Identification of Key Amino Acids Responsible for the Substantially Higher Affinities of Human Type 1 3β-Hydroxysteroid Dehydrogenase/Isomerase (3β-HSD1) for Substrates, Coenzymes, and Inhibitors Relative to Human 3β-HSD2*

Received for publication, February 3, 2005, and in revised form, March 14, 2005
Published, JBC Papers in Press, March 28, 2005, DOI 10.1074/jbc.M501269200

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The human type 1 (placenta, breast tumors, and prostate tumors) and type 2 (adrenals and gonads) isoforms of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD1 and 3β-HSD2) are encoded by two distinct genes that are expressed in a tissue-specific pattern. Our recent studies have shown that His156 contributes to the 14-fold higher affinity that 3β-HSD1 exhibits for substrate and inhibitor steroids compared with human 3β-HSD2 containing Tyr156 in the otherwise identical catalytic domain. Our structural model of human 3β-HSD localizes His156 or Tyr156 in the subunit interface of the enzyme homodimer. The model predicts that Gln105 on one enzyme subunit has a higher probability of interacting with His156 on the other subunit in 3β-HSD1 than with Tyr156 in 3β-HSD2. The Q105M mutant of 3β-HSD1 (Q105M1) shifts the Michaelis-Menten constant (Km) for 3β-HSD substrate and inhibition constants (Ki) for epoate and triostane to the much lower affinity profiles measured for wild-type 3β-HSD2 and H156Y1. However, the Q105M2 mutant retains substrate and inhibitor kinetic profiles similar to those of 3β-HSD2. Our model also predicts that Gln240 in 3β-HSD1 and Arg240 in 3β-HSD2 may be responsible for the 3-fold higher affinity of the type 1 isomerase activity for substrate steroid and cofactors. The Q240R1 mutation increases the isomerase substrate Km by 2.2-fold to a value similar to that of 3β-HSD2 isomerase and abolishes the allosteric activation of isomerase by NADH. The R240Q2 mutation converts the isomerase substrate, cofactor, and inhibitor kinetic profiles to the 4–14-fold higher affinity profiles of 3β-HSD1. Thus, key structural reasons for the substantially higher affinities of 3β-HSD1 for substrates, coenzymes, and inhibitors have been identified. These structure and function relationships can be used in future docking studies to design better inhibitors of the 3β-HSD1 that may be useful in the treatment of hormone-sensitive cancers and preterm labor.

The human type 1 (placenta, mammary gland, and prostate) and type 2 (adrenals, ovary, and testis) isoforms of 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145)/steroid Δ5-Δ4-isomerase (EC 5.3.3.1) (3β-HSD1 and 3β-HSD2) are encoded by two distinct genes that are expressed in a tissue-specific pattern (1). As shown in Fig. 1, human 3β-HSD1 catalyzes the conversion of 3β-hydroxy-5-ene-steroids (dehydroepiandrosterone (DHEA) and pregnenolone) to 3-oxo-4-ene-steroids (androstenedione and progesterone), and human 3β-HSD2 converts 17α-hydroxypregnenolone and pregnenolone to ultimately produce cortisol and aldosterone in the human adrenal, respectively (2). 17α-Hydroxylase/17,20 lyase (CYP17) in the human adrenal gland converts pregnenolone to DHEA, which is the major circulating steroid in humans as DHEA sulfate (2). In placenta, androstenedione is converted by aromatase and 17β-hydroxysteroid dehydrogenase (17β-HSD) to estradiol, which participates in the cascade of events that initiates labor in humans (2, 3). Placental 3β-HSD1 also converts pregnenolone to progesterone to help maintain the uterus in a quiescent state throughout human pregnancy (3). In addition to placenta and other human peripheral tissues, the 3β-HSD1 is selectively expressed in breast tumors (4) and prostate tumors (5, 6), where it catalyzes the first step in the conversion of circulating DHEA to estradiol or testosterone to promote tumor growth. Determination of the structure/function relationships of human 3β-HSD1 and 3β-HSD2 may lead to the development of highly specific inhibitors of 3β-HSD1 that can help control the timing of labor and slow the growth of hormone-sensitive tumors without inhibiting 3β-HSD2, so that steroidogenesis in the adrenal gland to produce cortisol and aldosterone is not compromised.

The two-step reaction of 3β-HSD/isomerase using DHEA as substrate is shown in Fig. 2. This reaction scheme shows the reduction of NAD⁺ to NADH by the rate-limiting 3β-HSD activity and the requirement of this NADH for the activation of isomerase on the same enzyme protein. Because the isomerase reaction is irreversible, the 3β-HSD/isomerase cannot convert androstenedione to DHEA (7, 8). According to our stopped-flow fluorescence spectroscopy study, NADH induces a time-dependent conformational change in the enzyme structure as the isomerase activity reaches a maximum over 1 min after the addition of the coenzyme (9). The intermediate steroid, 5-an...
Human 3β-Hydroxysteroid Dehydrogenase

FIG. 1. Human 3β-hydroxysteroid dehydrogenase is expressed as two tissue-specific isoforms (3β-HSD1 and 3β-HSD2) and is a key rate-limiting enzyme in the steroid biosynthetic pathways that produce estradiol, testosterone, cortisol, and aldosterone.

FIG. 2. 3β-HSD/isomerase catalyzes two sequential reactions on a single enzyme protein. The human 3β-HSD and isomerase activities are represented by using dehydroepiandrosterone as substrate.

Dehydroepiandrosterone (DHEA)

NAD⁺ → NADH

3β-HSD

5-Androstene-3,17-dione

Androstenedione

EXPERIMENTAL PROCEDURES

Materials—Dehydroepiandrosterone and pyridine nucleotides were purchased from Sigma; 5-androstene-3,17-dione was from Steraloids Inc. (Newport, RI); reagent grade salts, chemicals, and analytical grade solvents were from Fisher. Glass-distilled, deionized water was used for all aqueous solutions.

Site-directed Mutagenesis—By using the Advantage cDNA PCR kit (zco;clontechClontech) and pGEM-3/H9252HSD1 or pGEM-3/H9252HSD2 as template (12), double-stranded PCR-based mutagenesis was performed with the primers in Table I to create the cDNA encoding the Q105M1, Q105M2, Q240R1, and R240Q2 mutant enzymes. The presence of the mutated codon and integrity of the entire mutant 3β-HSD cDNA was verified by automated dideoxynucleotide DNA sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA). Chou-Fasman and Garnier-Osguthorpe-Robson analysis of each mutant enzyme was used to choose amino acid substitutions that produced no apparent changes in the secondary structure of the protein (Protylze program, Scientific and Educational Software, State Line, PA).

Expression and Purification of the Mutant and Wild-type Enzymes—The mutant 3β-HSD cDNA was introduced into baculovirus as de-
scribed previously (12). Recombinant baculovirus was added to 1.5 × 10⁶ Sf9 cells (1 liter) at a multiplicity of infection of 10 for expression of each mutant enzyme. The expressed mutant and wild-type enzymes were separated by SDS-12% PAGE, probed with our anti-3β-HSD polyclonal antibody, and detected using the ECL Western blotting system with anti-rabbit, peroxidase-linked secondary antibody (Amersham Biosciences). Each expressed enzyme was purified from the 100,000 × g pellet of the Sf9 cells (2 liters) by our published method (7, 8) using Igepal CO 720 (Rhodia, Inc., Cranbury, NJ) instead of the discontinued Emulgen 913 detergent (Kao Corp, Tokyo, Japan). Each expressed, purified mutant and wild-type enzyme produced a single major protein band (42.0 kDa) on SDS-12% PAGE that co-migrated with the human wild-type 1 control enzyme. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard (13).

**Kinetic Studies**—Michaelis-Menten kinetic constants for the 3β-HSD substrate were determined for the purified mutant and wild-type enzymes in incubations containing dehydroepiandrosterone (3–100 μM) plus NAD⁺ (0.2 mM) and purified enzyme (0.04 mg) at 27 °C in 0.02 M potassium phosphate, pH 7.4. The slope of the initial linear increase in absorbance at 340 nm/min (due to NADH production) was used to determine 3β-HSD activity. Kinetic constants for the isomerase substrate were determined at 27 °C in incubations of 5-androstene-3,17-dione (10–100 μM), with or without NADH (0.05 mM) and purified enzyme (0.01–0.04 mg) in 0.02 M potassium phosphate buffer, pH 7.4. Isomerase activity was measured by the initial absorbance increase at 241 nm (due to androstenedione formation) as a function of time. Blank assays (zero enzyme and zero substrate) ensured that specific isomerase activity was measured as opposed to non-enzymatic, “spontaneous” isomerization (8). In addition, isomerase incubations without added coenzyme (NADH) were used to measure any basal (zero coenzyme) isomerase activity in the mutants, and this basal activity was subtracted as a blank. Changes in absorbance were measured with a Varian (Sugar Land, TX) Cary 219 recording spectrophotometer. The Michaelis-Menten constants (Km, Vmax) were calculated from Lineweaver-Burk (1/VS versus 1/V) plots and verified by Hanes-Woolf (S versus VS) plots (14). The kcat values were determined from the Vmax values (nmol/min/mg) and represent the maximal turnover rate (nmol of product formed/min/mmol of enzyme dimer).

**Kinetic constants for the 3β-HSD cofactor** were determined for the purified mutant and wild-type enzymes in incubations containing NAD⁺ (10–100 μM), dehydroepiandrosterone (100 μM), and purified enzyme (0.04 mg) in 0.02 M potassium phosphate, pH 7.4, at 27 °C using the spectrophotometric assay at 340 nm. Kinetic constants for the isomerase cofactor were determined in incubations of NADH (0–50 μM), 5-androstene-3,17-dione (100 μM), and purified enzyme (0.01–0.04 mg) in 0.02 M potassium phosphate buffer, pH 7.4, at 27 °C using the spectrophotometric assay at 241 nm. Zero coenzyme blanks were used as described above for the substrate kinetics.

Inhibition constants (Ki) were determined for the inhibition of the wild-type 1, wild-type 2, Q240R1, and R240Q2 3β-HSD activities by epoane and trilostane using conditions that were appropriate for each enzyme species. For Q240R1 and 3β-HSD1, the incubations at 27 °C contained subsaturating concentrations of dehydroepiandrosterone (4.0 and 8.0 μM, relative to substrate Km values of 3.7–6.1 μM), epoane or trilostane (0–1.0 μM), NAD⁺ (0.1 mM), and purified human type 1 enzyme (0.03–0.04 mg) in 0.02 M potassium phosphate buffer, pH 7.4. For Q105M1, Q105M2, R240Q2, and 3β-HSD2, similar incubations contained 10.0 or 20.0 μM dehydroepiandrosterone, and the concentration ranges of epoane and trilostane were 0–10.0 μM. Dixon analysis (1 versus 1/V) was used to determine the type of inhibition and calculate the Ki values (14).

**Modeling and Sequence Alignment**—Amino acid and nucleotide sequences were retrieved from the Swiss-Prot protein sequence data base (15). Crystallographic coordinates were retrieved from the protein data bank (16). The three-dimensional structure of human type 1 3β-HSD/ isomerase was modeled using the crystal structure (17) of UDP-galactose 4-epimerase from Escherichia coli (Protein Data Bank accession code 1A9Z) as we previously described (10). The modeled images were produced using the Ribbons 2.0 program (18).

**RESULTS**

**Site-directed Mutagenesis, Expression, and Purification of the Mutant Enzymes**—The cDNA encoding the Q105M1 mutant of 3β-HSD1, the Q105M2 mutant of 3β-HSD2, the Q240R1 mutant of 3β-HSD1, and the R240Q2 mutant of 3β-HSD2 were produced by double-stranded, PCR-based mutagenesis and inserted into baculovirus. These amino acids were predicted to be critical residues by our modeled tertiary/quaternary structure for human 3β-HSD (Figs. 3 and 4). The locations of these targeted residues plus other key amino acids are indicated in the primary structures of 3β-HSD1 and 3β-HSD2 (Fig. 5). As shown by the immunoblots in Fig. 6A, the baculovirus system successfully expressed the mutant enzyme proteins in Sf9 cells. Each expressed enzyme was highly purified according to SDS-PAGE (Fig. 6B) by using our published method (7, 8).

**Substrate Kinetic Analyses of the Mutant Enzymes**—The Michaelis-Menten kinetic values measured for substrate utilization by purified Q105M1, Q105M2, 3β-HSD1, Q240R1, R240Q2, and 3β-HSD2 are summarized in Table II. For the dehydrogenase activity, the Q105M1 mutant demonstrates an 11-fold higher Kcat value for substrate and a 2-fold higher kcat value than 3β-HSD1, which are similar to those of 3β-HSD2 and to those reported previously for the H156Y1 mutant enzyme (DHEA Km = 42.4 μM, kcat = 7.2 min⁻¹) (11). The Q105M2 mutant exhibits DHEA kinetic constants that are similar to those of 3β-HSD2 with almost identical utilization efficiencies (kcat/Km) (7.1 min⁻¹). The Q105M1 and Q105M2 mutant enzymes retain isomerase substrate Km values similar to the appropriate 3β-HSD1 or 3β-HSD2 isoform. Both Q105M1 mutant enzymes exhibit reduced isomerase activity (kcat) compared with their wild-type enzyme counterpart. The Q240R1 mutant has a slightly elevated Km value for DHEA to yield a substrate utilization efficiency (kcat/Km) that is similar to wild-type 3β-HSD2. The R240Q2 mutant exhibits a decreased Km and elevated kcat for DHEA to yield a utilization efficiency (kcat/Km) that lies midway between wild-type 3β-HSD1 and 3β-HSD2. The Q240R1 mutation shifts the isomerase substrate Km value to a 2.2-fold higher level that is comparable with the isomerase substrate Km of wild-type 3β-HSD2. Similarly, the R240Q2 mutation shifts the isomerase substrate Km and kcat values from the wild-type 2 to the wild-type 1 profile.

**Coenzyme Kinetic Analyses of the Mutant Enzymes**—In agreement with our previously reported results obtained with the H156Y1 mutant of human 3β-HSD1 (11), the Q105M1 mutant enzyme exhibits the shift in 3β-HSD substrate kinetics to the 3β-HSD2 profile (Table II), but the mutation does not affect the Km values of NAD⁺ as the

| Mutation | Direction | Nucleotide sequence of primer* |
|----------|-----------|--------------------------------|
| Q105M1   | Forward   | 5'-GGTACCATGTTCCTGTATTAGGCGC-3' |
| Q105M1   | Reverse   | 5'-CAGGACCATGTACCTTCTACAGTG-3' |
| Q240R1   | Forward   | 5'-AAGTGACCAGCTCTAGTGGGAGGC-3' |
| Q240R1   | Reverse   | 5'-AAGTGACCAGCTCTAGTGGGAGGC-3' |
| R240Q2   | Forward   | 5'-GCCCTGGGACCCCGAAGGGC-3'    |
| R240Q2   | Reverse   | 5'-GCCCTGGGACCCCGAAGGGC-3'    |

*The mutated codons are underlined.
cofactor for the 3β-HSD1 reaction or of NADH as the allosteric activator of type 1 isomerase (Table III). The Q105M2 mutant enzyme retains $K_m$ and $k_{cat}$ values for NAD$^+$ utilization and for the NADH activations of isomerase that are similar to those measured for wild-type 3β-HSD2 (Table III). The Q240R1 mutation has little effect on the $K_m$ value of 3β-HSD1 for NAD$^+$ but disrupts the concentration-dependent activation of type 1 isomerase by NADH (Table III and Fig. 7). The basal isomerase activity of Q240R1 in the absence of NADH is 22% of the wild-type 3β-HSD1 isomerase after maximal activation by NADH. In contrast, the basal isomerase activities of 3β-HSD1, 3β-HSD2, R240Q2, Q105M1, and Q105M2 are only 5–10% of the $k_{cat}$ of NADH-activated isomerase, and NADH stimulates that basal activity in a concentration-dependent manner (Fig. 7). The R240Q2 mutant of 3β-HSD2 shifts the $K_m$ of NAD$^+$ utilization to a 2.3-fold lower value that is similar to the value measured for 3β-HSD1 and to a 3.9-fold lower $K_m$ for the NADH-activation of isomerase, which is comparable with that of type 1 isomerase (Table III).

**Kinetic Analyses of the Inhibition of the Mutant Enzymes—**

The inhibition constant values ($K_i$) derived from Dixon analyses (1 versus 1/$V$) show that epistane and trilostane inhibit the 3β-HSD activity of Q105M1 with 21–26-fold lesser affinities compared with the $K_i$ values measured using the wild-type 3β-HSD1 enzyme. However, Q105M2 retains the lower affinity profiles of inhibition by epistane and trilostane that are observed with wild-type 3β-HSD2 (Table IV). Although the Q240R1 mutant enzyme is inhibited by epistane and trilostane with the same high affinity profiles as measured for 3β-HSD1, the R240Q2 mutation of 3β-HSD2 shifts the inhibition constants ($K_i$) to values that are 5–14-fold lower for epistane and trilostane, respectively (Table IV). According to the Dixon plots (not shown), epistane and trilostane inhibited the 3β-HSD activities of Q105M1, Q105M2, Q240R1, R240Q2, 3β-HSD1, and 3β-HSD2 in a competitive manner.

**DISCUSSION**

Our studies of the structure/function of human 3β-HSD took a dramatic turn in 2002 when we reported (11) that it is possible to inhibit selectively human 3β-HSD1 without affecting the activity of human 3β-HSD2. Since that discovery, our primary focus has been on determining the structural basis for the 14–16-fold higher affinity of purified human 3β-HSD1 for substrate and inhibitor steroids compared with human 3β-HSD2. There is a strict tissue-specific distribution of the two human 3β-HSD isoforms. 3β-HSD1 is expressed in mammary gland, breast tumors, prostate, prostate tumors, and placenta, and 3β-HSD2 is expressed in the adrenals, testes, and ovaries. We also reported that 3β-HSD1 transfected into human breast tumor MCF-7 Tet-Off cells have a 12-fold greater affinity for substrate and inhibitor steroids compared with the 3β-HSD2 that we transfected into the MCF-7 Tet-Off cells (19). These recent discoveries identify human 3β-HSD1 as a target enzyme...
be effective as a therapy for prostate malignancies (24). Unlike the benign hyperplastic prostate, the growth of prostate tumors is stimulated by testosterone as well as by its 5α-reductase product steroid 5α-dihydrotestosterone (24). The selective inhibition human 3β-HSD1 activity to block testosterone production in prostate tissue and its tumors may yield a new treatment for prostate cancer that will avoid the initial flare of tumor growth seen with luteinizing hormone-releasing hormone agonists like leuprolide (25). Other enzyme targets for inhibition in the hormonal treatment of breast or prostate cancer include human sulfatase to block the conversion of circulating estrone sulfate or DHEA sulfate to the free steroids in tissues and isoforms of human 17β-hydroxysteroid dehydrogenase (17β-HSD1 and 17β-HSD5) that function as reductases to block the conversion of estrone to estradiol (26, 27). Thus, human 3β-HSD1 is one of several steroid-metabolizing enzymes currently under study as potential therapeutic targets for the treatment of hormone-sensitive cancers. Although our data obtained with epostane and trilostane demonstrate that it is possible to inhibit selectively 3β-HSD1 with them, we are not suggesting that these compounds be used to treat breast or prostate cancer. After the complete structural basis for the kinetic differences between human 3β-HSD2 and 3β-HSD1 near term to decrease estradiol production from DHEA of fetal origin (2, 3) be performed to design better inhibitors with greater specificity for 3β-HSD1 than epostane and trilostane.

The selective inhibition of steroid biosynthesis via 3β-HSD1 in human placenta has another clinical implication. The onset of labor in human pregnancy could be delayed by selectively inhibiting the activity of placental 3β-HSD1 near term to decrease estradiol production from DHEA of fetal origin (2, 3) without interfering with cortisol or aldosterone production by 3β-HSD2 in the maternal adrenal gland. Estradiol participates in the cascade of events leading to parturition in humans by stimulating the biosynthesis of oxytocin receptors in the myometrium (28). In addition, inhibition of placental 3β-HSD1 would inhibit progesterone production to diminish uterine quiescence (3).

In our recent structure/function study (11), the catalytic residues for human type 1 3β-HSD activity were identified as

**Human Type 1**

T G W S C L V T G A G F L G Q R I L V L K E K E R I V R L D K A F G P E L R E E

**Human Type 2**

F S K L Q N K T L X V L E G D I L E D E P F L K R A C Q D V S V I T H A C I I D V F G V

**Fig. 5. Amino acid sequence of the human type 1 3β-HSD/isomerase.** Single letter abbreviations for amino acids in the 42.0-kDa monomer of the homodimer are shown. The key amino acids discussed in this report are labeled with position numbers. The amino acids that form the two α-helices in the subunit interface are underlined.

**Fig. 6. Western immunoblots and SDS-PAGE of the mutant and wild-type enzymes by baculovirus.** A, for the Western blots, the S9 cell homogenate (3.0 μg) containing the Q105M1, Q105M2, Q240R1, or R240Q2 plus the purified control wild-type 3β-HSD (0.05 μg) were separated by SDS-12% PAGE. The 42.0-kDa band of the mutant enzymes currently under study as potential therapeutic targets for the treatment of hormone-sensitive cancers. Although our data obtained with epostane and trilostane demonstrate that it is possible to inhibit selectively 3β-HSD1 with them, we are not suggesting that these compounds be used to treat breast or prostate cancer. After the complete structural basis for the kinetic differences between human 3β-HSD2 and 3β-HSD1 near term to decrease estradiol production from DHEA of fetal origin (2, 3) be performed to design better inhibitors with greater specificity for 3β-HSD1 than epostane and trilostane.

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In our recent structure/function study (11), the catalytic residues for human type 1 3β-HSD activity were identified as
1. The critical His156/Tyr156 residue in the subunit interface of the enzyme dimer by homology modeling (Fig. 4), and we tested this prediction by mutagenesis of a potential key hydrogen-bonding partner, Glu102, on the other subunit of 3β-HSD1 and 3β-HSD2 to produce Q105M1 and Q105M2, respectively. The His156/Tyr156 difference between the two isozymes is responsible only for the higher affinity of 3β-HSD1 for dehydrogenase substrate and inhibitor steroids but is not involved in the higher affinity of 3β-HSD1 for coenzymes and for the isomerase substrate steroid (11).

2. The homology model predicts that Glu102 is positioned to hydrogen-bond to the imidazole ring of His156 across the subunit interface of 3β-HSD1 and to hydrogen-bond to the phenolic group of Tyr156 on the adjacent subunit of 3β-HSD2. In this model, the subunits are anti-parallel relative to each other.

3. Table II: Substrate kinetics for the 3β-HSD and isomerase activities of the purified mutant and wild-type enzymes

| Purified enzyme | 3β-HSD | Isomerase |
|-----------------|--------|----------|
|                 | $K_m$  | $k_{cat}$| $k_{cat}/K_m$ | $K_m$  | $k_{cat}$| $k_{cat}/K_m$ |
| Q105M1          | 40.5   | 6.8     | 0.17         | 33.9   | 34.8   | 1.06        |
| Q240R1          | 6.1    | 1.4     | 0.23         | 62.3   | 33.3   | 0.53        |
| 3β-HSD1         | 3.7    | 3.3     | 0.89         | 27.9   | 50.2   | 1.25        |
| Q105M2          | 25.0   | 4.5     | 0.18         | 80.8   | 66.7   | 0.82        |
| R240Q2          | 32.7   | 13.0    | 0.40         | 17.5   | 34.4   | 1.97        |
| 3β-HSD2         | 47.3   | 7.1     | 0.15         | 88.4   | 81.5   | 0.92        |

$^a$ Kinetic constants for the 3β-HSD substrate (DHEA) were determined in incubations at 27 °C containing NAD+ (200 μM), dehydroepiandrosterone (3–100 μM), and purified enzyme (0.04 mg) in 0.02 M potassium phosphate, pH 7.4. Each $K_m$ and $k_{cat}$ value represents the mean of triplicate measurements with an S.D. of 7% of single mutant enzyme preparations.

$^b$ Kinetic constants for the isomerase substrate (5-androsten-3,17-dione) were determined in incubations at 27 °C of NADH (50 μM), 5-androsten-3,17-dione (20–100 μM), and purified enzyme (0.01–0.04 mg) in 0.02 M potassium phosphate buffer, pH 7.4.

4. Table III: Cofactor kinetics for the 3β-HSD and isomerase activities of the purified mutant and wild-type enzymes

| Purified enzyme | 3β-HSD NAD+ | Isomerase NADH |
|-----------------|-------------|---------------|
|                 | $K_m$  | $k_{cat}$| $k_{cat}/K_m$ | $K_m$  | $k_{cat}$| $k_{cat}/K_m$ |
| Q105M1          | 38.8   | 2.3     | 0.06         | 4.9    | 28.8   | 5.88        |
| Q240R1          | 25.6   | 1.3     | 0.05         | ND     | ND     | ND          |
| 3β-HSD1         | 34.1   | 3.5     | 0.10         | 4.6    | 45.0   | 9.78        |
| Q105M2          | 61.4   | 8.7     | 0.14         | 7.2    | 69.8   | 9.69        |
| R240Q2          | 37.2   | 11.2    | 0.30         | 3.2    | 35.5   | 11.09       |
| 3β-HSD2         | 86.3   | 7.1     | 0.08         | 12.6   | 99.1   | 7.8         |

$^a$ Kinetic constants for the 3β-HSD cofactor were determined in incubations at 27 °C containing NAD+ (13 100 μM), dehydroepiandrosterone (100 μM), and purified enzyme (0.03–0.04 mg) in 0.02 M potassium phosphate, pH 7.4. Each $K_m$ and $k_{cat}$ value represents the mean of triplicate measurements with an S.D. of 6% of single mutant enzyme preparations.

$^b$ Kinetic constants for the isomerase cofactor were determined in incubations at 27 °C of NADH (3–50 μM), 5-androsten-3,17-dione (100 μM), and purified enzyme (0.01 mg) in 0.02 M potassium phosphate buffer, pH 7.4.

5. Table IV: Comparison of inhibition constants for the purified Q105M1, Q105M2, Q240R1, R240Q2, 3β-HSD1, and 3β-HSD2 activities

| Enzyme   | Inhibitor steroid | $K_i$ μM |
|----------|-------------------|----------|
|          | Epostane          | Trilostane |
| Q105M1   | 1.84              | 2.08     |
| Q240R1   | 0.97              | 0.13     |
| 3β-HSD1  | 0.07              | 0.10     |
| Q105M2   | 1.64              | 2.07     |
| R240Q2   | 0.21              | 0.12     |
| 3β-HSD2  | 0.98              | 1.66     |

$^a$ For 3β-HSD1, Q240R1, and R240Q2, the epoate and trilostane concentration ranges were 0–0.75 μM. For Q105M1, Q105M2, and 3β-HSD2, the epoate and trilostane concentration ranges were 0–7.5 μM. Inhibition constants ($K_i$) were determined in incubations at 27 °C containing the inhibitor plus dehydroepiandrosterone (4.0 or 8.0 μM for Q240R1 and 3β-HSD1; 10.0 or 20.0 μM for Q105M1, Q105M2, R240Q2, or 3β-HSD2), NAD+ (0.2 mM), and purified enzyme (0.03–0.04 mg) in 0.02 M potassium phosphate, pH 7.4. Each $K_i$ value represents the mean of triplicate measurements with an S.D. of 7% of single mutant enzyme preparations.
other at a helical interface, as has been reported for human 17β-HSD1 (29) and E. coli UDP-galactose-4-epimerase (17). Many members of the short chain dehydrogenase-reductase family are anti-parallel homodimers, which create a four-helix bundle subunit interface that is critical for the function of the adjacent active sites in each monomer (29). Based on a cataloging of interactions between Gln-His and Gln-Tyr residues in high resolution protein crystal structures, the Gln-Tyr side chain interactions are tightly clustered in only a few geometries compared with the extensive range of geometries observed for side chain interactions between Gln and His (30). Thus, the Q105M1 mutation may disrupt key hydrogen-bonding interactions between Gln105 and His156 in the subunit interface of 3β-HSD1. However, the Q105M2 mutation has little effect because the Gln105→Tyr156 hydrogen bond may either not exist or is relatively weak between the subunits of 3β-HSD2 because of the restrictive geometry of this interaction. Suboptimal hydrogen bonds are commonly observed in protein-protein interfaces, to the extent that over 17% of buried donors or acceptors do not participate in hydrogen bonds in high resolution structures (31). The 11-fold higher $K_m$ value for the dehydrogenase substrate, 2-fold lower dehydrogenase $k_{cat}$ and the 21–26-fold lower $K'_m$ values of trilostane and epistane measured for Q105M1 compared to 3β-HSD1 in this study are almost identical to the kinetic shifts reported previously for the H156Y mutant of 3β-HSD1 (11). As seen previously with the H156Y mutant, the $K_m$ values of coenzyme and isomerase substrate utilization for 3β-HSD1 are not modified by the Q105M1 mutation. In sharp contrast, the Q105M2 mutant exhibits substrate, coenzyme, or inhibitor kinetics of both the dehydrogenase and isomerase activities that are similar to those of 3β-HSD2, which emphasizes the different interactions between the subunits of the two isoforms. This interaction may be critical to the binding orientation of the substrate steroid in the adjacent catalytic sites. Thus, the interactions between Gln105 and His156 in the subunit interface of 3β-HSD1 appear to be key structural reasons for its 11–26-fold higher affinity for the DHEA dehydrogenase substrate and inhibitor steroids relative to 3β-HSD2. In addition, the Q105M1 mutant data support our model of the anti-parallel orientation of the two 3β-HSD1 monomers.

Human 3β-HSD1 utilizes NAD$^+$ as the cofactor for the dehydrogenase reaction and NADH as the allosteric activator of isomerase with 2.5–2.7-fold lower $K_m$ values than 3β-HSD2 (11). We showed recently that the 3β-HSD and isomerase activities share the same coenzyme domain on a single enzyme protein by shifting cofactor specificity from NAD(H) to NADP(H) for both activities with a two-point mutation, D36AK37R (10). In addition, 3β-HSD1 utilizes the isomerase substrate steroid with a 3.2-fold lower $K_m$ value but decreases the $K_m$ value of the isomerase activity of 3β-HSD1 by NADH. Most significantly, the Q240R1 mutant greatly diminishes the allosteric activation of the isomerase activity of 3β-HSD1 by NADH. The NADH versus velocity plot for Q240R1 in Fig. 7 shows that the isomerase is almost fully active at the 3β-HSD1 level without NADH. This suggests that the substitution of positively charged Arg$^{240}$ for uncharged Gln$^{240}$ shifts most of the enzyme into the isomerase conformation. Less enzyme in the 3β-HSD conformation explains the 2.4–2.8-fold lower $k_{cat}$ and unchanged $K'_m$ values measured for the utilization of DHEA and NAD$^+$ by the 3β-HSD activity of Q240R1.

The R240Q2 mutation of 3β-HSD2 shifts the $K_m$ value of the isomerase substrate steroid to a 2.4-fold lower value, shifts the $K_m$ value for NADH to a 4-fold lower value, shifts the $K_m$ of NAD$^+$ utilization to a 2.3-fold lower value, and lowers the inhibition constants ($K_i$) of epostane by 5-fold and of trilostane by 14-fold. These changes create a 3β-HSD2 mutant enzyme that is similar to 3β-HSD1 in these kinetic properties but that retains the high DHEA substrate $K_m$ value of 3β-HSD2. Because the $K_m$ values measured for epistane and trilostane of R240Q2 are shifted to the higher affinity kinetic profile of 3β-HSD1, these data show that the isomerase substrate and coenzyme binding properties are related to the competitive binding of 3β-HSD inhibitor steroids. The H156Y1 and Q105M1 mutations that affect only 3β-HSD DHEA substrate kinetics but not isomerase substrate or coenzyme kinetics shift the inhibition kinetics of 3β-HSD1 to the much lower affinity profile of 3β-HSD2. These opposing effects of R240Q2 and Q105M1 on the $K_i$ values of epostane and trilostane support our model of the two-step, sequential 3β-HSD and isomerase activities. In this model, the NADH produced by the 3β-HSD reaction induces a change in enzyme conformation around a single steroid-binding site, and this conformational change activates the isomerase reaction on the bifunctional enzyme protein (9).

In summary, three of the most important observations of this study are as follows: 1) the Q105M1 mutant decreases the affinity of 3β-HSD1 for DHEA substrate and inhibitor steroids so that it mimics 3β-HSD2; 2) Q240R1 abolishes the allosteric activation of the isomerase activity by NADH; and 3) the R240Q2 mutant increases the affinity of 3β-HSD2 for isomerase substrate, NADH, and inhibitor steroids so that it mimics the 3β-HSD1 enzyme. Determining the roles of Gln105, His156, and Gln240 in human 3β-HSD1 in comparison with those of Gln105, Tyr156, and Arg240 in 3β-HSD2 provides key structural data for use in future docking studies that may produce highly specific inhibitors of 3β-HSD1. Efforts are underway using our genetically engineered, soluble form of human type 1 3β-HSD to grow enzyme crystals that will provide a complete enzyme structure using x-ray diffraction for the future docking studies.

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