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

Received for publication, May 7, 2003, and in revised form, June 16, 2003
Published, JBC Papers in Press, June 27, 2003, DOI 10.1074/jbc.M304752200

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, and it characterized key amino acids that participated in coenzyme binding and the isomerase reaction. Homology modeling with UDP-galactose-4-epimerase predicts that Asp361 is responsible for the NAD(H) specificity of human 3β-HSD/isomerase and identifies the Rossman-fold coenzyme domain at the amino terminus. The D36A/K37R mutant in the potential coenzyme domain and the D241N, D257L, D258L, and D265N mutants in the potential isomerase domain (previously identified by affinity labeling) were created, expressed, and purified. The D36A/K37R mutant shifts the cofactor preference of both 3β-HSD and isomerase from NAD(H) to NADP(H), which shows that the two activities utilize a common coenzyme domain. The D257L and D258L mutations eliminate isomerase activity, whereas the D241N and D265N mutants have nearly full isomerase activity. Kinetic analyses and pH dependence studies showed that either Asp257 or Asp258 plays a catalytic role in the isomerization reaction. These observations further characterize the structure/function relationships of human 3β-HSD/isomerase and bring us closer to the goal of selectively inhibiting the type 1 enzyme in placenta (to control the timing of labor) or in hormone-sensitive breast tumors (to slow their growth).

The type 1 placenta, skin, mammary gland, prostate, endometrium) and type 2 (gonads, adrenals) isoforms of 3β-hydroxy-Δ5-steroid dehydrogenase (EC 1.1.1.145)/β-oxosteroid Δ5,Δ4-isomerase (EC 5.3.3.1) (3β-HSD1/isomerase) are encoded by two distinct genes, which are expressed in a tissue-specific pattern in humans (1). In human placenta, type 1 3β-HSD/isomerase catalyzes the conversion of 3β-hdroxy-5-ene steroids (dehydroepiandrosterone, pregnenolone) to 3-oxo-4-ene steroids (androstenedione, progesterone) on a single, dimeric protein containing both enzyme activities (2). Androstenedione is converted by placental aromatase and 17β-hydroxysteroid dehydrogenase (17β-HSD) to estradiol, which participates in the cascade of events that initiates labor in humans (3, 4). In addition to placenta and other human peripheral tissues, the type 1 enzyme is selectively expressed in breast tumors (5), prostate tumors (6), and choriocarcinomas (7), where it catalyzes the first step in the conversion of circulating dehydroepiandrosterone to estradiol or testosterone to promote tumor growth. In human adrenals, type 2 3β-HSD/isomerase is a key enzyme required for the production of cortisol and aldosterone (3). 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).
The importance of Asp and Arg residues for the NADP(H) specificity of human type 1 (10) and type 3 (11) 17β-HSD have been demonstrated. In addition, Asp functions as the proton acceptor in the Δ 3-isomerase activity of Pseudomonas testosteroni (12). The targeting of Asp residues for our current study is based on our homology modeling of human 3β-HSD/isomerase with other members of the short chain oxidoreductase family of proteins as well as on our previous affinity radiolabeled 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-HSD/isomerase that elucidates key structure/function relationships.

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 determined protein will produce a three-dimensional model useful for interpreting biochemical data, proposing and testing mechanisms 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 detergents (Kao Corp., Tokyo, Japan) instead of the discontinued Emulgen 913 detergent (Rhodia, Inc., Cranbury, NJ) of the continued 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-HSD substrate were determined for the purified mutant and wild-type enzymes in incubations containing dehydroepiandrosterone (20–100 μM) plus NADP or NADPH (0.1 mM), and purified enzyme (0.05 mM) at 27 °C in 0.02 M potassium phosphate buffer, pH 7.4. The slope of the initial linear increase in absorbance at 340 nm/min (due to NADPH 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 or NADPH (0.05 mM), and purified enzyme (0.03 mg) at 27 °C and 5-isomerase activity. Kinetic constants for the isomerase activity in the mutants, and this basal activity was subtracted 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 (Km, Vmax) were calculated from Lineweaver-Burke (Sversus 1/V) plots and verified by Hanes-Woolf (Sversus SV) plots (20). Km values (min−1) were calculated from the Vmax values (nmol/min/mg) and represent the maximal turnover rate (nmol of product formed/min/mmol of enzyme dimer).

Kinetic constants for the 3β-HSD cofactor were determined for the purified mutant and wild-type enzymes in incubations containing NADP or NADPH (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-

**Fig. 1.** 3β-HSD/Isomerase catalyzes two sequential reactions on a single enzyme protein. The human type 1 3β-HSD and isomerase activities are represented using DHEA as substrate.

![Diagram of 3β-HSD/Isomerase enzyme catalyzing two sequential reactions](image-url)
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-HSD/isomerase 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 as the preferred cofactor have an Asp36-Xaa37 sequence in the first turn of the Rossmann-fold, and many members that utilize NADP 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-HSD/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-HSD/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-HSD/isomerase (42-kDa monomer) and UDP-galactose-4-epimerase (39-kDa monomer) are homodimers that have strict NAD cofactor specificity (2, 28). In addition, the 3-HSD 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-HSD/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-HSD/isomerase that may be critical to cofactor specificity and, to test the model, targets the D36A/K37R mutant of 3-HSD/isomerase.

Site-directed Mutagenesis, Expression, and Purification of the Mutant Enzymes—The cDNA encoding the D36A/K37R mutant of wild-type 1 3-HSD/isomerase 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 (Fig. 2). As shown by the immunoblots in Fig. 3, the baculovirus system successfully expressed the mutant enzyme proteins in Sf9 cells. Each expressed enzyme was highly purified according to SDS-PAGE (Fig. 4) using our published method (2, 8).

Kinetic Analyses of the D36A/K37R Mutant—The Michaelis-Menten kinetic parameters for the wild-type and mutant enzymes are shown in Table III. As shown, both wild-type and mutant enzymes have a comparable affinity for NADH, with a 14-fold higher affinity for NADPH. The D36A/K37R mutant shows a 16-fold reduced apparent Kₘ for NADH, and a 14-fold increased apparent Kₘ for NADPH. These results agree with the predictions of the homology model, which identifies the Asp36 residue (identical in both NAD and NADP) as critical to cofactor specificity.

**Table II**

Comparison of the fingerprint residues in UDP-galactose 4-epimerase (1A9Z) and human 3-HSD1

| 1A9Z | % ID<a> | Notes<b> | 3-HSD<sup>c</sup> | 1A9Z | % ID<a> | Notes<b> | 3-HSD<sup>c</sup> | 1A9Z | % ID<a> | Notes<b> | 3-HSD<sup>c</sup> |
|------|--------|---------|-----------------|------|--------|---------|-----------------|------|--------|---------|-----------------|
| L    | 16     | 1.6     | 3.3             | V    | 28     | 1.5     | 6.6             | I    | 22     | 2.4     | 3.3             |
| V    | 16     | 1.6     | 3.3             | F    | 28     | 1.5     | 6.6             | Y    | 22     | 2.4     | 3.3             |
| T    | 16     | 1.6     | 3.3             | V    | 28     | 1.5     | 6.6             | I    | 22     | 2.4     | 3.3             |
| G    | 16     | 1.6     | 3.3             | G    | 28     | 1.5     | 6.6             | I    | 22     | 2.4     | 3.3             |
| A    | 16     | 1.6     | 3.3             | S    | 28     | 1.5     | 6.6             | S    | 22     | 2.4     | 3.3             |
| A    | 16     | 1.6     | 3.3             | S    | 28     | 1.5     | 6.6             | S    | 22     | 2.4     | 3.3             |
| A    | 16     | 1.6     | 3.3             | S    | 28     | 1.5     | 6.6             | S    | 22     | 2.4     | 3.3             |

*a* Percent conserved identity (% ID) in 124 proteins aligned with 1A9Z by the ClustalX program.

*b* Notes: 1, NAD contacts; 2, substrate contacts; 3, residues conserved between 1A9Z and 3-HSD1; 4, catalytic residues in 1A9Z.

*c* Identity of the 3-HSD residue (boldface) that aligns with the fingerprint residue of 1A9Z (identical; superscript s = similar).

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Human 3β-Hydroxysteroid Dehydrogenase 35485
Menten kinetic values measured for cofactor utilization by the purified D36A/K37R mutant and wild-type 1 3\beta-HSD/isomerase are summarized in Table III. In agreement with our previous results obtained for purified human type 1 placental 3\beta-HSD/isomerase (2), the wild-type 1 enzyme exhibits 3\beta-HSD 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 3\beta-HSD activity with NAD\(^+\) as the cofactor and now utilizes NADP\(^+\) as the cofactor. In fact, D36A/K37R has a lower \(K_m\) 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 3\beta-HSD 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 (\(K_{cat}\)) compared with the wild-type enzyme for the utilization of both substrate and coenzymes by both the 3\beta-HSD 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).

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**Table III**

| Cofactor Activity          | Wild-type 1 | D36A/K37R |
|---------------------------|-------------|-----------|
|                           | \(K_m\) \(\mu\)M | \(K_{cat}\) \(min^{-1}\) | \(K_m\) \(\mu\)M | \(K_{cat}\) \(min^{-1}\) |
| NAD\(^+\) 3\beta-HSD      | 34.1        | 3.9       | ND            | No activity |
| NADP\(^+\) 3\beta-HSD     | ND          | No activity | 20.6        | 1.3         |
| NADH Isomerase\(^b\)       | 2.4        | 34.9      | ND            | No activity |
| NADPH Isomerase            | ND          | No activity | 4.3         | 11.3        |

\(^a\) Kinetic constants for the 3\beta-HSD cofactor were determined in incubations containing NAD\(^+\) (20–100 \(\mu\)M), dehydroepiandrosterone (100 \(\mu\)M), and purified enzyme (0.03 mg) in 0.02M potassium phosphate, pH 7.4, 27 °C. Each \(K_m\) and \(K_{cat}\) value represents the mean of triplicate measurements with a S.D. \(\leq 6\%\). ND, not determined.

\(^b\) Kinetic constants for the isomerase cofactor were determined in incubations of NADH (2–50 \(\mu\)M), 5-androstene-3,17-dione (100 \(\mu\)M), and purified enzyme (0.01 mg) in 0.02 M potassium phosphate buffer, pH 7.4, 27 °C.

\(^c\) For D36A/K37R, there was a basal isomerase activity (1.0 nmol/min/nmol enzyme) without stimulation by coenzyme that was subtracted as a blank.
Kinetic Analyses of the D241N, D257L, D258L, and D265N Mutants—Based on the known reaction mechanism of the 3β-HSD/isomerase activity of P. testosteroni (12), Asp may function as the proton acceptor in the Δ5-isomerase activity of human 3β-HSD/isomerase. Our affinity labeling (13) and mutagenesis (14) studies identified the Gly250 tryptic peptide as part of the isomerase domain and Tyr253 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/isomerase (Table VI) show that the D257L and D258L mutations almost abolish the isomerase activity. In contrast, the D241N and D265N mutations have little effect on the $K_m$ and $K_{cat}$ values for the isomerase substrate compared with those of the wild-type 1 activity. 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). NADPH 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 NADH and NADPH, respectively. In addition, the D241N mutant has a basal isomerase activity in the absence of coenzyme that is 10% of the NADH-stimulated $K_{cat}$ 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/mmol 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/isomerase is expressed in a tissue-specific manner in peripheral tissues (placenta, mammary gland, prostate), whereas human type 2 3β-HSD/isomerase 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 Tyr156 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/isomerase by homology modeling and mutagenesis to determine the structure/function relationships responsible for coenzyme specificity, localized the coenzyme domain for 3β-HSD/isomerase, 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/isomerase 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 postmenopausal women with breast tumors, 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).
Kinetic constants for the 3β-HSD cofactor were determined in incubations containing NAD⁺ (20–100 μM), DHEA (100 μM), 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 NAD⁺. Each Kₘ and K₅₀ value represents the mean of triplicate measurements with a S.D. ≤ 5%. ND, not determined.

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, 27 °C. D257L and D258L isomerase activities were 0.8 nmol/min/nmol enzyme at 50 μM NADH.

For D241N, there was a measurable basal isomerase activity (2.7 nmol/min/nmol enzyme) in the absence of stimulation by coenzyme that was subtracted as a blank.

### Table VI

| Purified enzyme | 3β-HSD NAD⁺ | Isomerase NAD⁺ |
|----------------|-------------|---------------|
|                | Kₘ  | K₅₀ | Kₘ  | K₅₀ |
| Wild-type 1    | 34.1| 3.9 | 2.4 | 34.9 |
| D241N          | 22.3| 1.0 | 3.3 | 26.5 |
| D257L          | ND  | ND  | ND  | ND  |
| D258L          | ND  | ND  | ND  | ND  |
| D265N          | 33.7| 0.4 | 4.4 | 14.5 |

* Kinetic constants for the 3β-HSD cofactor were determined in incubations containing NAD⁺ (20–100 μM), DHEA (100 μM), and purified enzyme (0.03 mg) in 0.02 M potassium phosphate buffer, pH 7.4, 27 °C. D257L and D258L 3β-HSD activities were 0.1 nmol/min/nmol enzyme at 100 μM NAD⁺. Each Kₘ and K₅₀ 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 have supported this model by reporting physical evidence for the NADH-induced time-dependent conformational change (9). In our current study, homology modeling has identified the Rossmann-fold coenzyme domain in 3β-HSD/isomerase and targeted Asp³⁶ as the amino acid that may be responsible for the strict NAD⁺ specificity of the enzyme. This specificity may be because of hydrogen bonds between the carboxylate R-group of Asp and the 2',3'-hydroxyl groups of the adenosyl ribose of NAD⁺ (Fig. 6A). The D36A/K37R mutation removes Asp and introduces an Arg residue with guanidinium group nitrogens that hydrogen-bond and neutralize the 2'-phosphate group of NAD⁺ (Fig. 6B). This 2'-phosphate group is repelled by Asp³⁶ (10) and appears to be the reason for the strict preference of human 3β-HSD/ isomerase for NAD(H). Because the D36A/K37R mutant completely loses both 3β-HSD and isomerase activities in the presence of NAD(H), and because both are active with NAPD(H), our homology model and the role of Asp³⁶ is validated. In addition, because each of the two activities is equally affected by the D36A/K37R mutation (3-fold decrease in K₅₀ 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. Asp²⁴⁵ or Asp²⁵⁸ 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 P. testosteroni (12), Tyr²⁵⁵ may serve as the proton donor, and Asp²⁵⁷ or Asp²⁵⁸ may function as the proton acceptor for the isomerase activity of human type 1 3β-HSD/isomerase. The almost complete loss of isomerase activity by the D257L and D258L mutants appears to be a specific result of the substitutions rather than a general consequence of the lack of hydrogen bond donor groups in the active site.

**Fig. 5.** pH profiles of the isomerase activities of the wild-type and mutant enzymes. The purified wild-type 1 ( ), D241N ( ), D257L ( ), D258L ( ), or D265N mutant ( ) enzymes (0.03 mg) were incubated with 5-androstene-3,17-dione (100 μM) and NADH (50 μM) in 0.05 M sodium phosphate pyrophosphate buffer, pH 6.5–8.0, at 27 °C. Each point represents the mean of triplicate determinations. Error bars represent standard deviations.

**Fig. 6.** Interactions of the Asp³⁶ and Arg³⁷ residues with NAD and NADP. A, the carboxylate R-group of Asp⁳⁶ in wild-type 1 3β-HSD/isomerase hydrogen bonds to the 2',3'-hydroxyl groups of the adenosyl ribose group of NAD. B, the guanidinium R-group of Arg³⁷ in the D36A/K37R mutant enzyme hydrogen-bonds to the 2'-phosphate group of NADP. For both panels A and B, the oxygen atoms are orange, nitrogen atoms are purple, carbon atoms are gray, and phosphorus atoms are lavender.
The primary sequences of \( \beta \)-HSD based on homology modeling with key amino acids identified. The primary sequences of \( \beta \)-HSD/isomerase (green) and UDP-galactose-4-epimerase (yellow) were aligned as described under “Experimental Procedures.” The NAD and DHEA structures are included. The key Asp\(^{36} \) residue is shown hydrogen-bonding (gray dotted lines) to the 2',3'-hydroxyl groups of the adenosyl ribose group of NAD. The catalytic Tyr\(^{154} \) and Lys\(^{158} \) residues for human type 1 \( \beta \)-HSD activity \((32)\), the catalytic Tyr\(^{257} \) (30) and Asp\(^{257} \) residues for isomerase activity, and the Asp\(^{241} \) residue that bridges the upper isomerase domain with the lower coenzyme domain are also shown. This ribbon model represents the \( \beta \)-HSD/isomerase structure in the 3\( \beta \)-HSD conformation. The oxygen atoms are orange, nitrogen atoms are purple, carbon atoms are gray, and phosphorus atoms are lavender.

than a non-specific conformational event because of the different effects of the four mutations on the isomerase activity. If a non-specific conformational change because of the substitution of any Asp residue in this region may disrupt the ability of the enzyme to maintain the active \( \beta \)-HSD tertiary conformation.

The results of this mutagenesis study suggest key roles for Asp\(^{36} \) in the cofactor preference of both activities and for Asp\(^{257} \) or Asp\(^{258} \) in isomerase catalysis. Based on the homology data obtained for human type 1 \( \beta \)-HSD/isomerase and UDP-galactose-4-epimerase, a three-dimensional ribbon model has been constructed (Fig. 7). The highly homologous Rossmann-coenzyme domains of the two proteins allow the localization of coenzyme (NAD\(^{+} \)) in the model. The validity of this model has been strongly supported by using the D36A/K37R mutant to shift cofactor preference from NAD(H) to NADPH as described above. Because UDP-galactose-4-epimerase utilizes Tyr\(^{140} \) and Lys\(^{155} \) for epimerase catalysis \((29)\) just as the Tyr\(^{154} \) and Lys\(^{158} \) residues are used in \( \beta \)-HSD catalysis \((32)\), the \( \beta \)-HSD substrate steroid (DHEA) could also be added to the model. In the ribbon structure (Fig. 7), the Asp\(^{36} \) residue hydrogen-bonds with the 2',3'-hydroxy groups of NAD\(^{+} \), the nicotinamide group of NAD\(^{+} \) is positioned correctly with the 3\( \beta \)-HSD substrate steroid (DHEA) could also be added to the model. In the ribbon structure (Fig. 7), the Asp\(^{36} \) residue hydrogen-bonds with the 2',3'-hydroxy groups of NAD\(^{+} \), the nicotinamide group of NAD\(^{+} \) is positioned correctly with the 3\( \beta \)-HSD substrate steroid (DHEA) could also be added to the model. The distance between the isomerase and coenzyme domain (Rossmann-fold) is exaggerated somewhat in the model because UDP-galactose is a larger molecule than the steroid substrate.

Because our hypothesis for the sequential \( \beta \)-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 Asp\(^{241} \) in maintaining the \( \beta \)-HSD conformation by electrostatic repulsion of Asp\(^{36} \) 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 \( \beta \)-HSD conformation. After NADH is produced from the 3\( \beta \)-HSD reaction, we hypothesize that the Tyr\(^{257} \) and Asp\(^{257} \) (not shown) residues are brought by the well documented conformational change \((9)\) of the enzyme into contact with the 4\( \beta \)-proton of the bound intermediate steroid, 5-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 \( \beta \)-HSD isomerase based on homology analysis and tested by mutagenesis. Efforts are underway using our genetically engineered soluble form of human type 1 \( \beta \)-HSD/isomerase \((33)\) to grow enzyme crystals that will ultimately test the model using x-ray diffraction.

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