Structure/Function Relationships Responsible for Coenzyme Specificity and the Isomerase Activity of Human Type 1 3β-Hydroxysteroid Dehydrogenase/Isomerase*

James L. Thomas†‡§, William L. Duax+++§§, Anthony Addlaggera+++§§, Stacey Brandt‡, Robert R. Fuller†, and Wendy Norris‡

From the †Division of Basic Medical Sciences and ‡Department of Obstetrics-Gynecology, Mercer University School of Medicine, Macon, Georgia 31207, Hauptman-Woodward Medical Research Institute, Buffalo, New York 14203, +++State University of New York, Buffalo, New York 14203, and the ‡‡Institute of Molecular Biology, Howard Hughes Medical Institute and Department of Physics, University of Oregon, Eugene, Oregon 97403

Human type 1 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD/isomerase) catalyzes the two sequential enzyme reactions on a single protein that converts dehydroepiandrosterone or pregnenolone to androstenedione or progesterone, respectively, in placenta, mammary gland, breast tumors, prostate, prostate tumors, and other peripheral tissues. Our earlier studies show that the two enzyme reactions are linked by the coenzyme product, NADH, of the 3β-HSD activity. NADH activates the isomerase activity by inducing a time-dependent conformational change in the enzyme protein. The current study tested the hypothesis that the 3β-HSD and isomerase activities shared a common coenzyme domain. Homology modeling with UDP-galactose-4-epimerase predicts that Asp36 is responsible for the NAD(H) binding and the isomerase reaction. 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 without compromising the essential functions of the adrenal enzyme.

The two-step reaction of 3β-HSD/isomerase using dehydroepiandrosterone (DHEA) as substrate is shown in Fig. 1. 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 (2, 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-androstene-3,17-dione, remains bound during the reaction sequence (2, 9). This model suggests that the 3β-HSD and isomerase domains of the enzyme are linked by a shared coenzyme domain that functions both as the binding site for NAD+ during the 3β-HSD reaction and as the coenzyme domain for the allosteric activation of isomerase. Human type 1 (placental) 3β-HSD/isomerase has a strict preference for NAD+ as the 3β-HSD cofactor and for NADH as the activator of isomerase. Substitution of NADP(H) for NAD(H) abolishes both enzyme activities in the native placental enzyme (2).

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* This work was supported by National Institutes of Health Grants HD20055 (to J. L. T.) and DK26546 (to W. L. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Mercer University School of Medicine, 1550 College St., Macon, GA 31207, Tel.: 478-301-4177; Fax: 478-301-5489; E-mail: Thomas_J@Mercer.edu. This is an Open Access article under the CC BY license. This paper is available online at http://www.jbc.org

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Printed in U.S.A.
The mutant 3β-HS
does not appear to modify the secondary protein structure.

The adjacent Asp257 and Asp258 residues required substitution by (Protylze program, Scientific and Educational Software, State Line, Ind.), and 2D41N mutations in the isomerase region have been correlated with our homology data to conclude that other members of the short chain oxidoreductase family of proteins as well as on our previous affinity radiola-
beled peptide and mutagenesis studies that identified critical amino acids in the putative isomerase domain of the enzyme (13, 14). Characterization of the mutant enzymes with the D36A/K37R substitutions in the coenzyme domain and with the D241N, D257L, D258L, or D241N mutations in the isomerase region has been correlated with our homology data to produce the first three-dimensional model of human 3β-HS
d/iso
erase that elucidates key structure/function relationship.

It has been shown that if the sequence of a protein of uncertain three-dimensional structure has 30% identity with the sequence of a protein for which the three-dimensional structure has been determined by x-ray crystallographic analysis, the unknown protein will have a similar fold. The fold will be similar enough that energy minimization of a model of the unknown protein based upon the crystallographically deter-
mined protein will produce a three-dimensional model useful for interpreting biochemical data, proposing and testing mechan-
isms of action, and designing enzyme inhibitors (15–17).

**EXPERIMENTAL PROCEDURES**

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

**Site-directed Mutagenesis**—Using the Advantage cDNA PCR kit (BD Biosciences) and pGEM-3βHSD1 as a template (14), double-stranded PCR-based mutagenesis was performed with the primers in Table I to create the cDNA encoding the D36A/K37R, D241N, D257L, D258L, and D265N mutant enzymes. The presence of the mutated codon and integ-
rity of the entire mutant 3β-HS cDNA were verified by automated dieoxyxynucleotide 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 pro-
duced no apparent changes in the secondary structure of the protein (Protylze program, Scientific and Educational Software, State Line, PA). The adjacent Aspβ and Aspγ residues required substitution by Leu to avoid predicted changes in secondary structure, whereas the standard Asn substitution (12, 18) for the Aspα and Aspγ residues produces no apparent modification of secondary protein structure.

**Expression and Purification of the Mutant and Wild-type Enzymes**—The mutant 3β-HS cDNA was introduced into baculovirus as de-
scribed previously (14). Recombinant baculovirus was added to 1.5 × 10^6 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-polyacrylamide (12%) gel elec-
rophoresis, probed with our anti-3β-HSD polyclonal antibody, and de-
tected using the ECL Western blotting system with antirabbit, a 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 (2, 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 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 (19).

**Kinetic Studies**—Michaelis-Menten kinetic constants for the 3β-HS substrate were determined for the purified mutant and wild-type en-
zymes in incubations containing dehydroepiandrosterone (2–100 μM) plus NAD+ or NADP+ (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/min (due to NADH production) was used to determine 3β-HS activity. Kinetic constants for the isomerase substrate were determined at 27 °C in incubations of NADH or NADPH (2–50 μM), dehydroepiandrosterone (100 μM), and purified enzyme (0.01 mg) in 0.02 M potassium phosphate buffer, pH 7.4. Isomer-
ase 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) ensured that specific isomerase activity was measured as opposed to nonenzymatic “spontaneous” isomerization (8). In addition, isomerase incubations without added coenzyme (NADH, NADPH) were used to measure any basal (zero-coenzyme) isomerase activity in the mutants, and this basal activity was sub-
tracted as a blank and reported in the kinetic table legends. Changes in absorbance were measured with a Varian (Sugar Land, TX) Cary 219 recording spectrophotometer. The Michaelis-Menten constants (K_m, V_max) were calculated from Lineweaver-Burke (1/S versus 1/V) plots and verified by Hanes-Woolf (S versus SV) plots (20). K_m values (min−1) were calculated from the V_max values (nmol/min/mg) and represent the maximal turnover rate (nmol of product formed/min/mg of enzyme dimer). Kinetic constants for the 3β-HS cofactor were determined for the purified mutant and wild-type enzymes in incubations containing NAD+ or NADP+ (20–100 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 or NADPH (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. Zero-coenzyme blanks were used as described above for the substrate kinetics.

**pH Dependence Studies**—The pH profiles of the isomerase activities of the wild-type control, D241N, D257L, D258L, and D265N mutant enzymes were measured by the isomerase assay in incubations containing 5-androstene-3,17-dione (50 μM), NADH (50 μM), and purified en-

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**TABLE I**

Oligonucleotide primers used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Direction</th>
<th>Nucleotide sequence of primer⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>D36A/K37R</td>
<td>Forward</td>
<td>5’TCTTG GCCAG GCCCTGACACA-3’</td>
</tr>
<tr>
<td>D241N</td>
<td>Reverse</td>
<td>5’GAAAGC CTCTGGC AAACAGCTTA-3’</td>
</tr>
<tr>
<td>D257L</td>
<td>Forward</td>
<td>5’TTCGTT GCCCTCGCTCCCTOA-3’</td>
</tr>
<tr>
<td>D258L</td>
<td>Reverse</td>
<td>5’GGTGTT CTTCGACGCCCTCCAACAGG-3’</td>
</tr>
<tr>
<td>D265N</td>
<td>Reverse</td>
<td>5’TACAGT AGGGCCTCAACACCAGC-3’</td>
</tr>
</tbody>
</table>

⁴The mutated codons are in the bold italic font.
zyme (0.03 mg) in 0.05M sodium phosphate pyrophosphate buffer, pH 6.5–8.0, at 27 °C.

Modeling and Sequence Alignment—Amino acid and nucleotide sequences were retrieved from the Swiss Protein Database (21). Crystallographic coordinates were retrieved from the Protein Data Bank (22). ClustalX was used for sequence alignments (23). The three-dimensional structure of human type 1 3-HSD1 was modeled using the crystal structure (24) of UDP-galactose 4-epimerase from Escherichia coli (Protein Data Bank accession code 1A9Z) as a template using the default settings of the Center for Biological Sequence Analysis web server (25). The modeled images were produced using the Ribbons 2.0 program (26).

RESULTS

Homology Modeling Targets the Coenzyme Domain—Homology analysis has revealed that many members of the short-chain oxidoreductase family of enzymes that utilize NAD/H as the preferred cofactor have an Asp36-Xaa37 sequence in the first turn of the Rossmann-fold (βαβαβαβ), and many members that utilize NADP/H have an Xaa36-Arg37 sequence in those positions (10, 27). Based upon analysis of the alignments, a fingerprint of 35 amino acid residues that are conserved at 70% or better in the entire family have been identified. This fingerprint has been used to predict fold, cofactor preference, aggregation properties, and substrate specificity of subsets of the unknown members of the short-chain oxidoreductase family (27). Human type 1 3-HSD1 is a member of the short-chain oxidoreductase family. As shown in Table II, it has 30% overall sequence identity and 40% of the fingerprint residues that are conserved with E. coli UDP-galactose-4-epimerase, a short-chain oxidoreductase family member for which the x-ray crystal structure determination has been reported (24). Although homology modeling of 3-HSD1 isomerase using an enzyme such as UDP-galactose-4-epimerase may seem surprising, the smaller size of other hydroxysteroid dehydrogenases (e.g. 17β-HSD1, monomeric molecular mass 34 kDa (10)) was a factor in the homology analysis. Both 3-HSD1/isomerase (42-kDa monomer) and UDP-galactose-4-epimerase (39-kDa monomer) are homodimers that have strict NAD/H cofactor specificity (2, 28). In addition, the 3-HSD1 and epimerase catalytic events are closely related in that both use a YXXXK motif at the same position in the primary structure for the oxidation of a hydroxyl group (29). In addition, 3-HSD1/isomerase shares amino acid identity with 10 of the 24 residues in the Rossmann-fold cofactor-binding domain of UDP-galactose-4-epimerase. Analysis of the conserved residues of the fingerprint of UDP-galactose-4-epimerase identifies the Asp36 residue in 3-HSD1 that may be critical to cofactor specificity, and, to test the model, targets the D36A/K37R mutant of 3-HSD1.

Site-directed Mutagenesis, Expression, and Purification of the Mutant Enzymes—The cDNA encoding the D36A/K37R mutant of wild-type 3-HSD1 and the cDNA encoding the D241N, D257L, D258L, and D265N mutants (targeted by affinity radioalkylation (13) and mutagenesis (14)) were produced by double-stranded, PCR-based mutagenesis and inserted into baculovirus. The locations of these targeted residues plus other key amino acids are indicated in the primary structure of the protein (27). Human type 1 3-HSD1 isomerase is a member of the short-chain oxidoreductase family. As shown in Table II, it has 30% overall sequence identity and 40% of the fingerprint residues that are conserved with E. coli UDP-galactose-4-epimerase, a short-chain oxidoreductase family member for which the x-ray crystal structure determination has been reported (24). Although homology modeling of 3-HSD1 isomerase using an enzyme such as UDP-galactose-4-epimerase may seem surprising, the smaller size of other hydroxysteroid dehydrogenases (e.g. 17β-HSD1, monomeric molecular mass 34 kDa (10)) was a factor in the homology analysis. Both 3-HSD1/isomerase (42-kDa monomer) and UDP-galactose-4-epimerase (39-kDa monomer) are homodimers that have strict NAD/H cofactor specificity (2, 28). In addition, the 3-HSD1 and epimerase catalytic events are closely related in that both use a YXXXK motif at the same position in the primary structure for the oxidation of a hydroxyl group (29). In addition, 3-HSD1/isomerase shares amino acid identity with 10 of the 24 residues in the Rossmann-fold cofactor-binding domain of UDP-galactose-4-epimerase. Analysis of the conserved residues of the fingerprint of UDP-galactose-4-epimerase identifies the Asp36 residue in 3-HSD1/isomerase that may be critical to cofactor specificity, and, to test the model, targets the D36A/K37R mutant of 3-HSD1/isomerase.

Kinetic Analyses of the D36A/K37R Mutant—The Michaelis-
Menten kinetic values measured for cofactor utilization by the purified D36A/K37R mutant and wild-type 1-3HSD/3HSD-isomerase are summarized in Table III. In agreement with our previous results obtained for purified human type 1 placental 3HSD/isomerase (2), the wild-type 1 enzyme exhibits 3HSD activity with NAD but not with NADP as the cofactor, and the wild-type 1 isomerase is activated only by NADH with NADPH producing no isomerase activity. In sharp contrast, the D36A/K37R mutant of the wild-type 1 enzyme has no measurable 3HSD activity with NAD as the cofactor and utilizes NADP as the cofactor. In fact, D36A/K37R has a lower Km value for NADP than that measured for utilization of NAD by the wild-type 1 enzyme. Similarly, NADPH activates the D36A/K37R mutant, and NADH specificity of the wild-type 1 isomerase is completely lost.

Analogous shifts in cofactor specificity from NAD(H) to NADP(H) are shown in the substrate kinetic profiles of the 3HSD and isomerase activities of D36A/K37R compared with those of the wild-type 1 enzyme (Table IV). The D36A/K37R mutant exhibits the same 3-fold lower turnover rate (Kcat) compared with the wild-type enzyme for the utilization of both substrate and coenzymes by both the 3HSD and isomerase activities. Unlike wild-type isomerase that has no basal activity in the absence of NADH, the D36A/K37R mutant enzyme exhibits a basal isomerase activity in the absence of coenzyme that is 10% of the NADPH-stimulated activity (Tables III and IV, footnotes c).
Kinetic Analyses of the D241N, D257L, D258L, and D265N Mutants—Based on the known reaction mechanism of the D3-isoform, activity of P. testosteroni (12), Asp may function as the proton acceptor in the D3-isoform activity of human 3β-HSD/isoform. Our affinity labeling (13) and mutagenesis (14) studies identified the Gly256 tryptic peptide as part of the isomerase domain and Tyr258 as a critical residue (possible proton donor) for the isomerase activity (30). The substrate kinetics of the purified D241N, D257L, D258L, and D265N mutants of human type 1 3β-HSD/isoform (Table V) show that the D257L and D258L isomerase activities were 0.8 nmol/min/nmol enzyme at 100 μM), NADH or phosphate buffer, pH 7.4, 27 °C. The substantial decrease in 3β-HSD activity for D241N, D257L, D258L, and D265N suggests that substitution of any Asp residue in this domain disrupts the 3β-HSD conformation of the enzyme.

The cofactor kinetic profiles mirror the substrate kinetics with respect to the lack of activity of the D257L and D258L mutants (Table VI). NADP+ and NADPH were tested as coenzymes for the D241N, D257L, D258L, and D265N mutants, but the 3β-HSD and isomerase activities of these mutants retain a strict preference for NAD+ and NADH, respectively. In addition, the D241N mutant has a basal isomerase activity in the absence of coenzyme that is 10% of the NADH-stimulated Kcat value (Table VI, footnote c). The wild-type 1, D257L, D258L, and D265N isomerases completely lack activity in the absence of coenzyme.

Dependence of the Mutant and Wild-type Isomerase Activities on pH—For a pH-dependent enzyme activity (if the mutant enzyme bearing a substitution for a potentially catalytic residue retains residual pH-dependent activity), the substituted amino acid must not be catalytic, because the residual activity has to be due to a different amino acid in the enzyme. Conversely, a catalytic role for the substituted amino acid is supported by the absence of pH dependence for the residual activity of the mutant enzyme (31). The extremely low residual isomerase activities of D257L and D258L (0.8 nmol/min/nmol of enzyme) exhibit little change over the pH 6.5–8.0 range (Fig. 5), which supports a catalytic role for Asp257 or Asp258 in the isomerization reaction. Similar to native human type 1 3β-HSD in placenta (2), the isomerase activities of the wild-type 1, D241N, and D265N enzymes vary with pH and exhibit an optimum value at pH 7.5 (Fig. 5).

**DISCUSSION**

Human type 1 3β-HSD/isoform is expressed in a tissue-specific manner in peripheral tissues (placenta, mammary gland, prostate), whereas human type 2 3β-HSD/isoform is expressed in endocrine glands (adrenals, gonads) (1, 5, 6, 7). In our recent structure/function study (32), the catalytic residues for human type 1 3β-HSD activity were identified as Tyr154 and Lys158 (Fig. 2). The presence of His156 in human type 1 3β-HSD versus Tyr156 in type 2 3β-HSD was also shown to be responsible for the 14–17-fold greater affinity of the type 1 enzyme for 3β-HSD substrate steroids and inhibitors compared with the type 2 enzyme (32). In this report, we have further characterized human type 1 3β-HSD/isoform by homology modeling and mutagenesis to determine the structure/function relationships responsible for coenzyme specificity, localized the coenzyme domain for 3β-HSD/isoform, identified critical residues for the isomerase activity, and refined our model of the sequential 3β-HSD and isomerase reaction mechanism. A more complete understanding of the structure/function of human type 1 3β-HSD/isoform may lead to the selective inhibition of steroid biosynthesis in human placenta and breast tumors in a clinical setting. The onset of labor in human pregnancy could be delayed by selectively inhibiting the activity of placental type 1 3β-HSD near term to decrease estradiol production from fetal DHEA (4) without interfering with cortisol or aldosterone production by type 2 3β-HSD in the maternal adrenal gland. In post-menopausal women with a breast tumor, the high levels of circulating DHEA (3) could be blocked from conversion to estradiol by the selective inhibition of type 1 3β-HSD in the tumor and the surrounding mammary gland tissue (5).

**TABLE IV**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cofactor</th>
<th>Activity</th>
<th>Wild-type 1</th>
<th>D36A/K37R</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEA</td>
<td>NAD+</td>
<td>3β-HSDa</td>
<td>K&lt;sub&gt;m&lt;/sub&gt; 3.7</td>
<td>K&lt;sub&gt;cat&lt;/sub&gt; 3.3</td>
</tr>
<tr>
<td>DHEA</td>
<td>NADP+</td>
<td>3β-HSDa</td>
<td>ND</td>
<td>No activity</td>
</tr>
<tr>
<td>5-AND</td>
<td>NADH</td>
<td>Isomeraseb</td>
<td>26.8</td>
<td>33.4</td>
</tr>
<tr>
<td>5-AND</td>
<td>NADPH</td>
<td>Isomeraseb</td>
<td>ND</td>
<td>No activity</td>
</tr>
</tbody>
</table>

<sup>a</sup> Kinetic constants for the 3β-HSD substrate were determined in incubations containing DHEA (2–100 μM), NAD+ (0.1 mM), NADP+ (0.1 mM), and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4, 27 °C. Each K<sub>m</sub> and K<sub>cat</sub> value represents the mean of triplicate measurements with a S.D. ≤ 6%, ND, not determined.

<sup>b</sup> Kinetic constants for the isomerase substrate were determined in NADPH (0.05 mM), and purified enzyme (0.01 mg) in 0.02 M potassium phosphate, pH 7.4, 27 °C.

<sup>c</sup> For D36A/K37R, there was a measurable basal isomerase activity without stimulation by coenzyme at each substrate concentration that was subtracted as a blank.

**TABLE V**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>3β-HSD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Isomerase&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Purified enzyme</td>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>K&lt;sub&gt;cat&lt;/sub&gt;</td>
</tr>
<tr>
<td>Wild-type 1</td>
<td>3.7</td>
<td>3.3</td>
</tr>
<tr>
<td>D241N</td>
<td>11.8</td>
<td>1.3</td>
</tr>
<tr>
<td>D257L</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D258L</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D265N</td>
<td>10.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Kinetic constants for the 3β-HSD substrate were determined in incubations containing DHEA (2–100 μM), NAD+ (0.1 mM), and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4, 27 °C. D257L and D258L 3β-HSD activities were 0.1 nmol/min/nmol enzyme at 100 μM) DHEA. Each K<sub>m</sub> and K<sub>cat</sub> value represents the mean of triplicate measurements with a S.D. ≤ 4%, ND, not determined.

<sup>b</sup> Kinetic constants for the isomerase substrate were determined in incubations of 5-androstene-3,17-dione (15–100 μM), and purified enzyme (0.01 mg) in 0.02 M potassium phosphate, pH 7.4, 27 °C.
TABLE VI
Cofactor kinetics for the 3β-HSD and isomerase activities of the purified D241N, D257L, D258L, D265N, and wild-type enzymes

<table>
<thead>
<tr>
<th>Purified enzyme</th>
<th>3β-HSD NAD (^+)</th>
<th>3β-HSD NAD (^+)</th>
<th>Isomerase NADPH</th>
<th>Isomerase NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) ((\mu)M)</td>
<td>(K_m) (s(^-1))</td>
<td>(K_m) ((\mu)M)</td>
<td>(K_m) (s(^-1))</td>
</tr>
<tr>
<td>Wild-type 1</td>
<td>34.1</td>
<td>3.9</td>
<td>2.4</td>
<td>34.9</td>
</tr>
<tr>
<td>D241N</td>
<td>223</td>
<td>1.0</td>
<td>3.3</td>
<td>26.5</td>
</tr>
<tr>
<td>D257L</td>
<td>ND</td>
<td>ND(^a)</td>
<td>ND</td>
<td>ND(^a)</td>
</tr>
<tr>
<td>D258L</td>
<td>ND</td>
<td>ND(^a)</td>
<td>ND</td>
<td>ND(^a)</td>
</tr>
<tr>
<td>D265N</td>
<td>33.7</td>
<td>0.4</td>
<td>4.4</td>
<td>14.5</td>
</tr>
</tbody>
</table>

\(^a\) Kinetic constants for the 3β-HSD cofactor were determined in incubations containing NAD\(^+\) (20–100 \(\mu\)M), DHEA (100 \(\mu\)M), and purified enzyme (0.03 mg) in 0.02 \(\mu\)L potassium phosphate buffer, pH 7.4, 27 °C. D257L and D258L 3β-HSD activities were 0.1 nmol/min/nmol enzyme at 100 \(\mu\)M NAD\(^+\). Each \(K_m\) and \(K_m\) value represents the mean of triplicate measurements with a S.D. ≤ 5%. ND, not determined.

The question of whether the 3β-HSD and isomerase activities share the same coenzyme domain on the enzyme protein is central to our hypothesis for the mechanism of the sequential enzyme reactions. According to our model, NADH formed by the 3β-HSD reaction induces the enzyme protein to assume the active isomerase conformation so that a single coenzyme domain serves both activities. Our previous studies with the Y253F mutant (13, 14) and on the known reaction mechanism of the isomerase activity of 3β-HSD/isomerase and the role of Asp\(^{36}\) is validated. In addition, because each of the two activities is equally affected by the D36A/K37R mutation (3-fold decrease in \(K_m\) with NADP(H)), 3β-HSD and isomerase appear to share the same coenzyme domain, and our hypothesis of sequential activities linked by an NADH-induced conformational change is supported.

K37R mutant completely loses both 3β-HSD and isomerase activities in the presence of NAD(H), and because both are active with NADP(H), our homology model and the role of Asp\(^{36}\) is validated. In addition, because each of the two activities is equally affected by the D36A/K37R mutation (3-fold decrease in \(K_m\) with NADP(H)), 3β-HSD and isomerase appear to share the same coenzyme domain, and our hypothesis of sequential activities linked by an NADH-induced conformational change is supported.

Kinetic analyses of the D241N, D257L, D258L, and D265N mutants support our hypothesis that this region is part of the isomerase substrate domain. Asp\(^{357}\) or Asp\(^{258}\) may function as a catalytic residue for isomerase (according to the kinetic data) and for the lack of pH dependence of the residual isomerase activity of the D257L and D258L mutants. Based on our previous studies with the Y253F mutant (13, 14) and on the known reaction mechanism of the isomerase activity of 3β-HSD/isomerase and the role of Asp\(^{36}\) is validated. In addition, because each of the two activities is equally affected by the D36A/K37R mutation (3-fold decrease in \(K_m\) with NADP(H)), 3β-HSD and isomerase appear to share the same coenzyme domain, and our hypothesis of sequential activities linked by an NADH-induced conformational change is supported.

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shift cofactor preference from NAD(H) to NADP(H) as described above. Because UDP-galactose-4-epimerase utilizes Tyr146 and Lys155 for epimerase catalysis (29) just as the Tyr154 and Lys158 residues are used in β-HSD catalysis (32), the β-HSD substrate steroid (DHEA) could also be added to the model. In the ribbon structure (Fig. 7), the Asp256 residue hydrogen-bonds with the '2', '3'-hydroxy groups of NAD+, the nicotinamide group of NAD+, is positioned correctly with the β-hydroxyl group of DHEA as well as with the catalytic Tyr154 and Lys158 residues, and Asp241 bridges the coenzyme domain with the isomerase domain (Asp256 ‘‘Tyr253’’). The distance between the isomerase and coenzyme domain (Rossman-fold) is exaggerated somewhat in the model because UDP-galactose is a larger molecule than the steroid substrate.

Because our hypothesis for the sequential β-HSD/isomerase activity centers on an NADH-induced conformational change that activates isomerase, the basal isomerase activities of the purified wild-type 1, D36A/K37R, D241N, D257L, D258L, and D265N enzymes without stimulation by NADH were measured. Only the D36A/K37R and D241N mutants exhibited isomerase activity in the absence of coenzyme (both 10% of coenzyme-activated activity). This finding and the positions of these residues in the ribbon model suggest a function for Asp241 in maintaining the β-HSD conformation by electrostatic repulsion of Asp256 plus other negatively charged residues in the Rossmann-fold domain and possibly by electrostatic attraction to the positively charged nicotinamide group of NAD+.

The ribbon structure in Fig. 7 shows the enzyme in the β-HSD conformation. After NADH is produced from the β-HSD reaction, we hypothesize that the Tyr253 and Asp256 (not shown) residues are brought by the well documented conformational change (9) of the enzyme into contact with the β-proton of the bound intermediate steroid, 3-androsten-3,17-dione, to catalyze isomerization (12, 18) to 4-androsten-3,17-dione (androstenedione). The ribbon diagram represents the first three-dimensional model of β-HSD isomerase based on homology analysis and tested by mutagenesis. Efforts are underway using our genetically engineered soluble form of human type 1 β-HSD/isomerase (33) to grow enzyme crystals that will ultimately test the model using x-ray diffraction.

REFERENCES


