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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ABSTRACT

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 Y\textsuperscript{154}-P-H\textsuperscript{156}-S-K\textsuperscript{158} motif as well as the Y269S and K273Q mutants from a second motif, Y\textsuperscript{269}-T-L-S-K\textsuperscript{273}, 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 (Y\textsuperscript{154}-P-Y\textsuperscript{156}-S-K\textsuperscript{158}) in the type 1 enzyme. The mutant and wild-type enzymes have been expressed and purified. The \(K_m\) value of dehydroepiandrosterone is 13-fold greater and the maximal turn-over rate (\(K_{cat}\)) is 2-fold greater for wild-type 2 3β-HSD compared to 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 epistane inhibits the 3β-HSD activity of the wild-type 1 enzyme with 14 to 17-fold greater affinity compared to 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 \(K_m\) 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 Tyr\textsuperscript{154} and Lys\textsuperscript{158} 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.
INTRODUCTION

The human type 1 (placenta, skin, mammary gland, prostate, endometrium) and type 2 (gonads, adrenals) isoforms of 3ß-hydroxysteroid dehydrogenase (EC 1.1.1.145)/steroid Δ5-Δ4-isomerase (EC 5.3.3.1) (3ß-HSD/isomerase^1^) are encoded by two distinct genes which are expressed in a tissue-specific pattern (~1). In human placenta, type 1 3ß-HSD/isomerase catalyzes the conversion of 3ß-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, Figure ~1~). Because the fetal adrenal lacks significant 3ß-HSD/isomerase activity, the placental type 1 enzyme converts the fetal dehydroepiandrosterone to androstenedione ~3~. Androstenedione is converted by placental aromatase and 17ß-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 the tumor growth-promoting hormone, estradiol or testosterone. Determination of the structure/function relationships of the type 1 enzyme may lead to the development of specific inhibitors of type 1 3ß-HSD/isomerase that can help control the timing of labor and slow the growth of hormone-sensitive tumors.

The Y-X-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 11$\chi$-HSD (~8~), *Streptomyces hydrogenans* 3",20$\chi$-HSD (~8~), *Drosophila* alcohol dehydrogenase (~9~), *E. Coli* 7"$\chi$-HSD (~10~), and human 17$\chi$-HSD (~11~). In each of these enzymes, there is a single Y-X-X-X-K motif associated with the dehydrogenase activity, but there are two of these motifs (~154Y-X-X-X-K~).
K$_{158}$ and Y$_{269}$X-X-X-K$_{273}$) in the primary structures of human type 1 and type 2 $3\beta$-HSD/isomerase (Figure 2) (12). $3\beta$-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 $3\beta$-HSD/isomerase that target the potentially critical residues in the two Y-X-X-X-K motifs (Y$_{154}$F, K$_{158}$Q, Y$_{269}$S, K$_{273}$Q). In addition, the H$_{156}$Y mutant was created to produce a chimera of the human type 2 enzyme motif (Y$_{154}$P-Y$_{156}$S-K$_{158}$) in the corresponding human type 1 enzyme motif (Y$_{154}$P-H$_{156}$S-K$_{158}$). 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 $3\beta$-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 $3\beta$-HSD/isomerases.

**EXPERIMENTAL PROCEDURES**

**Materials**

Dehydroepiandrosterone, pregnenolone and pyridine nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO); 5-androstene-3,17-dione and 17$\alpha$-hydroxypregnenolone from Steraloids Inc. (Newport, RI); reagent grade salts, chemicals and analytical grade solvents from Fisher Scientific Co. (Pittsburg, PA). 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-3HSD1 as template (13), double-stranded PCR-based mutagenesis was performed with the primers in Table 1 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 3$\beta$-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 (Protyle program, Scientific and Educational Software, State Line, PA).

**Expression and purification of the mutant and wild-type enzymes**

The mutant 3$\beta$-HSD cDNA was introduced into baculovirus as previously described (13,14). Recombinant baculovirus was added to 1.5 x 10⁹ 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-3$\beta$-HSD polyclonal antibody and detected using the ECL western blotting system with antirabbit, peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Each expressed enzyme was purified from the 100,000 g pellet of the Sf9 cells (2 L) by our published method (2). The Emulgen 913 detergent (polyoxyethylene(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 Rhodia, 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 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 (15).

**Kinetic and pH-dependency studies**
Michaelis-Menten kinetic constants for the 3ß-HSD substrate were determined for the purified mutant and wild-type enzymes in incubations containing dehydroepiandrosterone (2-100 µM), pregnenolone (2-20 : M) or 17"-hydroxypregnenolone (2-20 : M) plus NAD⁺ (0.1 mM) and purified enzyme (0.03 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 per min (due to NADH production) was used to determine 3ß-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 0.02 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, zero-substrate) assured that specific isomerase activity was measured as opposed to non-enzymatic, "spontaneous" isomerization (16). 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-Burke (1/S vs. 1/V) plots and verified by Hanes-Woolf (S vs. S/V) plots (17). Kₐₜ values (min⁻¹) were calculated from the Vₘₐₓ values (nmol/min/mg) and represent the maximal turnover rate (nmol product formed/min/nmol enzyme dimer).

Kinetic constants for the 3ß-HSD cofactor were determined for the purified mutant and wild-type enzymes in incubations containing NAD⁺ (13-100 µM), dehydroepiandrosterone (100 µM) and purified enzyme (0.03 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 (2-50 µM), 5-androstene-3,17-dione (100 µM) and purified enzyme (0.01 mg) in 0.02 M potassium phosphate buffer, pH 7.4 at 27°C using the spectrophotometric assay at 241 nm.
Inhibition constants ($K_i$) were determined for the inhibition of the wild-type 1, wild-type 2 and H156Y 3$\beta$-HSD activities by epostane using conditions that were appropriate for each enzyme species. For type 1 3$\beta$-HSD, the incubations at 27°C contained sub-saturating concentrations of dehydroepiandrosterone (1.9 and 2.8 $\mu$M, relative to $K_m$ of 3.7 $\mu$M), epostane (0–1.0 $\mu$M), NAD$^+$ (0.1 mM) and purified human type 1 enzyme (0.03 mg) in 0.02 M potassium phosphate buffer, pH 7.4. For type 2 3$\beta$-HSD and the H156Y mutant of type 1 3$\beta$-HSD, the incubations at 27°C contained sub-saturating concentrations of dehydroepiandrosterone (15.6 or 23.0 $\mu$M, relative to $K_m$ values of 47.3 $\mu$M and 42.4 $\mu$M, respectively), epostane (0–7.5 $\mu$M), NAD$^+$ (0.1 mM) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate buffer, pH 7.4. Dixon analysis ($1/V$ vs. $I$) was used to determine the type of inhibition and calculate the $K_i$ values (17).

The effects of pH on the residual 3$\beta$-HSD activities of the Y154F, K158Q, Y269S and K273Q mutants were measured in incubations at 27°C containing 5"-androstan-3$\beta$-ol-17-one (100 $\mu$M), NAD$^+$ (0.2 mM) and purified enzyme (0.08 mg) in 0.05 sodium phosphate pyrophosphate buffer, pH 7.4 or 9.7, using the 3$\beta$-HSD assay at 340 nm. The complete pH profiles of the fully active wild-type 1, wild-type 2 and H156Y enzymes were measured by the 3$\beta$-HSD assay in incubations containing pregnenolone (20 $\mu$M), NAD$^+$ (0.1 mM) and purified enzyme (0.03 mg) in 0.05 M sodium phosphate pyrophosphate buffer, pH 6.5-10.5, at 27°C.

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 in Methods. As shown by the immunoblot in Figure 3, the baculovirus system successfully expressed the mutant enzyme proteins as well as the human wild-type 1 and wild-type 2 3\$-HSD/isomerase in Sf9 cells. Each expressed enzyme was purified using our published method (2) to apparent homogeneity according to SDS-PAGE (Figure 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 variations of human 3\$-HSD/isomerase are summarized in Table 2. Purified human type 1 placental 3\$-HSD/isomerase has been previously shown to have Michaelis-Menten constants for substrate steroid and cofactors that were almost identical to those measured for expressed, purified wild-type 1 enzyme (13). The wild-type 1 3\$-HSD activity has 13-fold lower $K_m$, 2-fold lower $K_{cat}$ and 7-fold higher $K_{cat}/K_m$ values for DHEA as substrate compared to the purified wild-type 2 3\$-HSD. The isomerase 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 3\$-HSD (containing the $Y^{154}-P-H^{156}-S-K^{158}$ motif) to values which are similar to those of wild-type 2 3\$-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 3\$-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 steroid 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 3 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 3$\beta$-HSD. Similar to DHEA, pregnenolone utilization by wild-type 1 3$\beta$-HSD exhibits a 16 to 17-fold lower $K_m$ value and 2-fold lower maximal activity compared to the 3$\beta$-HSD activities of the wild-type 2 and the H156Y mutant. However, 17"$\beta$-hydroxypregnenolone, the precursor steroid for cortisol production by human adrenal type 2 3$\beta$-HSD, is utilized by the wild-type 1 3$\beta$-HSD with only a 4 to 5-fold lower $K_m$ and 30-40% lower $V_{max}$ than the wild-type 2 and H156Y 3$\beta$-HSD activities. The wild-type 2 3$\beta$-HSD utilization efficiency ($V_{max}/K_m$) for 17"$\beta$-hydroxypregnenolone ($3.4 \text{ min}^{-1}\text{mg}^{-1}\text{ml}^{-1}$) is 2-fold higher than for DHEA ($1.7 \text{ min}^{-1}\text{mg}^{-1}\text{ml}^{-1}$) or pregnenolone ($1.7 \text{ min}^{-1}\text{mg}^{-1}\text{ml}^{-1}$) as substrate.

The cofactor kinetics of the purified mutant and wild-type enzymes (Table 4) mirror the substrate kinetics in Table 2 with some important distinctions. Wild-type 1 3$\beta$-HSD has 2-fold lower $K_m$ and $K_{cat}$ values than those of the wild-type 2 3$\beta$-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 3$\beta$-HSD (Tables 2 and 3). Unlike the substrate kinetic comparison in which the H156Y mutant exhibited the 3$\beta$-HSD kinetics of the wild-type 2 enzyme, H156Y utilizes NAD$^+$ as a cofactor for 3$\beta$-HSD activity with $K_m$, $K_{cat}$ and $K_{cat}/K_m$ values which are similar to those of the wild-type 1 3$\beta$-HSD activity. The mutants of the potentially catalytic amino acids for 3$\beta$-HSD (Y154F, K158Q, Y269S, K273Q) have no measurable 3$\beta$-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.

**Dependency of the mutant and wild-type 3$\beta$-HSD activities on pH**

Similar to native human type 1 placental 3$\beta$-HSD (2), the expressed wild-type 1 3$\beta$-HSD has an optimal pH of 9.7. However, the wild-type 2 3$\beta$-HSD and the H156Y mutant of type 1 3$\beta$-HSD exhibit a pH optimum of 9.0 (Figure 5). The presence of His$^{156}$ in the Y$^{154}$-P-H$^{156}$-S-K$^{158}$ motif of type 1 3$\beta$-HSD as opposed to Tyr$^{156}$ in the Y$^{154}$-P-Y$^{156}$-S-K$^{158}$ motif of type 2 3$\beta$-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 3$\beta$-HSD, the pH-dependency of the residual 3$\beta$-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 3$\beta$-HSD activity is undetectable for each of these mutant enzymes using DHEA as substrate (Table 2), the use of 5"-androstan-3$\beta$-ol-3-one as substrate enhances residual 3$\beta$-HSD by 3-fold to detectable levels. The 3$\beta$-hydroxy-5"-reduced steroid is not a substrate for isomerase activity, so feedback inhibition by a 3-keto-4-ene product steroid (e.g., androstenedione) of 3$\beta$-HSD/isomerase does not limit the 3$\beta$-HSD activity (2,19). The extremely low residual 3$\beta$-HSD activities of these mutants allow a meaningful comparison only between pH 7.4 and the optimal pH 9.7. As shown in Table 5, the Y269S and K273Q mutants exhibit low residual 3$\beta$-HSD activity at pH 7.4, and that activity is doubled at the optimal pH 9.7. The 3$\beta$-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-androstan-3$\beta$-ol-3-one is used as substrate to enhance any potential 3$\beta$-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 3$\beta$-HSD activities by epostane**

Dixon analysis (Figure 6) shows that epostane competitively inhibits the 3$\beta$-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 2 and 3), epostane 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 3$\beta$-HSD for epostane.

**DISCUSSION**

Because of the tissue-specific distribution of human type 1 3$\beta$-HSD in peripheral tissues (placenta, tumors) and type 2 3$\beta$-HSD in endocrine tissues (adrenals, gonads) (1,5,6,7), determination of the structure/function relationships of the two isoforms 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 3$\beta$-HSD near term to decrease estradiol production without interfering with cortisol or aldosterone production by type 2 3$\beta$-HSD in the maternal adrenal gland. When the human type 2 3$\beta$-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 3$\beta$-HSD had lower $K_m$ values for DHEA and pregnenolone compared to the type 2 3$\beta$-HSD (1). Unlike the 1991 study using HeLa cell homogenates (1), our study
uses over-expressed, purified human wild-type 1 and 2 enzymes to determine the kinetics of substrate and cofactor utilization, employs enzyme assays that differentiate between the 3$\$-HSD and isomerase activities, compares the kinetics of inhibition of the two isoforms by epostane and characterizes a structural basis for the kinetic differences by mutagenesis. Finally, amino acids involved in the catalysis of the type 1 3$\$-HSD activity are identified in this report.

Human 3$\$-HSD/isomerase is a unique member of the short-chain hydroxysteroid dehydrogenase family. The enzyme sequentially catalyzes a dehydrogenase activity (3$\$-HSD) followed by an isomerase activity on a single, dimeric protein. The coenzyme product of the 3$\$-HSD reaction, NADH, induces a conformational change in the enzyme protein that activates the isomerase reaction (20). In contrast to other short-chain dehydrogenases with a single catalytic Y-X-X-X-K motif (8-11), there are two potentially catalytic motifs (Y$^{154}$-P-H-S-K$^{158}$ and Y$^{269}$-T-L-S-K$^{273}$) in the primary structure of human 3$\$-HSD/isomerase. In our previous studies (21-23), two tryptic peptides associated with the 3$\$-HSD and isomerase activities have been localized in the primary structure of the type 1 enzyme using affinity radiolabeled steroids (Figure 2). One radiolabeled tryptic peptide, $^{250}$GQFY$^{253}$YISDDTPHQSYDNLY$^{269}$TLSK$^{273}$, was identified by the affinity radioalkylators, $^{2\pi}$-bromo[2$'$-14C]acetoxyprogesterone ($^{2\pi}$-BAP) (21,22) and 5,10-secoestr-4-yne-3,10,17-trione (tritiated by sodium [3H]borohydride after covalently binding to the enzyme) (23). When the isomerase substrate steroid, 5-androstone-3,17-dione, or the allosteric activator of isomerase, NADH, was co-incubated with the secosteroid or $^{2\pi}$-BAP, respectively, the radioalkylation of the first tryptic peptide was blocked, and a second radiolabeled tryptic peptide was identified: $^{136}$EIIQNGHEEPLETPAY$^{154}$PHSK$^{158}$ (22,23). The Tyr$^{253}$ residue in the $^{250}$GQFY$^{253}$YISDDTPHQSYDNLY$^{269}$TLSK$^{273}$ peptide was shown by mutagenesis (13) and spectral shifts (24) to be critical to the isomerase activity. Thus, the Y$^{269}$-
T-L-S-K^{273} motif is part of a tryptic peptide that is associated with the isomerase site of the enzyme, and the Y^{154}-P-H-S-K^{158} motif resides outside of the isomerase domain. In this report, we use site-directed mutagenesis to produce the key mutant enzymes (Y154F, H156Y, K158Q, Y269S and K273Q) in the two motifs. Characterizing the properties of the expressed, purified mutant enzymes has determined which of the two motifs participates in the 3\$-HSD reaction.

The Y^{154}-P-H-S-K^{158} motif appears to contain critical residues for 3\$-HSD catalysis based on the kinetic profiles of the mutant enzymes and on the pH-dependency studies. The Y154F and K158Q mutant enzymes lack measurable 3\$-HSD activity even using conditions that allowed detection of pH-dependent 3\$-HSD activity in the Y269S and K273Q mutants. The Y154F and K158Q 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^+ kinetics of the K158Q mutant could not be measured, the 15-fold higher K_m value measured for the allostERIC activation of K158Q isomerase by NADH suggests that Lys^{158} may bind cofactor. The Lys^{159} residue in the catalytic Y^{155}-X-X-X-K^{159} motif of human type 1 17\$-HSD binds the 2' and 3' hydroxyls of the nicotinamide ribose group of NADP^+ (25). In contrast to the specific modifications produced by the Y154F and K158Q mutations, the Y269S and K273Q mutants have drastically reduced isomerase activity with extremely high K_m values for the isomerase substrate, which suggest that Tyr^{269} and Lys^{273} reside in the isomerase site and that the enzyme conformation may have been altered by these mutations to interfere with the 3\$-HSD activity catalyzed by the Tyr^{154} and Lys^{158} residues.

The most compelling evidence in support of the catalytic role of the Y^{154}-P-H^{156}-S-K^{158} motif in human type 1 3\$-HSD is the kinetic data obtained with the H156Y mutant. The human type 2 enzyme is 93% homologous with the human type 1 3\$-HSD and possesses an analogous
motif, Y$^{154}$-P-Y$^{156}$-S-K$^{158}$, with a single amino acid difference: Tyr$^{156}$ in place of His$^{156}$. 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 $V_{\text{max}}$ and $K_{\text{cat}}$ 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 1 $3\beta$-HSD activity is shifted from pH 9.7 to the pH 9.0 optimum of the wild-type 2 $3\beta$-HSD by the H156Y mutation. However, the different pH optima are not directly responsible 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 3$\beta$-HSD activity was equivalent for all three enzyme species.

The 14 to 17-fold higher $K_i$ values measured for the inhibition of the H156Y and wild-type 2 $3\beta$-HSD activities by epostane in comparison to the $K_i$ obtained for the wild-type 1 enzyme is an exciting and novel observation. This shift in $K_i$ values suggests that His$^{156}$ is a key residue that significantly enhances the binding of inhibitor and substrate steroids to the human type 1 enzyme compared to the type 2 enzyme containing Tyr$^{156}$. The importance of His$^{156}$ to the binding of steroids plus the kinetic and pH profiles of the mutant enzymes discussed above strongly support a catalytic role for the Tyr$^{154}$ and Lys$^{158}$ residues in the Y$^{154}$-P-H$^{156}$-S-K$^{158}$ motif of human type 1 $3\beta$-HSD.

The dramatic kinetic differences between the human type 1 and type 2 $3\beta$-HSD activities may produce novel clinical applications. The ability of epostane to inhibit the type 1 $3\beta$-HSD at concentrations ($K_i=0.07$ : M) that spare the adrenal and gonadal type 2 $3\beta$-HSD from inhibition ($K_i=0.98$ : M) suggests that steroidogenesis can be selectively blocked in breast tumors, prostate tumors, choriocarcinomas and in placenta near term. Knowledge of the structural basis (His$^{156}$ in
type 1 3\$-HSD vs Tyr$^{156}$ in type 2 3\$-HSD) for the kinetic differences between the isoenzymes may lead to development of inhibitors with much greater specificity for the human type 1 3\$-HSD. The clinical therapeutics made possible by the selective inhibition of type 1 3\$-HSD are enormous. Combination therapy with a selective type 1 3\$-HSD inhibitor and an aromatase inhibitor could increase the effectiveness of both drugs in the treatment of breast cancer (26). A type 1 3\$-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 3\$-HSD inhibitor would be a useful tool in the management of premature labor in humans. Although human 3\$-HSD purified from microsomes has not been crystallized, efforts are underway with our genetically-engineered, soluble form of human type 1 3\$-HSD (28) that will ultimately correlate these mutagenesis results with the tertiary/quaternary structure of the enzyme.

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REFERENCES


FIGURE LEGENDS

**Figure 1.** 3β-HSD/isomerase is a key enzyme in the biosynthesis of steroid hormones.  
The roles of human type 1 3$\beta$-HSD in steroidogenesis in placenta and peripheral tissues (notably hormone sensitive-tumors) and of human type 2 3$\beta$-HSD in adult adrenal cortex are illustrated.

**Figure 2.** 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 Y$^{154}$-X-H$^{156}$-X-K$^{158}$ and Y$^{269}$-X-X-X-K$^{273}$ motifs for 3β-HSD activity as well as the initial amino acid of each affinity radiolabeled peptide are indicated. The sequence of human type 2 3β-HSD/isomerase is also shown. Homologous amino acids in the human type 2 enzyme are
indicated by dots, and the non-identical amino acids are identified by single-letter abbreviations.

**Figure 3. Western immunoblot showing the expression of the mutant and wild-type enzymes by baculovirus.** The Sf9 cell homogenate (1.5 µg) containing the Y269S, K273Q, Y154F, K158Q, H156Y, or wild-type 2 enzyme plus the purified control wild-type 1 3$\text{HSD}$ (0.05 : g) were separated by SDS-polyacrylamide (12%) gel electrophoresis. The 42.0 kDa band of the enzyme monomer was detected using our anti-3$\text{HSD}$ antibody.

**Figure 4. SDS-Polyacrylamide gel electrophoresis of the purified mutant and wild-type enzymes.** Each lane was over-loaded with 2.0 µg of purified protein, and the bands were visualized by coomassie blue staining.

**Figure 5. pH profiles of the 3$\text{HSD}$ activities of the wild-type and H156Y enzymes.** The purified wild-type 1 (■), wild-type 2 (○) or H156Y mutant (▲) enzymes (0.03 mg) were incubated with pregnenolone (20 : M) and NAD$^+$ (0.1 mM) in 0.05 M sodium phosphate pyrophosphate buffer, pH 6.5-10.5, at 27°C. Each point represents the mean of triplicate determinations. Error bars represent standard deviations.

**Figure 6. Inhibition of the 3$\text{HSD}$ activities of the wild-type and H156Y enzymes by epostane.** For wild-type 1 3$\text{HSD}$, the incubations contained 1.9 uM (▲) or 2.8 : M (▽) of DHEA as substrate, epostane (0–1.0 : M), NAD$^+$ (0.1 mM) and purified wild-type 1 enzyme (0.03 mg) in 0.02 M potassium phosphate buffer, pH 7.4, at 27°C. For wild-type 2 3$\text{HSD}$, similar incubations contained 15.6 : M (◆, dashed line) or 23.0 : M (▲, dashed line) of DHEA, epostane (0–7.5 : M) and the appropriate purified enzyme. Identical incubations for the H156Y mutant contained 15.6 : M (○) or 23.0 : M (□) DHEA. Each point on the Dixon plot (I/V vs. I) represents the mean of triplicate determinations, and the error bars represent
standard deviations. $K_i$ values were calculated from the intersection of the Dixon plots obtained for each enzyme preparation.

---

1 The abbreviations used are: 3$\beta$-HSD, 3$\beta$-hydroxysteroid dehydrogenase; 17$\alpha$-HSD, 17$\alpha$-hydroxysteroid dehydrogenase; CYP11B1, 11$\beta$-hydroxylase, CYP11B2, aldosterone synthase; CYP17, 17$\alpha$-hydroxylase/17-20 lyase; CYP19, aromatase, CYP21, 21-hydroxylase; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; 17$\alpha$-OH-Pregnenolone, 17$\alpha$-hydroxypregnenolone; 2$\beta$-BAP, 2$\beta$-bromoacetoxyprogesterone.
Table 1. Oligonucleotide primers used for site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Direction</th>
<th>Nucleotide sequence of primer$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y154F</td>
<td>Forward</td>
<td>5’-CCGCTCCA <strong>TTC</strong> CCACACAGCAA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CTGTGTGGA <strong>GAA</strong> TGGAGCGGGCCA-3’</td>
</tr>
<tr>
<td>K158Q</td>
<td>Forward</td>
<td>5’-ACACAGGC <strong>CAA</strong> AAGCTTGCTGAGAA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-AAGCTT <strong>TTG</strong> CGTGTGGGGGTATGG-3’</td>
</tr>
<tr>
<td>H156Y</td>
<td>Forward</td>
<td>5’-CATACCCA <strong>TAC</strong> AGCAAAAAGCTTGCT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GCTTTTTGCT <strong>GTA</strong> TGGGTATGAGGC-3’</td>
</tr>
<tr>
<td>Y269S</td>
<td>Forward</td>
<td>5’-ACCTTAAT <strong>TCC</strong> ACCCTGAGCAAAGA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GCTCAGGCTT <strong>GGA</strong> ATTAAGGTTATCA-3’</td>
</tr>
<tr>
<td>K273Q</td>
<td>Forward</td>
<td>5’-CCCTGAGC <strong>CAA</strong> GAGTTCGGCCTCCTC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CCGAACTC <strong>TTG</strong> GCTCAGGGTGTAATT-3’</td>
</tr>
</tbody>
</table>

$^1$ The mutated codons are in the bold, italic font.
Table 2. Substrate kinetics for the 3β-HSD and isomerase activities of the purified mutant and wild-type enzymes.

<table>
<thead>
<tr>
<th>Purified Enzyme</th>
<th>3β-HSD&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Isomerase&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ µM</td>
<td>$K_{cat}$ min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wild-type 1</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Wild-type 2</td>
<td>47.3</td>
<td>6.9</td>
</tr>
<tr>
<td>H156Y</td>
<td>42.4</td>
<td>7.2</td>
</tr>
<tr>
<td>Y154F</td>
<td>N.D.</td>
<td>No Activity Detected</td>
</tr>
<tr>
<td>K158Q</td>
<td>N.D.</td>
<td>No Activity Detected</td>
</tr>
<tr>
<td>Y269S</td>
<td>N.D.</td>
<td>No Activity Detected</td>
</tr>
<tr>
<td>K273Q</td>
<td>N.D.</td>
<td>No Activity Detected</td>
</tr>
</tbody>
</table>

<sup>1</sup> Michaelis-Menten kinetic constants for the 3β-HSD substrate were measured in incubations containing dehydroepiandrosterone (2-100 µM), NAD<sup>+</sup> (0.1 mM) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4, at 27°C. $K_{cat}$ values (nmol product formed/min/nmol enzyme dimer) were calculated from the $V_{max}$ values. N.D., not determined.

<sup>2</sup> 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 standard deviation ≤ 6%.
Table 3. Substrate kinetic constants for the 3β-HSD activities of purified wild-type 1, wild-type 2 and H156Y.

<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) (µM)</td>
<td>(V_{max}) (nmol/min/mg)</td>
<td>(K_m) (µM)</td>
</tr>
<tr>
<td>DHEA(^1)</td>
<td>3.7</td>
<td>43.3</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>
</tbody>
</table>

\(^1\) Kinetic constants for the 3β-HSD substrate were determined in incubations at 27°C containing dehydroepiandrosterone (DHEA, 2-100 µM), pregnenolone (2-20 µM) or 17α-hydroxypregnenolone (17α-OH-Pregnenolone, 2-20 µM), NAD\(^+\) (0.1 mM) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4. These values are the means of triplicate determinations with standard deviations of ≤ 7%.
Table 4. Cofactor kinetics for the 3β-HSD and isomerase activities of the purified mutant and wild-type enzymes.

<table>
<thead>
<tr>
<th>Purified Enzyme</th>
<th>3β-HSD$^1$ NAD$^+$</th>
<th>Isomerase$^2$ NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$K_{cat}$ (min$^{-1}$)</td>
</tr>
<tr>
<td>Wild-type 1</td>
<td>34.1</td>
<td>3.9</td>
</tr>
<tr>
<td>Wild-type 2</td>
<td>86.3</td>
<td>7.1</td>
</tr>
<tr>
<td>H156Y</td>
<td>25.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Y154F</td>
<td>N.D.</td>
<td>No Activity Detected</td>
</tr>
<tr>
<td>K158Q</td>
<td>N.D.</td>
<td>No Activity Detected</td>
</tr>
<tr>
<td>Y269S</td>
<td>N.D.</td>
<td>No Activity Detected</td>
</tr>
<tr>
<td>K273Q</td>
<td>N.D.</td>
<td>No Activity Detected</td>
</tr>
</tbody>
</table>

$^1$ Kinetic constants for the 3β-HSD cofactor were determined in incubations containing NAD$^+$ (13-100 µM), dehydroepiandrosterone (100 µM) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4, at 27°C. $K_{cat}$ values (nmol product formed/min/nmol enzyme dimer) were calculated from the $V_{max}$ values. N.D., not determined.

$^2$ Kinetic constants for the activation of isomerase by NADH were determined in incubations of NADH (2-50 µM), 5-androstene-3,17-dione (100 µM) and purified enzyme (0.01 mg) in 0.02 M potassium phosphate buffer, pH 7.4, at 27°C. Each value represents the mean of triplicate measurements with standard deviations of ≤ 10%.
Table 5. Dependency of the residual 3β-HSD activities of the purified mutant enzymes on pH.

<table>
<thead>
<tr>
<th>Purified Enzyme</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></td>
<td>nmol/min/mg(^1)</td>
<td></td>
<td>nmol/min/mg</td>
<td></td>
<td></td>
</tr>
<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>N.D.</td>
<td>None detected</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>K158Q</td>
<td>None detected</td>
<td>N.D.</td>
<td>None detected</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

\(^1\) 3β-HSD activities were measured in incubations at 27\(^\circ\)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. N.D., not determined. Values are the means ± standard deviations of triplicate determinations.
HUMAN 3β-HYDROXYSTEROID DEHYDROGENASE

CYP17

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP17</td>
<td>Conversion of pregnenolone to 17α-OH-pregnenolone</td>
</tr>
</tbody>
</table>

Figure 1

Type 2 3β-HSD in adrenal cortex

Type 1 3β-HSD in placenta and peripheral tissues

- Pregnenolone is converted to 17α-OH-pregnenolone by CYP17.
- 17α-OH-pregnenolone is converted to 17β-hydroxyprogrenolone by CYP21.
- 17β-hydroxyprogrenolone is converted to progesterone by CYP21.
- Progesterone is converted to aldosterone by CYP21 and CYP11B2.
- Progesterone is converted to cortisol by CYP21 and CYP11B1.
- Cortisol is converted to 17β-estradiol by CYP19 and 17β-HSD.
- 17β-estradiol is converted to testosterone by CYP19 and 17β-HSD.
- DHEA-S is converted to DHEA by sulfatase.
- DHEA is converted to pregnenolone by sulfotransferase.
Figure 2
Figure 3

Wild-type 1

Wild-type 2

> 42.0 kDa

Y269S  K273Q  Y154F  K158Q  H156Y
Y269S  K273Q  Y154F  K158Q  H156Y  Wild-type 1  Wild-type 2

> 42.0 kDa

Figure 4
Figure 5

Percent maximal $\beta$-HSD activity vs pH
Figure 6
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

James L. Thomas, J. Ian Mason, Stacey Brandt, Byron R. Spencer, Jr. and Wendy Norris

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