Structure/Function Relationships Responsible for the Kinetic Differences between Human Type 1 and Type 2 3β-Hydroxysteroid Dehydrogenase and for the Catalysis of the Type 1 Activity*

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Two distinct genes encode the 93% homologous type 1 (placenta, peripheral tissues) and type 2 (adrenals, gonads) 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD/isomerase) in humans. Mutagenesis studies using the type 1 enzyme have produced the Y154F and K158Q mutant enzymes in the Y154-P-Y156-S-K158 motif as well as the Y269S and K273Q mutants from a second motif, Y269S, T-L-S-K273, both of which are present in the primary structure of the human type 1 3β-HSD/isomerase. In addition, the H156Y mutant of the type 1 enzyme has created a chimera of the type 2 enzyme motif (Y154-P-Y156-S-K158) in the type 1 enzyme. The mutant and wild-type enzymes have been expressed and purified. The Km value of dehydroepiandrosterone is 13-fold greater, and the maximal turnover rate (kcat) is 2-fold greater for wild-type 2 3β-HSD compared with the wild-type 1 3β-HSD activity. The H156Y mutant of the type 1 enzyme has substrate kinetic constants for 3β-HSD activity that are very similar to those of the wild-type 2 enzyme. Dixon analysis shows that epoestane inhibits the 3β-HSD activity of the wild-type enzyme with 14–17-fold greater affinity compared with the wild-type 2 and H156Y enzymes. The Y154F and K158Q mutants exhibit no 3β-HSD activity, have substantial isomerase activity, and utilize substrate with Km values similar to those of wild-type 1 isomerase. The Y269S and K273Q mutants have low, pH-dependent 3β-HSD activity, exhibit only 5% of the maximal isomerase activity, and utilize the isomerase substrate very poorly. From these studies, a structural basis for the profound differences in the substrate and inhibition kinetics of the wild-type 1 and 2 3β-HSD, plus a catalytic role for the Tyr154 and Lys158 residues in the 3β-HSD reaction have been identified. These advances in our understanding of the structure/function of human type 1 and 2 3β-HSD/isomerase may lead to the design of selective inhibitors of the type 1 enzyme not only in placenta to control the onset of labor but also in hormone-sensitive breast, prostate, and choriocarcinoma tumors to slow their growth.

The human type 1 (placenta, skin, mammary gland, prostate, endometrium) and type 2 (gonads, adrenals) isoforms of 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145)/sterol Δ5,Δ4-isomerase (EC 5.3.3.1) (3β-HSD/isomerase) are encoded by two distinct genes, which are expressed in a tissue-specific pattern (1). In human placenta, type 1 3β-HSD/isomerase catalyzes the conversion of 3β-hydroxy-5-ene-steroids (dehydroepiandrosterone, pregnenolone) to 3-oxo-4-ene-steroids (androstenedione, progesterone) on a single, dimeric protein containing both enzyme activities (2). During human pregnancy, the placental enzyme catalyzes the conversion of pregnenolone to progesterone, which maintains the uterus in a quiescent state. Near term, however, the fetal zone adrenal gland produces large amounts (200 mg/day) of dehydroepiandrosterone (DHEA, Fig. 1). Because the fetal adrenal lacks significant 3β-HSD/isomerase activity, the placental type 1 enzyme converts the fetal dehydroepiandrosterone to androstenedione (3). Androstenedione is converted by placental aromatase and 17β-hydroxysteroid dehydrogenase to estradiol, which participates in the cascade of events that initiates labor in humans (4). The type 1 enzyme also is selectively expressed in human breast tumors (5), prostate tumors (6), and choriocarcinomas (7), where it catalyzes the first step in the conversion of circulating dehydroepiandrosterone to androstenedione. Determination of the structure/function relationships of the type 1 enzyme may lead to the development of specific inhibitors of type 1 3β-HSD/isomerase that can help control the timing of labor and slow the growth of hormone-sensitive tumors.

The Y-X-X-K motif has been conserved in the short chain dehydrogenase/reductase family of enzymes and participates in the catalytic mechanisms of rat liver 11β-HSD (8), Streptomyces hygroscopicus 3α,20β-HSD (8), Drosophila melanogaster alcohol dehydrogenase (9), Escherichia coli 7α-HSD (10), and human 17β-HSD (11). In each of these enzymes, there is a single Y-X-X-K motif associated with the dehydrogenase activity, but there are two of these motifs (154-Y-X-X-K158 and 268-Y-X-X-K273) in the primary structures of human type 1 and type 2 3β-HSD/isomerase (Fig. 2) (12). 3β-HSD/isomerase is an ideal enzyme system to study using site-directed mutagenesis, because an appropriately targeted point mutation will primarily affect only one of the two activities. If both activities are abolished, the mutation most likely has induced a nonspecific conformational change in the enzyme protein. In the current study, we have used site-directed mutagenesis to produce four mutated forms of human type 1 3β-HSD/isomerase that target the potentially critical residues in the two Y-X-X-K motifs (Y154F, K158Q, Y269S, K273Q). In addition, the H156Y mutant was

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1 The abbreviations used are: HSD, hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; 17α-OH-pregnenolone, 17α-hydroxy-pregnenolone; 2α-BAP, 2α-bromoacetoxyprogesterone.
created to produce a chimera of the human type 2 enzyme motif (Y154-P-Y156-S-K158) in the corresponding human type 1 enzyme motif (Y154-P-H156-S-K158). Our characterization of the purified mutant, wild-type 1, and wild-type 2 enzymes have elucidated the roles of the targeted amino acid residues in the 3β-H9252-HSD reaction mechanism and identified a structural basis for the profound differences in the substrate and inhibition kinetics of the human type 1 and type 2 3β-H9252-HSD/isomerases.

EXPERIMENTAL PROCEDURES

Materials—Dehydroepiandrosterone, pregnenolone, and pyridine nucleotides were purchased from Sigma; 5-androstene-3,17-dione and 17α-hydroxypregnenolone from Steraloids Inc. (Newport, RI); and reagent grade salts, chemicals, and analytical grade solvents from Fisher Scientific Co. Epostane was a gift from Sterling-Winthrop Research Institute (Rensselaer, NY). Glass-distilled, deionized water was used for all aqueous solutions.

Site-directed Mutagenesis—Using the Advantage cDNA PCR kit (BD Biosciences Clontech, Palo Alto, CA) and pGEM-3βHSD1 as template (13), double-stranded PCR-based mutagenesis was performed with the primers in Table I to create the cDNA encoding the Y154F, H156Y, K158Q, Y269S, and K273Q mutant enzymes. The presence of the mutated codon and integrity of the entire mutant 3β-H9252-HSD cDNA were 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-H9252-HSD cDNA was introduced into baculovirus as described previously (13, 14). Recombinant baculovirus was added to 1.5 × 10⁶ SF9 cells (1L) at a multiplicity of infection of 10 for expression of each mutant enzyme. The expressed mutant and wild-type enzymes
were separated by SDS-polyacrylamide (12%) gel electrophoresis, 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 x g pellet of the Sf9 cells (2 liters) by our published method (2). The Emulgen 193 detergent (polyoxyethyylene (13–14) nonylphenyl ether, Kao Corp., Tokyo) used in the original purification is no longer available. In its place, Igepal CO 720 (polyoxyethylene (12) nonylphenyl ether) was obtained as gift from Rhone, Inc. (Cranbury, NJ). Each expressed, purified mutant and wild-type enzyme produced a single band (42.0 kDa) on SDS-polyacrylamide (12%) gel electrophoresis that comigrated with the human wild-type 1 control enzyme. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard (15).

**Kinetic and pH Dependence Studies**—The Michaelis-Menten kinetic constants for the 3β-HSD substrate were determined for the purified mutant and wild-type enzymes in incubations containing dehydroepiandrosterone (2–100 μM), pregnenolone (2–20 μM), or 17α-hydroxyprogrenenolone (2–20 μM) plus NAD⁺ (0.1 mM) and purified enzyme (0.03 mg) at 27 °C in 0.2 M potassium phosphate buffer, 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 (17–150 μM), NADH (0.05 mM), and purified enzyme (0.01 mg) in 0.2 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, zero substrate) assured that specific isomerase activity (due to androstenedione formation) as a function of time. Blank assays (zero enzyme, zero substrate) were performed to determine the maximal turnover rate (nmol product formed per minute per mg of purified protein, and the bands were visualized by Coomassie Blue staining.

**RESULTS**

### Site-directed Mutagenesis, Expression, and Purification of the Wild-type and Mutant Enzymes

The wild-type 1 cDNA mutants (Y154F, H156Y, K158Q, Y269S, K273Q) were produced by double-stranded, PCR-based mutagenesis and inserted into baculovirus as described under “Experimental Procedures.” As shown by the immunoblot in Fig. 3, the baculovirus system successfully expressed the mutant enzyme proteins as well as the human wild-type 1 and wild-type 2 3β-HSD/isoforms in SF9 cells. Each expressed enzyme was purified using our published method (2) to apparent homogeneity according to SDS-PAGE (Fig. 4). An additional mutant, Y269F, was created and expressed, but Western immunobLOTS showed that this mutant enzyme was unstable.

### Kinetic Analyses of the Wild-type and Mutant Enzymes

The Michaelis-Menten kinetic values measured for substrate utilization by purified wild-type 1, wild-type 2, and mutant variants of human 3β-HSD/isoform are summarized in Table II. Purified human type 1 placental 3β-HSD/isoform has been previously shown to have Michaelis-Menten constants for substrate and cofactors that were almost identical to those measured for expressed, purified wild-type 1 enzyme (13). The wild-type 1 3β-HSD activity has 13-fold lower Km values for the isomerase cofactor compared with the purified wild-type 2 3β-HSD. The kinetic values of the two isoenzymes follow the same pattern but with less dramatic differences. The H156Y mutant shifts the kinetic constants of wild-type 1 3β-HSD (containing the Y154-P-H156-S-K158 motif) to values that are similar to those of

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*The mutated codons are in the bold, italic font.*
### Table II

<table>
<thead>
<tr>
<th>Purified enzyme</th>
<th>3β-HSD (^a)</th>
<th>Isomerase (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m)</td>
<td>(K_{cat})</td>
</tr>
<tr>
<td>Wild-type 1</td>
<td>3.7 μM</td>
<td>3.7 min⁻¹</td>
</tr>
<tr>
<td>Wild-type 2</td>
<td>47.3 μM</td>
<td>6.9 min⁻¹</td>
</tr>
<tr>
<td>H156Y</td>
<td>4.2 μM</td>
<td>7.2 min⁻¹</td>
</tr>
<tr>
<td>Y154F</td>
<td>ND</td>
<td>No activity detected</td>
</tr>
<tr>
<td>K158Q</td>
<td>ND</td>
<td>No activity detected</td>
</tr>
<tr>
<td>Y269S</td>
<td>ND</td>
<td>No activity detected</td>
</tr>
<tr>
<td>K273Q</td>
<td>ND</td>
<td>No activity detected</td>
</tr>
</tbody>
</table>

\( ^a \) Michaelis-Menten kinetic constants for the 3β-HSD substrate were measured in incubations containing dehydroepiandrosterone (2–100 μM), NAD\(^+\) (0.1 mM), and purified enzyme (0.03 mg) in 0.02 M potassium phosphate buffer, pH 7.4, and that activity is doubled at the optimal pH 9.7. The 3β-HSD activities of the wild-type 2 and the H156Y mutant were calculated from the \(V_{\max}\) values. ND, not determined.

\( ^b \) Kinetic constants for the isomerase substrate were determined in incubations of 5-androstene-3,17-dione (17–150 μM), NADH (0.05 mM), and purified enzyme (0.01 mg) in 0.02 M potassium phosphate buffer, pH 7.4, at 27 °C. Each \(K_m\) and \(K_{cat}\) value represents the mean of triplicate measurements with a S.D. \(\pm 6\%\).

wild-type 2 3β-HSD (containing the Y^154-P-Y^156-S-K^158 motif). However, the H156Y mutant retains the kinetic profile of the wild-type 1 isomerase activity. None of the Y154F, K158Q, Y269S, or K273Q mutants has detectable 3β-HSD activity using DHEA as substrate. The Y154F and K158Q mutant enzymes exhibit somewhat reduced isomerase activities (\(K_{cat}\)) with \(K_m\) values for the isomerase substrate that are almost identical to those of the control wild-type 1 isomerase activity. In contrast, the Y269S and K273Q mutants of the type 1 enzyme have extremely high \(K_m\) values for the isomerase substrate and utilize isomerase substrate with efficiencies (\(K_{cat}/K_m\)) that are 70-fold lower than the control wild-type 1 isomerase activity.

Table III uses the more familiar Michaelis-Menten constants, \(K_m\) and \(V_{\max}\), to compare the utilization kinetics measured for the three common steroid substrates of human type 1 and type 2 3β-HSD. Similar to DHEA, pregnenolone utilization by wild-type 1 3β-HSD exhibits a 16–17-fold lower \(K_m\) value and 2-fold lower maximal activity compared with the 3β-HSD activities of the wild-type 2 and the H156Y mutant. However, 17α-hydroxyprogrenolone, the precursor steroid for cortisol production by human adrenal type 2 3β-HSD, is utilized by the wild-type 1 3β-HSD with only a 4–5-fold lower \(K_m\) and 30–40% lower \(V_{\max}\) than the wild-type 2 and H156Y 3β-HSD activities. The wild-type 2 3β-HSD utilization efficiency (\(V_{\max}/K_m\)) for 17α-hydroxyprogrenolone (3.4 min⁻¹ mg⁻¹ ml⁻¹) is 2-fold higher than for DHEA (1.7 min⁻¹ mg⁻¹ ml⁻¹) or pregnenolone (1.7 min⁻¹ mg⁻¹ ml⁻¹) as substrate.

The cofactor kinetics of the purified mutant and wild-type enzymes (Table IV) mirror the substrate kinetics in Table II with some important distinctions. Wild-type 1 3β-HSD has 2-fold lower \(K_m\) and \(K_{cat}\) values than those of the wild-type 2 3β-HSD. Clearly, these differences in NAD\(^+\) kinetics are much less dramatic than the differences in the substrate kinetics measured for the wild-type 1 and 2 3β-HSD (Tables II and III). Unlike the substrate kinetic comparison in which the H156Y mutant exhibited the 3β-HSD kinetics of the wild-type 2 enzyme, H156Y utilizes NAD\(^+\) as a cofactor for 3β-HSD activity with \(K_m\), \(K_{cat}\), and \(K_{cat}/K_m\) values, which are similar to those of the wild-type 1 3β-HSD activity. The mutants of the potentially catalytic amino acids for 3β-HSD (Y154F, K158Q, Y269S, K273Q) have no measurable 3β-HSD activity in the cofactor study, and the kinetic values for the NADH activation of isomerase are mostly in agreement with the kinetic data for the isomerase substrate. However, the \(K_m\) of K158Q for the NADH activation of isomerase is 15-fold higher than the \(K_m\) of the control wild-type 1 isomerase activity for NADH.

**Dependence of the Mutant and Wild-type 3β-HSD Activities on pH**—Similar to native human type 1 placental 3β-HSD (2), the expressed wild-type 1 3β-HSD has an optimal pH of 9.7. However, the wild-type 2 3β-HSD and the H156Y mutant of type 1 3β-HSD exhibit a pH optimum of 9.0 (Fig. 5). The presence of His^156 in the Y^154-P-H^156-S-K^158 motif of type 1 3β-HSD as opposed to Tyr^156 in the Y^154-P-Y^156-S-K^158 motif of type 2 3β-HSD appears to be responsible for this shift in the pH optimum.

Because there are two Y-X-X-X-K motifs in human type 1 3β-HSD, the pH dependence of the residual 3β-HSD activities of the Y154F, K158Q, Y269S, and K273Q mutants can help assess which motif is involved in catalysis. If the residual dehydrogenase activity of the mutant enzyme is pH-dependent, the substituted amino acid must not be catalytic, because the residual activity has to be due to a different amino acid in the enzyme (18). Although 3β-HSD activity is undetectable for each of these mutant enzymes using DHEA as substrate (Table II), the use of 5α-androstane-3β-ol-3-one as substrate enhances residual 3β-HSD by 3-fold to detectable levels. The 3β-hydroxy-5α-reduced steroid is not a substrate for isomerase activity, so feedback inhibition by a 3-keto-4-ene product steroid (e.g. androstenedione) of 3β-HSD/isomerase does not limit the 3β-HSD activity (2, 19). The extremely low residual 3β-HSD activities of these mutants allow a meaningful comparison only between pH 7.4 and the optimal pH 9.7. As shown in Table V, the Y269S and K273Q mutants exhibit low residual 3β-HSD activity at pH 7.4, and that activity is doubled at the optimal pH 9.7. The 3β-HSD activity of the control wild-type 1 enzyme also doubles as the pH increases from 7.4 to 9.7. However, even when 5α-androstane-3β-ol-3-one is used as substrate to enhance any potential 3β-HSD activity, the Y154F and K158Q mutant enzymes have undetectable activity at either pH.

**Inhibition of the Wild-type 1, Wild-type 2, and H156Y 3β-HSD Activities by Epostane**—Dixon analysis (Fig. 6) shows that epostane competitively inhibits the 3β-HSD activity of each of the three enzyme species and illustrates dramatic differences in the inhibition kinetics. Similar to the comparison of substrate kinetics (Tables II and III), epoate inhibits the wild-type 1 enzyme (\(K_i = 0.07 \mu M\)) with a 14-fold higher affinity than the wild-type 2 enzyme (\(K_i = 0.98 \mu M\)). The H156Y mutant (\(K_i = 1.18 \mu M\)) of the type 1 enzyme shares the much lower affinity of the wild-type 2 3β-HSD for epoate.

**DISCUSSION**

Because of the tissue-specific distribution of human type 1 3β-HSD in peripheral tissues (placenta, tumors) and type 2 3β-HSD in endocrine tissues (adrenals, gonads) (1, 5, 6, 7), determination of the structure/function relationships of the two isozymes may lead to clinical applications based on exploiting differences between the enzyme proteins. For example, it may
be possible to delay the onset of labor in human pregnancy by selectively inhibiting the activity of placental type 1 3β-HSD near term to decrease estradiol production without interfering with cortisol or aldosterone production by type 2 3β-HSD in the maternal adrenal gland. When the human type 2 3β-HSD cDNA was identified two years after the human type 1 cDNA, preliminary kinetic analysis of the crude type 1 and type 2 enzymes expressed in HeLa cells suggested that the type 1 3β-HSD had lower \( K_a \) values for DHEA and pregnenolone compared with the type 2 3β-HSD (1). Unlike the 1991 study using HeLa cell homogenates (1), our study uses overexpressed, purified human wild-type 1 and 2 enzymes to determine the kinetics of substrate and cofactor utilization, employs enzyme assays that differentiate between the 3β-HSD and isomerase activities, compares the kinetics of inhibition of the two isoforms by epostane, and characterizes a structural basis for the kinetic differences by mutagenesis. Finally, amino acids involved in the catalysis of the type 1 3β-HSD activity are identified in this report.

**Human 3β-HSD/isomerase** is a unique member of the short chain hydroxysteroid dehydrogenase family. The enzyme sequentially catalyzes a dehydrogenase activity (3β-HSD) followed by an isomerase activity on a single, dimeric protein. The coenzyme product of the 3β-HSD reaction, NADH, induces a conformational change in the enzyme protein that activates the isomerase reaction (20). In contrast to other short chain dehydrogenases with a single catalytic Y-X-X-K motif (8–11), there are two potentially catalytic motifs (Y154-P-H-S-K158 and Y269-T-L-S-K273) in the primary structure of human 3β-HSD/isomerase. In our previous studies (21–23), two tryptic peptides associated with the 3β-HSD and isomerase activities have been localized in the primary structure of the type 1 enzyme using affinity radiolabeled steroids (Fig. 2). One radiolabeled tryptic peptide, \(^{25}\text{O}Q\text{FYISDTPHQSYDNLN}^{269\text{O}}\text{TLSK}^{273}\), was identified by the affinity radioalkylators, 2α-bromo-[2\(^{14}\text{C}\)]ace
toxyprogesterone (2α-BAP) (21, 22) and 5,10-secoestr-3,17-dione (23), two tryptic peptides that are associated with the isomerase site of the enzyme (23). When the isomerase substrate steroid, 5-androstene-3,17-dione, or the allosteric activator of isomerase, NADH, was covalently bound to the enzyme (23). When the isomerase substrate steroid, 5-androstene-3,17-dione, or the allosteric activator of isomerase, NADH, was covalently bound to the enzyme (23).

**Table III**

<table>
<thead>
<tr>
<th>Substrate steroid</th>
<th>Wild-type 1</th>
<th>Wild-type 2</th>
<th>H156Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m )</td>
<td>( V_{max} )</td>
<td>( K_m )</td>
</tr>
<tr>
<td>DHEA(^a)</td>
<td>3.7</td>
<td>45.3</td>
<td>47.3</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>2.8</td>
<td>45.7</td>
<td>49.5</td>
</tr>
<tr>
<td>17α-OH-pregnenolone</td>
<td>3.5</td>
<td>42.5</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>( \mu M )</td>
<td>nmol/min/mg</td>
<td>( \mu M )</td>
</tr>
</tbody>
</table>

\(^a\) Kinetic constants for the 3β-HSD substrate were determined in incubations at 27 °C containing dehydroepiandrosterone (DHEA, 2–100 \( \mu M \)), pregnenolone (2–20 \( \mu M \)), or 17α-hydroxyprogrenolone (17α-OH-pregnenolone, 2–20 \( \mu M \)), NAD\(^+\) (0.1 mM), and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4. These values are the mean of triplicate determinations with S.D. of \( \pm 7\% \).
**Human 3β-Hydroxysteroid Dehydrogenase**

**TABLE V**

<table>
<thead>
<tr>
<th>Purified enzyme (mg</th>
<th>pH 7.4</th>
<th>WT1</th>
<th>pH 9.7</th>
<th>WT1</th>
<th>pH 9.7/pH 7.4 activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type 1</td>
<td>56.3 ± 0.7</td>
<td>100</td>
<td>108.1 ± 0.8</td>
<td>100</td>
<td>1.9</td>
</tr>
<tr>
<td>Y269S</td>
<td>0.7 ± 0.2</td>
<td>1.0</td>
<td>1.4 ± 0.3</td>
<td>1.1</td>
<td>2.0</td>
</tr>
<tr>
<td>K273Q</td>
<td>1.7 ± 0.2</td>
<td>3.2</td>
<td>3.4 ± 0.3</td>
<td>3.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Y154F</td>
<td>None detected</td>
<td>ND</td>
<td>None detected</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>K158Q</td>
<td>None detected</td>
<td>ND</td>
<td>None detected</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* 3β-HSD activities were measured in incubations at 27 °C containing 5α-androstan-3β-ol-17-one (100 μM), NAD⁺ (0.2 mM), and purified enzyme (0.08 mg) in 0.05 M sodium phosphate pyrophosphate buffer, pH 7.4 or pH 9.7. WT1 is human wild-type 1 3β-HSD. ND, not determined. Values are the means ± S.D. of triplicate determinations.

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**Fig. 6. Inhibition of the 3β-HSD activities of the wild-type and H156Y enzymes by epostane.** For wild-type 1 3β-HSD, the incubations contained 1.9 μM (●) or 2.8 μM (○) of DHEA as substrate, epoasteone (0–1.0 μM), NAD⁺ (0.1 mM), and purified wild-type 1 enzyme (0.05 mg) in 0.02 M potassium phosphate buffer, pH 7.4, at 27 °C. For wild-type 2 3β-HSD, similar incubations contained 15.6 μM (♦, dashed line) or 23.0 μM (▲, dashed line) of DHEA, epoasteone (0–7.5 μM), and the appropriate purified enzyme. Identical incubations for the H156Y mutant contained 15.6 μM (□) or 23.0 μM (▲) DHEA. Each point on the Dixon plot (1/V _versus_ I) represents the mean of triplicate determinations, and the error bars represent S.D. values. Kᵢ values were calculated from the intersection of the Dixon plots obtained for each enzyme preparation.

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determined which of the two motifs participates in the 3β-HSD reaction.

The Y₁₁⁵⁴-P-H-S-K₁₁⁵⁸ motif appears to contain critical residues for 3β-HSD catalysis based on the kinetic profiles of the mutant enzymes and on the pH dependence studies. The Y₁₁⁴F and K₁₁⁸Q mutant enzymes lack measurable 3β-HSD activity, even using conditions that allowed detection of pH-dependent 3β-HSD activity in the Y₂₆₉S and K₂₇₃Q mutants. The Y₁₁⁴F and K₁₁⁸Q mutants exhibit significant isomerase activity and utilize the isomerase substrate steroid with K_m values that are similar to the control values for the wild-type 1 enzyme. Although the NAD⁺ /NADH activi of the K₁₁⁸Q mutant could not be measured, the 14-fold higher K_m value measured for the allosteric activation of K₁₁⁸Q isomerase by NADH suggests that Lys₁₁⁵⁸ may bind cofactor. The Lys₁₁⁵⁸ residue in the catalytic Y₁₁⁵⁴-X-X-X-K₁₁⁵⁸ motif of human type 1 17β-HSD binds the 2'- and 3'-hydroxyls of the nicotinamide ribose group of NAD⁺(25). In contrast to the specific modifications produced by the Y₁₁⁴F and K₁₁⁸Q mutations, the Y₂₆₉S and K₂₇₃Q mutants have drastically reduced isomerase activity with extremely high K_m values for the isomerase substrate, which suggest that Tyr₂₆₉ and Lys₂₇₃ reside in the isomerase site and that the enzyme conformation may have been altered by these mutations to interfere with the 3β-HSD activity catalyzed by the Tyr₁₁⁴ and Lys₁₁⁵⁸ residues.

The most compelling evidence in support of the catalytic role of the Y₁₁⁵⁴-P-H₁₁⁵⁶-S-K₁₁⁵⁸ motif in human type 1 3β-HSD is the kinetic data obtained with the H156Y mutant. The human type 2 enzyme is 93% homologous with the human type 1 3β-HSD and possesses an analogous motif, Y₁₁⁴P-Y₁₁⁵⁶-S-K₁₁⁵⁸, with a single amino acid difference: Tyr₁₁⁴ in place of His₁₁⁵⁸. The H156Y mutant form of the type 1 enzyme shifts the substrate kinetics for DHEA and pregnenolone to the same 13-fold higher K_m and 2-fold higher V_max and K_cat values exhibited by the type 2 enzyme. The isomerase substrate and coenzyme kinetic profiles of the H156Y mutant retain the characteristics of the wild-type 1 enzyme. In addition, the optimal pH of the wild-type 3β-HSD activity is shifted from pH 9.7 to the pH 9.0 optimum of the wild-type 2 3β-HSD by the H156Y mutation. However, the different pH optima are not directly responsible for the differences in the kinetic profiles of the wild-type 1, wild-type 2, and H156Y enzymes. All substrate and inhibition kinetic studies were performed at pH 7.4, at which the percent maximal 3β-HSD activity was equivalent for all three enzyme species.

The 14–17-fold higher Kᵢ values measured for the inhibition of the H156Y and wild-type 2 3β-HSD activities by epoasteone in comparison with the Kᵢ obtained for the wild-type 1 enzyme is an exciting and novel observation. This shift in Kᵢ values suggests that His₁₁⁵⁸ is a key residue that significantly enhances the binding of inhibitor and substrate steroids to the human type 1 enzyme compared with the type 2 enzyme containing Tyr₁₁⁴. The importance of His₁₁⁵⁸ to the binding of steroids plus the kinetic and pH profiles of the mutant enzymes discussed above strongly support a catalytic role for the Tyr₁₁⁴ and Lys₁₁⁵⁸ residues in the Y₁₁⁴-P-H₁₁⁵⁶-S-K₁₁⁵⁸ motif of human type 1 3β-HSD.

The dramatic kinetic differences between the human type 1 and type 2 3β-HSD activities may produce novel clinical applications. The ability of epoasteone to inhibit the type 1 3β-HSD at concentrations (Kᵢ = 0.07 μM) that spare the adrenal and gonadal type 2 3β-HSD from inhibition (Kᵢ = 0.98 μM) suggests...
that steroidogenesis can be selectively blocked in breast tumors, prostate tumors, choriocarcinomas, and in placenta near term. Knowledge of the structural basis (His156 in type 1 3β-HSD versus Tyr156 in type 2 3β-HSD) for the kinetic differences between the isoenzymes may lead to development of inhibitors with much greater specificity for the human type 1 3β-HSD. The clinical therapeutics made possible by the selective inhibition of type 1 3β-HSD are enormous. Combination therapy with a selective type 1 3β-HSD inhibitor and an aromatase inhibitor could increase the effectiveness of both drugs in the treatment of breast cancer (26). A type 1 3β-HSD inhibitor could block the intracrine biosynthesis of estradiol and thereby lower the doses of highly toxic methotrexate needed for the treatment of hormone-sensitive choriocarcinomas (27). A type 1 3β-HSD inhibitor would be a useful tool in the management of premature labor in humans. Although human 3β-HSD purified from microsomes has not been crystallized, efforts are underway with our genetically engineered, soluble form of human type 1 3β-HSD (28) that will ultimately correlate these mutagenesis results with the tertiary/quaternary structure of the enzyme.

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Structure/Function Relationships Responsible for the Kinetic Differences between Human Type 1 and Type 2 3β-Hydroxysteroid Dehydrogenase and for the Catalysis of the Type 1 Activity
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