enzymes in binary complex with NADPH and by proceeding to site-directed mutagenesis experiments, we have recently shown that the number and strength of the hydrogen bonds involved the safety belt could be directly related to the binding constant of NADPH, even if no residue of the safety belt makes any direct interaction with the cofactor (23, 24, 41). Briefly, the weaker the stabilization of the two loops of the safety belt, the lower is the binding constant. If the loops are less stabilized as observed in m17 α -HSD, they are more prone to be in an open conformation and to facilitate the NADPH binding (or release of the product). On the other hand, a strong network of H-bonds in the safety belt, as observed for h3 α -HSD3, can impede the binding of NADPH or the release of the oxidized product. In the h5 β -red binary complex structure reported here, there is only one hydrogen bond at the interface of loop B and loop $\beta 1-\alpha 1$ (between residues Glu₂₈ and Asn₂₂₇), a quite similar structural arrangement to that found in the m17 α -HSD structure but one that differs from that observed in the h3 α -HSD3 structure (Figure 3A–C). Interestingly, this finding is again in perfect agreement with the K_d values determined for these three enzyme–NADPH complexes (Table 1). On the other hand, the position of the single hydrogen bond joining loop B and loop $\beta 1-\alpha 1$ in h5 β -red (Figure 3A) differs from that found in the m17 α -HSD safety belt (Figure 3C). Indeed, in the h5 β -red safety belt, the interloop hydrogen bond is located near the top extremity of the loops, while it is located at the bottom portion in the m17 α -HSD structure. The main structural consequence of this difference is that the two loops are slightly more distant from each other in h5 β -red compared with m17 α -HSD or h3 α -HSD (Figure 3D). As a result, the steroid-binding cavity of h5 β -red is substantially larger than that of the two other enzymes (Figure 3A–C, right panels).

Crystallization of h5 β -Reductase in the Presence of NADPH and Androstenedione and Overall Description of the Structure. As mentioned above, h5 β -red demonstrates a strong substrate inhibition in the presence of low concentrations of $\Delta 4$. We characterized, at the molecular level, this inhibition mechanism by determining the crystal structure of h5 β -red in the presence of $\Delta 4$. Ternary complex crystals of h5 β -red were obtained by adding a 4-fold molar excess of $\Delta 4$ to the concentrated protein prior to its crystallization (see Table 2 for crystallization conditions). The near complete data set (see Table 3 for data collection and refinement statistics) allowed us to build a model with two protein molecules per asymmetric unit at 2.0 Å resolution. The cofactor molecules (one for each monomer) were unambiguously fit in the electronic density. We also found a large electronic density that corresponded very well to the shape

Table 3: Summary of Data Collection and Refinement Statistics

	h5 β -reductase in complex with NADPH	h5 β -reductase in complex with NADP ⁺ and androst-4-ene-dione
Data Collection		
resolution (Å)	19.7–2.2 (2.3–2.2)	19.6–2.0 (2.1–2.0)
unit cell params		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	50.38, 110.63, 130.65	50.41, 110.87, 130.28
α , β , γ (deg)	90	90
space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
total reflns	132357 (15704)	384886 (21805)
unique reflns	36835 (4411)	47996 (5657)
completeness (%)	97.2 (95)	95.6 (83.8)
<i>I</i> / σ <i>I</i>	16.2 (4.8)	21.3 (5.1)
<i>R</i> _{merge} (%)	10.2 (28.1)	7.4 (23.3)
redundancy	3.6 (3.6)	8.0 (3.9)
Refinement		
reflns used, <i>R</i> work set/ <i>R</i> _{free} set	35278/1854	45704/2435
<i>R</i> _{cryst} (%)	21.6	20.8
<i>R</i> _{free} (%)	25.1	23.8
rmsd from ideal, bondlength (Å)/angle (deg)	0.007/1.2	0.007/1.2
non-hydrogen atoms		
all atoms	5693	5923
protein	5283	5296
NADPH	96	96
steroids	N/A	42
water	278	461
heterogeneous atoms	36	28
overall B factors (Å ²)	21.1	14.0
cofactor B factors (Å ²)	17.8	10.4
steroids B factors (Å ²)	N/A	23.5
Verification with PROCHECK (%)		
most favored regions	88.8	89.7
allowed regions	12.2	10
disallowed regions	0	0.3

of an all-*trans* steroid skeleton, suggesting that the steroid bound to the enzyme had not been 5 β -reduced. After close examination, we found that the bound steroid very likely corresponded to the Δ 4 added to the protein at the moment of its crystallization. However, the Δ 4 molecule was not profoundly inserted into the steroid-binding cavity with its A ring toward the catalytic site in a position allowing its 5 β -reduction. It was instead positioned at the entry of the cavity, firmly tethered by hydrogen bonds at both its extremities (Figure 4). Indeed, the C3-keto oxygen atom of Δ 4 established strong hydrogen bonds with the side chain of Ser₂₂₅ and backbone of Asn₂₂₇, two residues belonging to loop B, while its C17-keto oxygen atom was H-bonded to the hydroxyl group of Tyr₁₃₂ residue. In addition, the steroid nucleus was also very well stabilized by hydrophobic interactions involving Val₃₀₉ and Trp₂₃₀, whose indole ring side chains were perfectly stacked against rings C and D. In this position and orientation, the bound Δ 4 obviously hinders access to the catalytic site of the h5 β -red enzyme for the incoming steroid substrates. It also prevents a substrate molecule from binding h5 β -red in a catalytically productive position (Figure 5). Finally, because its stabilization in this subsite involves the Asn₂₂₇ residue of loop B, Δ 4 also certainly interferes with the formation or the opening of the safety belt and, consequently, with the binding or release of the cofactor.

In addition to the obstruction created by the steroid bound in this alternative position, the Trp₂₃₀ side chain also contributes to diminishing the size of the opening of the steroid-binding cavity. In the 5 β -red/NADPH binary complex structure, the indole ring of Trp₂₃₀ is turned approximately

90° and is positioned in the space occupied by the steroid A-ring in the h5 β -red/NADPH/ Δ 4 ternary complex structure (Figure 6). This suggests that Trp₂₃₀ acts as a swinging door, controlling the passage of steroid substrates toward the catalytic site of h5 β -red. Thus, in the binary complex, the side chain of Trp₂₃₀ has sufficient space to flip and to clear the opening of the steroid-binding cavity, allowing the passage of a steroid molecule toward the catalytic site of the enzyme. However, when a steroid molecule is bound in the alternative binding site, Trp₂₃₀ can no longer flip or rotate, contributing to a barrier able to efficiently impede any other steroid molecules from reaching the active site of h5 β -red. It is thus more than likely that this subsite, or alternative binding site, within the substrate-binding site where the Δ 4 molecule is bound in the present ternary complex structure corresponds to the inhibitor-binding site responsible for the competitive substrate inhibition phenomenon observed.

Characterization of the Alternative Binding Site of h5 β -Reductase. We used site-directed mutagenesis to verify this hypothesis and to better characterize this alternative steroid-binding site in order to determine its importance in the mechanism of substrate inhibition of h5 β -red. We first mutated residues directly involved in Δ 4 stabilization in the alternative binding site. At the O3 extremity, the replacement of Asn₂₂₇ was useless since this residue establishes a hydrogen bond with Δ 4 via its backbone amino group (-NH). Consequently, it was impossible to completely eliminate the interaction made by the enzyme with this part of the steroid, even when removing the other hydrogen bond made by the Ser₂₂₅ residue. We instead mutate the residue that maintains the other extremity of Δ 4 (O17 atom), Tyr₁₃₂, and change it

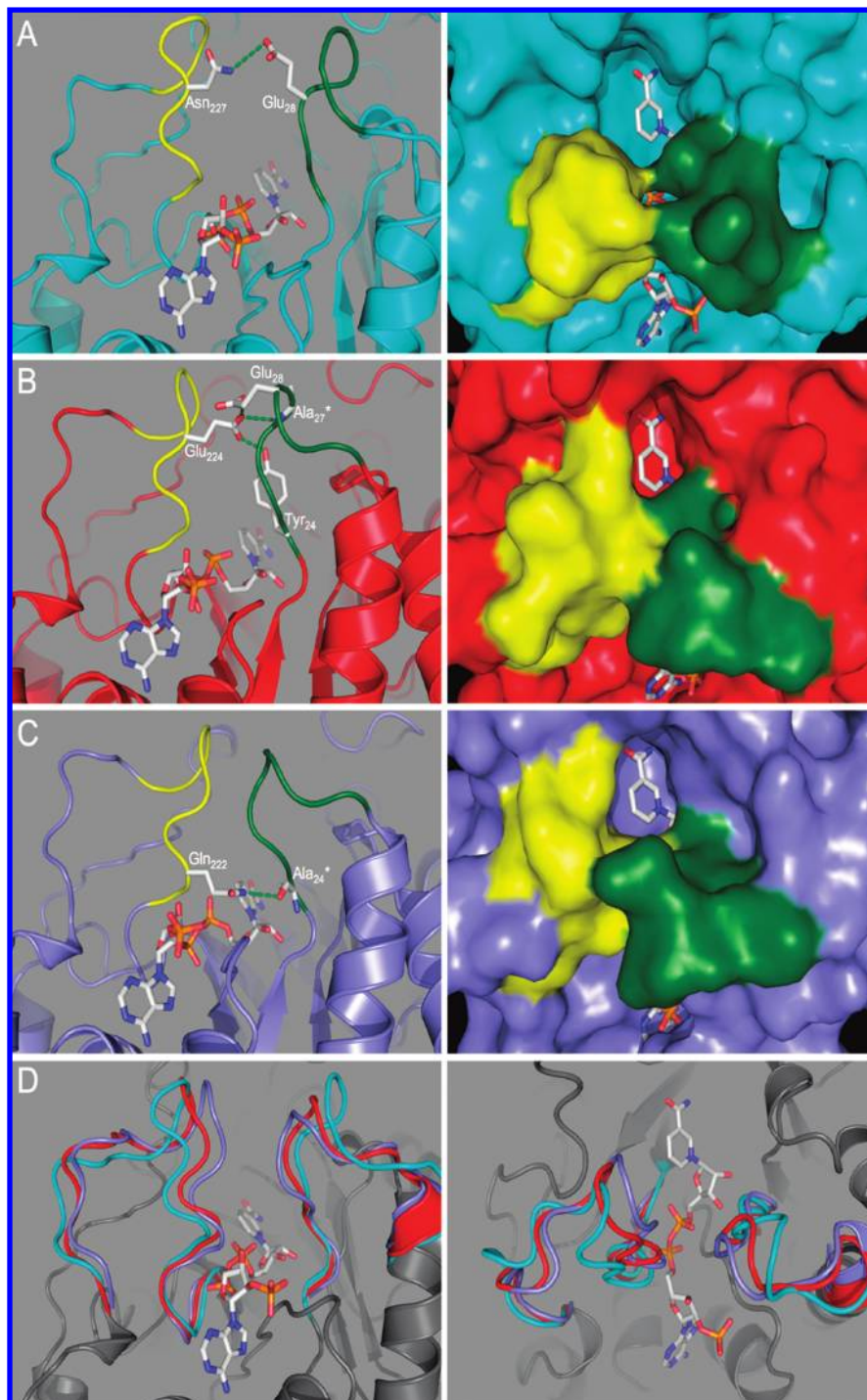


FIGURE 3: Comparison of the “safety belt” formation following NADPH binding in (A) h5 β -reductase-NADPH, (B) human type 3 3 α -HSD-NADPH, and (C) mouse 17 α -HSD-NADPH binary complexes (left panels). For simplification, only residues involved in the formation of hydrogen bonds (dashed green lines) connecting the mobile loop B and the small β 1- α 1 loop are depicted. Asterisks (*) indicate that the interaction is with the main chain. The impact of the position of the mobile loop B (in yellow) and the small β 1- α 1 loop (in green) on the shape and size of the enzyme’s steroid-binding cavity is also shown (right panels). Panels D show two orthogonal views of a superimposition of the three safety belts: h5 β -red in cyan, h3 α -HSD3 in red, and m17 α -HSD in purple. The figure was generated with Swiss-PDB viewer (45) and Pymol (Delano Scientific).

to a phenylalanine, a residue frequently found at this position in the structure of many other AKR enzymes. Astonishingly, kinetic assays showed that the h5 β -red(Y₁₃₂F) mutant enzyme was still substrate-inhibited by Δ 4, albeit the transition zone of Δ 4 concentrations at which the substrate inhibition phenomenon occurs was slightly shifted, now ranging from 1.0 to 2.0 μ M (Figure 1C). The catalytic efficiency of the mutated enzyme was also similar to that of the wild-type

h5 β -red enzyme (Table 4). Consequently, if the alternative binding site identified in the h5 β -red/NADPH/ Δ 4 ternary complex structure really corresponds to the inhibitor-binding site, this result indicates that the loss of the interaction between Tyr132 and the O17 atom of Δ 4 has a very limited impact on the binding of the steroid substrates to this site.

Following this unsuccessful attempt to diminish the affinity of Δ 4 for the alternative binding site, we increased steric

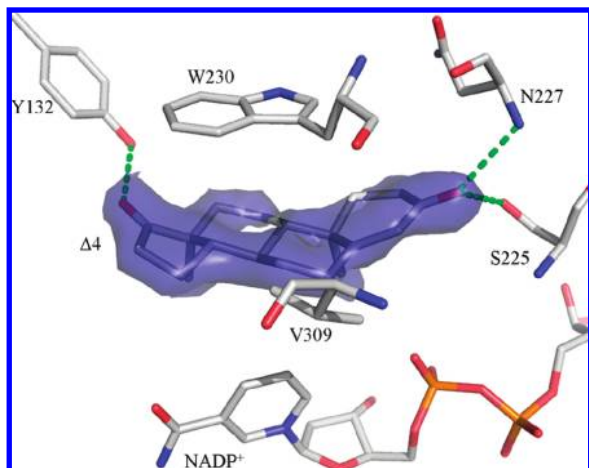


FIGURE 4: Close-up view of residues interacting with the androstenedione ($\Delta 4$) molecule in the alternative binding site of monomer A. The figure shows residues making H-bonds (dashed green lines) with $\Delta 4$, namely, Tyr₁₃₂, Ser₂₂₅, and Asn₂₂₇. Residues making hydrophobic contacts with the steroid, namely, Trp₂₃₀ and Val₃₀₉, are also represented. The $2F_o - F_c$ map for $\Delta 4$ is contoured at 1 σ level. Atoms are colored using standard CPK color set, and the figure was generated with PyMol (Delano Scientific).

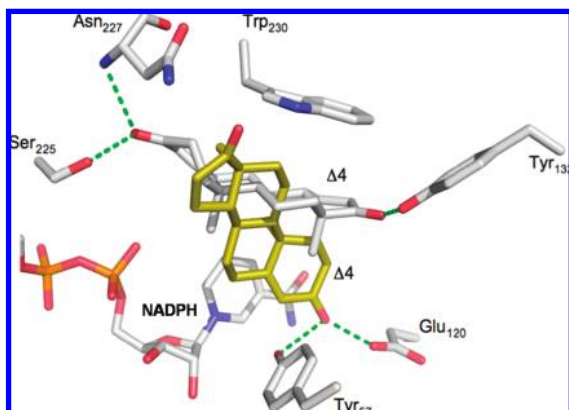


FIGURE 5: View of the two mutually exclusive positions for a $\Delta 4$ molecule in the steroid-binding site of $h5\beta$ -red. The figure shows $\Delta 4$ bound in a productive or catalytic orientation (in gold) and in a nonproductive orientation (in standard CPK color set). Residues making H-bonds (dashed green lines) with $\Delta 4$ when bound in the alternative site (Tyr₁₃₂, Ser₂₂₅, and Asn₂₂₇) or in the catalytic site (Tyr₅₇ and Glu₁₂₀) are also represented. The figure was generated with PyMol (Delano Scientific).

hindrance directly inside the alternative binding site in order to more drastically disturb the enzyme- $\Delta 4$ interaction. As observed in Figure 6, when $\Delta 4$ occupies the alternative binding site, the Val₃₀₉ side chain makes a hydrophobic contact with the steroid nucleus around its C6 and C7 atoms. By replacing this small residue with a bulky phenylalanine, we made the $h5\beta$ -red enzyme unable to bind $\Delta 4$ in the alternative position. This was confirmed by kinetic assays made on the $h5\beta$ -red(V₃₀₉F) mutant enzyme, which showed that the enzyme is no longer inhibited by the substrate, even at high $\Delta 4$ concentrations (Figure 1D). It thus appears that the $\Delta 4$ substrate now has free access to the catalytic site of $h5\beta$ -red(V₃₀₉F), since the mutation has made its high-affinity alternative steroid-binding site inaccessible. However, $h5\beta$ -red(V₃₀₉F) displays a 13.6-fold reduced catalytic efficacy compared with the wild-type enzyme, probably because the steric hindrance generated by the presence of a phenylalanine residue at position 309 also restrains the capacity of the side

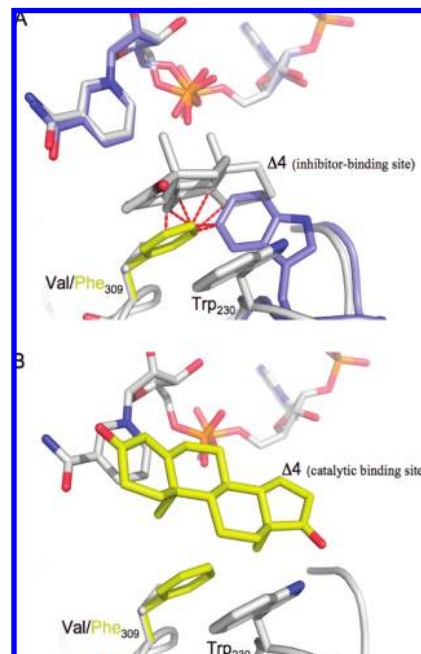


FIGURE 6: Likely impact of the replacement of the valine residue at position 309 by a phenylalanine on the binding of a $\Delta 4$ molecule in the steroid-binding site of $h5\beta$ -red. (A) The presence of a bulky side chain at position 309 very likely impedes (indicated by red broken lines) both binding of $\Delta 4$ in the inhibitor-binding site (in standard CPK color set) and Trp₂₃₀ from occupying the position observed in the binary complex structure (in blue). (B) On the other hand, it seems that the Val₃₀₉Phe mutation always allows the passage of a $\Delta 4$ molecule (in gold) into the active site of $h5\beta$ -red and its binding in a productive or catalytic orientation. The figure was generated with PyMol (Delano Scientific).

Table 4: Steady-State Kinetic Parameters for Androstenedione Reduction Catalyzed by the Wild-Type and Mutated Forms of $h5\beta$ -Reductase

enzyme	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)
$h5\beta$ -reductase	0.366 ± 0.094	0.78	2.13
$h5\beta$ -red(Y132F)	0.974 ± 0.22	2.43	2.49
$h5\beta$ -red(V309F)	16.29 ± 2.12	2.55	0.156

chain of the Trp₂₃₀ residue to flip and to completely clear the passage toward the catalytic site of the enzyme. This is supported by the fact that Val₃₀₉ and Trp₂₃₀ residues are in close proximity. It is likely that the phenyl ring of the Phe₃₀₉ fills the space occupied by the ring B of $\Delta 4$ bound in the alternative binding site and can therefore interfere with the indole ring of the Trp₂₃₀ residue (Figure 6A). In addition, in this position the bulky side chain of Phe₃₀₉ considerably reduces the size of the steroid binding cavity, explaining why the K_m value of $h5\beta$ -red(V₃₀₉F) for $\Delta 4$ (Table 4) is significantly increased (44.5-fold over that of the wild-type enzyme). Indeed, in the manual docking experiments we have made for this enzyme (see above), the side chain of a phenylalanine residue placed at this position pointed inside the steroid binding cavity and interacted very closely with the angular methyl groups (C18 and C19 atoms) of a $\Delta 4$ molecule positioned in the catalytic site in an optimal position for its 5β -reduction (Figure 6B). Nevertheless, considering the plasticity of residues delineating the steroid-binding site, a well-known characteristic of the AKR enzyme family (42, 43), it is also possible that the position of other residues is affected by the presence of Phe₃₀₉, therefore also potentially contrib-

uting to the change in substrate affinity observed in the mutant enzyme.

Concluding Remarks. During the review of this work, crystal structures of the human 5 β -red in complex with various substrates have been published by another group. Similar to the result discussed here, Di Costanzo et al. (44) published a structure of h5 β -red in complex with testosterone in the same alternative binding site we have identified. It is noteworthy that the coordinates of our structures were deposited to the RCSB PDB before the published structure of Di Costanzo et al. (44). In addition, these authors did not characterize this subsite by performing site-directed mutations or kinetic assays. The interactions made by the testosterone molecule observed in their complex are identical to what we observed for Δ 4. Furthermore, contrary to the published structures by Di Costanzo et al., we have identified a structural rearrangement of Trp₂₃₀ between our h5 β -red/NADPH and h5 β -red/NADPH/ Δ 4 complexes. The presence of a glycerol molecule in the vicinity of Trp₂₃₀ of the Di Costanzo et al. (44) binary complex certainly impedes this residue from adopting the conformation we have identified in our binary complex. We have suggested that this residue can act as a swinging door to allow the steroid to enter in the active site or in the substrate inhibition site depending upon its conformation.

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