Pergamon

J. Steroid Biochem. Molec. Biol. Vol. 66, No. 5–6, pp. 327–334, 1998
© 1998 Elsevier Science Ltd. All rights reserved
Printed in Great Britain
0960-0760/98 $19.00 + 0.00

PII: S0960-0760(98)00058-2

Site-directed Mutagenesis Identifies Amino Acid Residues Associated with the Dehydrogenase and Isomerase Activities of Human Type I (Placental) 3β-Hydroxysteroid Dehydrogenase/Isomerase

James L. Thomas,[1]* Brett W. Evans,[1] Gustavo Blanco,[2] Robert W. Mercer,[2] J. Ian Mason,[3] Stuart Adler,[1,2] William E. Nash,[1] Keith E. Isenberg[4] and Ronald C. Strickler[1]

[1]Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.; [2]Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.; [3]Department of Clinical Biochemistry, Royal Infirmary of Edinburgh NHS Trust, Edinburgh, U.K. and [4]Department of Psychiatry, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

3β-Hydroxysteroid dehydrogenase/steroid $\Delta^{5\rightarrow4}$-isomerase (3β-HSD/isomerase) was expressed by baculovirus in *Spodoptera fungiperda* (Sf9) insect cells from cDNA sequences encoding human wild-type I (placental) and the human type I mutants – H261R, Y253F and Y253,254F. Western blots of SDS-polyacrylamide gels showed that the baculovirus-infected Sf9 cells expressed the immunoreactive wild-type, H261R, Y253F or Y253,254F protein that co-migrated with purified placental 3β-HSD/isomerase (monomeric M_r=42,000 Da). The wild-type, H261R and Y253F enzymes were each purified as a single, homogeneous protein from a suspension of the Sf9 cells (5.0 l). In kinetic studies with purified enzyme, the H261R mutant enzyme had no 3β-HSD activity, whereas the K_m and V_{max} values of the isomerase substrate were similar to the values obtained with the wild-type and native enzymes. The V_{max} (88 nmol/min/mg) for the conversion of 5-androstene-3,17-dione to androstenedione by the Y253F isomerase activity was 7.0-fold less than the mean V_{max} (620 nmol/min/mg) measured for the isomerase activity of the wild-type and native placental enzymes. In microsomal preparations, isomerase activity was completely abolished in the Y253,254F mutant enzyme, but Y253,254F had 45% of the 3β-HSD activity of the wild-type enzyme. In contrast, the purified Y253F, wild-type and native enzymes had similar V_{max} values for substrate oxidation by the 3β-HSD activity. The 3β-HSD activities of the Y253F, Y253,254F and wild-type enzymes reduced NAD^+ with similar kinetic values. Although NADH activated the isomerase activities of the H261R and wild-type enzymes with similar kinetics, the activation of the isomerase activity of H261R by NAD^+ was dramatically decreased. Based on these kinetic measurements, His^{261} appears to be a critical amino acid residue for the 3β-HSD activity, and Tyr^{253} or Tyr^{254} participates in the isomerase activity of human type I (placental) enzyme. © 1998 Elsevier Science Ltd. All rights reserved.

J. Steroid Biochem. Molec. Biol., Vol. 66, No. 5–6, pp. 327–334, 1998

Supported by NIH Grant HD20055 (JLT).
*Correspondence to J. L. Thomas. Tel. 314-362 3630; Fax: 314-362 3328; e-mail: thomasj@medicine.wustl.edu
Present address: Department of Obstetrics and Gynecology, Henry Ford Health System, Area K-9, 2799 West Grand Boulevard, Detroit, MI 48202, U.S.A.
Received 13 Jan. 1998; accepted 10 Mar. 1998.
Abbreviations: 3β-HSD/isomerase, 3β-hydroxysteroid dehydrogenase/steroid $\Delta^{5\rightarrow4}$-isomerase; secosteroid, 5,10-secoestr-4-yne-3,10,17-trione; 2α-BAP, 2α-bromoacetoxyprogesterone; pregnenolone, 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; androstenedione, 4-androstene-3,17-dione.

INTRODUCTION

Human placental 3β-hydroxysteroid dehydrogenase (3β-HSD, EC 1.1.1.145) and steroid $\Delta^{5\rightarrow4}$-isomerase (EC 5.3.3.1) catalyze the sequential conversion of 3β-hydroxy-5-ene steroids (pregnenolone, dehydroepiandrosterone) to 3-oxo-4-ene steroids (progesterone, androstenedione) on a single, dimeric protein containing both enzyme activities [1]. The bifunc-

tional enzyme catalyzes a key biosynthetic step that is required for each of the pathways that produce active steroid hormones. In the human, the type I enzyme (placenta, skin, mammary gland) and the type II enzyme (gonads, adrenals) are expressed in a tissue-specific pattern, are encoded by two distinct genes, and have 93% homology in the amino acid sequences [2]. The 3β-HSD and isomerase activities co-purify as the single type I protein from human term placenta [1, 3].

Tryptic peptides associated with both catalytic activities have been localized using affinity radiolabeling steroids. One tryptic peptide, ^{250}GQFYYISDDTP*H*QSYDNLNYTLSK^{273}, was modified at His^{261} by both the 3β-HSD-site-directed alkylator, 2α-bromoacetoxyprogesterone [4, 5] and by the isomerase-site-directed alkylator, 5,10-secoestr-4-yne-3,10,17-trione [6]. Additional studies using stopped-flow fluorescent spectroscopy [7] supported the following model for the sequential reaction mechanism: NADH, the coenzyme product of the rate-limiting 3β-HSD reaction, induces a conformational change around the bound 3-oxo-5-ene-steroid (the 3β-HSD product and isomerase substrate) to activate isomerase in the single, bifunctional enzyme protein. Thus, 3β-HSD/isomerase offers a unique enzyme system to study using site-directed mutagenesis because an appropriately targeted point mutation may modify only one of the two activities. In the current study, we have used site-directed mutagenesis to produce three mutated forms of human type I (placental) 3β-HSD/isomerase (H261R, Y253F and Y253,254F) and compared the kinetics of the purified mutant, wild-type, and native placental enzymes to investigate the roles of the targeted amino acid residues in the 3β-HSD and isomerase reaction mechanisms.

MATERIALS AND METHODS

Materials

Pregnenolone, dehydroepiandrosterone and pyridine nucleotides were purchased from Sigma (St. Louis, MO); 5-androstene-3,17-dione and 5-pregnene-3,20-dione from Steraloids (Wilton, NH); reagent grade salts, chemicals and analytical grade solvents from Fisher (Pittsburg, PA). Glass distilled, deionized water was used for all aqueous solutions.

Site-directed mutagenesis

The 1,555 base pair (bp) cDNA that encodes human type I (placental) 3β-HSD/isomerase was cut from the host plasmid, pCMV5H3βHSD [8], using *EcoR*1/*PspA*1 and ligated into pGEM-3Z (Promega, Madison, WI) to produce pGEM-3βHSD (4298 bp). A double stranded, polymerase chain reaction (PCR) method of site-directed mutagenesis [9, 10] produced the mutant cDNA using the high-fidelity Klentaq DNA polymerase (supplied by W. Barnes, Washington University, St. Louis, MO). To create the H261R mutant cDNA, the codon for His^{261}, CAC, was converted to the codon for Arg^{261}, CGC, using the forward mutated primer, 5′-TGACACGCCT*CGC*CAAAGCTATGAT-3′, and the abutting reverse primer, 5′-TCTGAGATATAGTAGAACTGTCCTCG-3′. To produce the Y253F mutant cDNA, the codon for Tyr^{253}, TAC, was converted to the codon for Phe^{253}, TTC, using the forward mutated primer, 5′-GAGGACAGTTC*TTC*TATATCTCAGATGAC-3′, and the abutting reverse primer, 5′-GGATGCTTGGGGCCTT-3′. To produce the Y253,254F mutant cDNA, the codons for Tyr^{253}, TAC, and Tyr^{254}, TAT, were converted to the codons for Phe^{253}, TTC, and Phe^{254}, TTT, using the forward mutated primer, 5′-GAGGACAGTTC*TTCTTT*ATCTCAGATGAC-3′ and the same abutting reverse primer described above. The correct mutations were confirmed using a PCR-based dideoxynucleotide sequencing method (Thermo Sequenase kit, Amersham Life Science, Cleveland, OH). In addition, the entire 1555 bp cDNA was sequenced to confirm the integrity of the coding region after PCR-based mutagenesis.

Expression of the mutant and wild-type enzymes

The mutant 3β-HSD cDNA was cut from pGEM-3βHSD using *EcoR*1/*PspA*1 and ligated into the baculovirus transfer vector pVL1392 (Invitrogen, San Diego, CA). The recombinant baculovirus was produced by transfecting *Spodoptera fungiperda* (Sf9) insect cells with linearized AcMNPV DNA and the pVL1392-3βHSD using the Invitrogen Bac-N-Blue transfection kit. Screening for the recombinant baculovirus by plaque assay and infection of Sf9 cells were performed as previously described for the wild-type 3β-HSD/isomerase [8]. The H261R mutant, Y253F mutant, Y253,254F mutant or wild-type enzyme was expressed in suspension cultures of Sf9 cells.

Western blots

The expressed enzyme was separated by SDS-polyacrylamide (7.5%) gel electrophoresis, electroblotted onto a nitrocellulose membrane, probed with primary antibody raised against human placental 3β-HSD/isomerase, and incubated with goat [^{125}I]-antirabbit IgG (1.0 μCi, ICN Biomedicals) as described previously for the wild-type enzyme [8]. The immunoreactive bands were visualized using a Molecular Dynamics (Sunnyvale, CA) Model 400 PhosphorImager.

Enzyme purification

3β-HSD and steroid Δ-isomerase were co-purified from human placental microsomes or from the

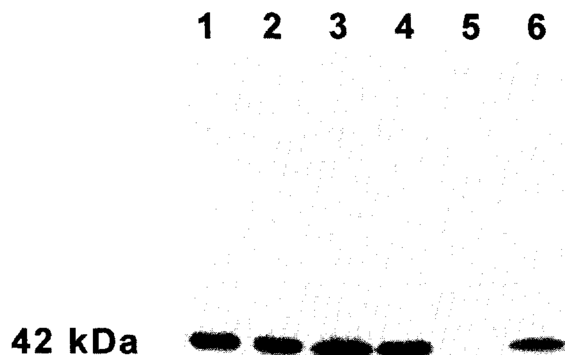

Fig. 1. Western blot of an SDS-polyacrylamide gel showing the expression of the mutant and wild-type 3β-HSD/isomerase in insect Sf9 cells infected with recombinant baculovirus. Lane 1: purified human placental (type I) 3β-HSD/isomerase (1.0 μg). Lane 2: purified H261R mutant enzyme (0.5 μg). Lane 3: Y253,254F mutant enzyme in the Sf9 cell homogenate (10.0 μg). Lane 4: purified Y253F mutant enzyme (0.5 μg). Lane 5: uninfected Sf9 cell homogenate (10.0 μg). Lane 6: purified wild-type I enzyme (0.5 μg).

$100{,}000 \times g$ pellet of the homogenized Sf9 cells (5.0 l) by our published method. The purified placental enzyme is a homogeneous protein (monomeric M_r=42,000) according to SDS-polyacrylamide gel electrophoresis and NH_2-terminal sequence analysis [1]. The purified H261R mutant, Y253F mutant, or wild-type enzyme exhibited a single band that co-migrated with pure placental enzyme on SDS-polyacrylamide gel electrophoresis (silver stain) and had a single NH_2-terminal sequence of the expected nine amino acids: NH_2-TGWSCLVTG... [1, 11]. The microsomal Y253,254F mutant enzyme was prepared from the $110{,}000 \times g$ pellet of the baculovirus-infected Sf9 cell homogenate as previously described [8]. Protein concentrations were determined by the Coomassie blue method using bovine serum albumin as standard [12].

Kinetic studies

Kinetic constants for substrates of the 3β-HSD activity of the purified Y253F and wild type enzymes were determined in incubations at 37°C containing dehydroepiandrosterone (1.9–6.0 μM) or pregnenolone (3.0–20.0 μM), NAD^+ (0.1 mM), and purified enzyme (0.03 mg) in 0.02 M potassium phosphate buffer, pH 7.4, 4.0% final methanol content (buffer A). 3β-HSD cofactor kinetics were determined by incubating NAD^+ (13.0–100.0 μM), dehydroepiandrosterone (30.0 μM for wild-type and native enzymes, 80.0 μM for Y253F), and purified enzyme (0.03 mg) in buffer A at 37°C. The 3β-HSD kinetic study of the purified H261R enzyme used the maximal soluble concentration of dehydroepiandrosterone (100.0 μM) and the highest NAD^+ concentration (200.0 μM) that could be measured spectrophotometrically. The slope of the initial linear increase in absorbance at 340 nm (due to NADH production) per unit time was used to determine 3β-HSD activity.

Kinetic constants for substrates of the isomerase activity of the purified H261R, Y253F, wild-type and native enzymes were determined in incubations of 5-androstene-3,17-dione (17.0–42.0 μM) or 5-pregnene-3,20-dione (5.0–30.0 μM), NADH (0.05 mM), and purified enzyme (0.01 mg) in buffer A at 37°C. The coenzyme activation kinetics of isomerase were determined in incubations of NAD^+ (13.0–100 μM for wild-type and Y253F, 40.0–200 μM for H261R) or NADH (3.0–15.0 μM), 5-androstene-3,17-dione (30.0 μM), and purified enzyme (0.01 mg) in buffer A at 37°C. Isomerase activity was measured by the initial absorbance increase at 241 nm (due to 3-oxo-4-ene product formation from the 3-oxo-5-ene steroid substrate) 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 [13].

The microsomal Y253,254F mutant enzyme was assayed for the 3β-HSD and isomerase activities as described above, except that the substrate concentrations and protein content were maximized in an effort to measure each of the enzyme activities.

Changes in absorbance were measured with a Varian (Sugar Land, TX) Cary 219 recording spectrophotometer. The Michaelis–Menten constants (K_m and V_{max}) were calculated from Lineweaver–Burke ($1/S$ *vs.* $1/V$) plots and verified by Hanes–Woolf (S *vs.* S/V) plots [14].

RESULTS

Expression of the mutant and wild-type enzymes

A Western blot of an SDS-polyacrylamide gel (Fig. 1) showed that Sf9 cells infected with the appropriate recombinant baculovirus expressed the immunoreactive H261R (lane 2), Y253,254F (lane 3), Y253F (lane 4) and wild type (lane 6) protein, all of which co-migrated with the purified, human placental 3β-HSD/isomerase (lane 1). The absence of a band in lane 5 shows that uninfected Sf9 cells do not express endogenous 3β-HSD/isomerase.

Kinetic analysis of the purified mutants

Table 1 summarizes the results of the substrate kinetic studies that compared the purified H261R and Y253F mutants with the wild-type and native placental enzymes. The H261R mutation completely abolished the 3β-HSD activity but only modestly affected the K_m and V_{max} values measured for the isomerase activities of the wild-type and native placental enzymes. In contrast, the Y253F mutant had a 7-fold lower isomerase V_{max} but had the same 3β-HSD V_{max} as the wild type and native enzymes. The kinetic data in Table 1 were calculated from the

Table 1. Substrate kinetics for the 3β-HSD and isomerase activities of the purified H261R, Y253F, wild-type and native placental enzymes

	3β-HSD[a]		Isomerase[b]	
Purified enzyme	K_m (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)
H261R	ND	0	63.9	435
Y253F	20.7	48.3	48.3	88[c]
Wild-type	3.7	43.3	27.9	598
Native[d]	2.6	48.5	25.6	626

[a]Kinetic constants for the 3β-HSD substrate were determined in incubations containing dehydroepiandrosterone (1.9–6.0 μM), NAD^+ (0.1 mM), and purified enzyme (0.03 mg) in 0.02 M potassium phosphate buffer, pH 7.4, 4.0% methanol (buffer A) at 37°C. The 3β-HSD assay for the H261R mutant contained 100.0 μM dehydroepiandrosterone and 0.2 mM NAD^+.
[b]Kinetic constants for the isomerase substrate were determined in incubations of 5-androstene-3,17-dione (17.0–42.0 μM), NADH (0.05 mM), and purified enzyme (0.01 mg) in 0.02 M potassium phosphate buffer, pH 7.4, 4.0% methanol (buffer A) at 37°C.
[c]The microsomal Y253,254F mutant had no isomerase activity but had 45% of the microsomal wild-type 3β-HSD activity.
[d]Identical 3β-HSD and isomerase assays determined these kinetic constants that were previously published for the native enzyme purified from human placental microsomes [1, 22].

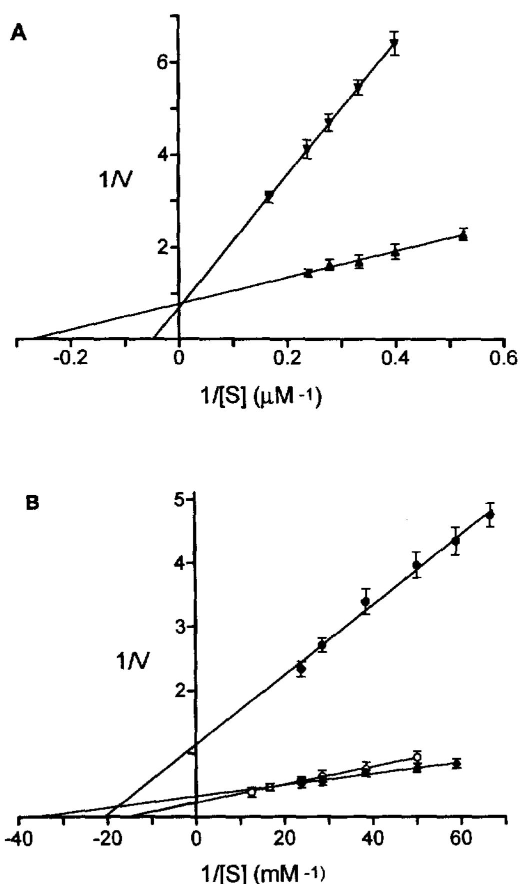

Fig. 2. Lineweaver–Burke plots determined the kinetic constants for the 3β-HSD and isomerase substrates using the purified mutant and wild-type enzymes. (A) The 3β-HSD activity was measured in duplicate incubations containing dehydroepiandrosterone (1.9–6.0 μM) and NAD^+ (0.1 mM) in 0.02 M potassium phosphate buffer, pH 7.4, 4.0% methanol at 37°C for the purified Y253F mutant (▼) and wild-type (▲) enzymes (0.03 mg). (B) The isomerase activity was measured in duplicate incubations containing 5-androstene-3,17-dione (17.0–42.0 μM) and NADH (0.05 mM) in 0.02 M potassium phosphate buffer, pH 7.4, 4.0% methanol at 37°C for the Y253F mutant (•), H261R mutant (○) and wild-type (♦) enzymes (0.01 mg). Variation in the duplicate assays is represented by the S.E.M. bars on the plots.

Lineweaver–Burke plots in Fig. 2A and B. The standard error of the mean (S.E.M.) bars in the plots show the low variability encountered in these kinetic studies with the pure enzymes. The intersection of the Lineweaver–Burke plots depicts the equal V_{max} values (reciprocal of the $1/V$ intercept) measured for the 3β-HSD activities of the Y253F mutant and wild-type enzymes (Fig. 2A). The similar V_{max} values measured for the isomerase activities of the H261R and wild-type enzymes compared to the greatly reduced V_{max} of the Y253F mutant can be visualized on the $1/V$ axis in Fig. 2B. Similar changes in the kinetic constants for the H261R, Y253F, wild-type, and native enzymes were measured with the pregnene 3β-HSD and isomerase substrates, pregnenolone and 5-pregnene-3,20-dione, respectively (data not shown).

The microsomal Y253,254F mutant enzyme (0.04–0.08 mg, 100 μM 5-androstene-3,17-dione) exhibited no isomerase activity. In identical assays, the microsomal wild-type enzyme (0.04 mg) had substantial isomerase activity (48.0 nmol 5-androstene-3,17-dione converted/min/mg). However, the microsomal Y253,254F mutant enzyme (0.04–0.08 mg, 100 μM dehydroepiandrosterone) had measurable 3β-HSD activity (3.0–5.0 nmol/min/mg) that was 45% of the 6.7 nmol/min/mg measured for the 3β-HSD activity of the microsomal wild-type enzyme (0.04 mg). The microsomal Y253,254F was not studied further because of the complete absence of isomerase activity and the inability to dissolve the high concentrations of dehydroepiandrosterone necessary to obtain meaningful 3β-HSD kinetics.

The kinetic constants (K_m, V_{max}) measured for the 3β-HSD cofactor, NAD^+, were very similar for the Y253F mutant, wild-type and native enzymes, but the H261R mutant exhibited no 3β-HSD activity even with maximal substrate and NAD^+ concentrations (Table 2).

We have previously shown that either NAD^+ or NADH can activate isomerase, with NADH being the preferred activator in terms of the K_m and V_{max}

Table 2. Cofactor kinetics for the 3β-HSD activity of the purified H261R, Y253F, wild-type and native placental enzymes

	NAD$^+$ reduction by 3β-HSD[a]	
Purified enzyme	K_m (μM)	V_{max} (nmol/min/mg)
H261R	ND	0
Y253F	38.5	44.3
Wild-type	34.1	45.9
Native[b]	26.2	58.2

[a]3β-HSD cofactor kinetics were determined by incubating NAD$^+$ (13.0–100.0 μM), dehydroepiandrosterone (30.0 μM for wild-type and native enzymes, 80.0 μM for Y253F), and purified enzyme (0.03 mg) in buffer A at 37°C. The 3β-HSD assay for the H261R mutant contained 100.0 μM dehydroepiandrosterone and 200.0 μM NAD$^+$.

[b]An identical 3β-HSD assay determined these kinetic constants for the native enzyme purified from human placental microsomes [1, 22].

values measured [15]. The ratio of the NADH/NAD$^+$ V_{max} values range from 1.4 to 1.8 for the native placental, wild type and Y253F mutant enzymes. However, NAD$^+$ activates the isomerase activity of the H261R mutant very poorly. The NADH/NAD$^+$ V_{max} ratio is 25 for the H261R mutant enzyme (Table 3). These data were calculated from the Lineweaver–Burke plots that are shown in Fig. 3A and B. The similar V_{max} values measured for the NADH activation of the H261R and wild-type isomerase (Fig. 3B) can be compared to the vastly different V_{max} obtained for the NAD$^+$ activation of the H261R or wild-type isomerase (Fig. 3A).

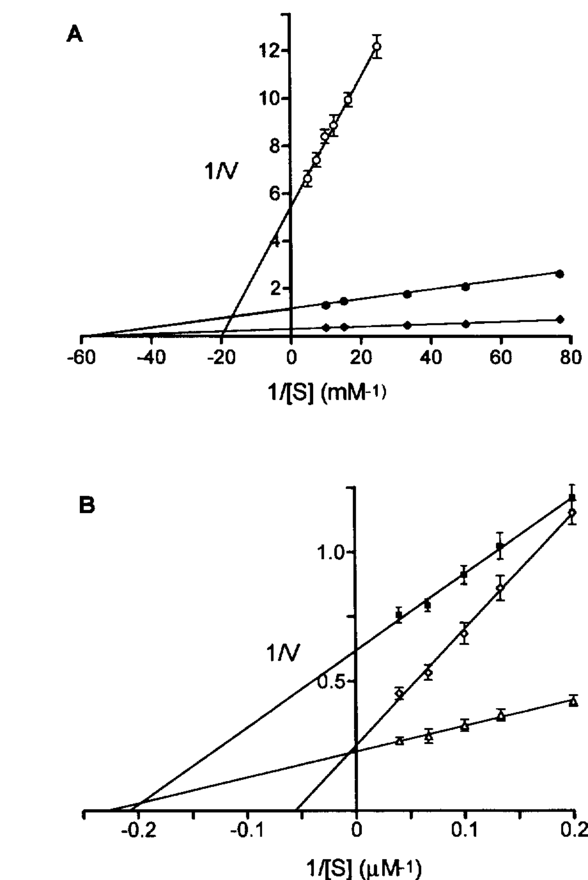

Fig. 3. Lineweaver–Burke plots determined the kinetic constants for the activation of isomerase in the mutant and wild-type enzymes by NAD$^+$ and NADH. (A) The isomerase activity was measured for purified H261R mutant (○), Y253F mutant (•) or wild-type (♦) enzyme (0.01 mg) in duplicate incubations containing NAD$^+$ (13.0–100 μM for wild-type and Y253F, 40.0–200 μM for H261R) and 5-androstene-3,17-dione (30.0 μM) in buffer A at 37°C. (B) Identical incubations measured the activation of the isomerase activities of the purified H261R (◇), Y253F (■) and wild-type (△) enzymes by NADH (3.0–15.0 μM). Variation in the duplicate assays is represented by S.E.M. bars except where the points obscure the error bars.

DISCUSSION

The 3β-HSD/isomerase enzymes present an interesting mechanistic question: Are the catalytic centers and critical amino acids for each of the two coupled, biosynthetic reactions located in unique and separate domains, in overlapping but distinct sites, or in the same, inseparable site? We have identified the active site region for 3β-HSD and isomerase in the primary structure of the enzyme using a substrate-protection technique that enhances the specificity of affinity radiolabeling steroids [4–6]. The codons for potentially critical amino acids in the identified region were then changed to create the cDNA that expressed the mutant enzymes. The mutant enzymes were purified so that meaningful kinetic analyses could be per-

Table 3. Comparison of the NAD$^+$ and NADH activation of the isomerase activity of the purified H261R, Y253F, wild-type and native placental enzymes

	Activation[a] by NAD$^+$		Activation by NADH		
Purified enzyme	K_m (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)	NADH/NAD$^+$ V_{max} ratio
H261R	50.1	18	22.0	451	25.2
Y253F	17.1	89	4.8	161	1.8
Wild-type	14.5	389	4.6	536	1.4
Native[b]	14.4	394	2.4	609	1.5

[a]The coenzyme kinetic constants were determined in incubations of NAD$^+$ (13.0–200.0 μM) or NADH (3.0–15.0 μM), 5-androstene-3,17-dione (30.0 μM), and purified enzyme (0.01 mg) in buffer A at 37°C.

[b]Identical isomerase assays determined these kinetic constants for the native enzyme purified from human placental microsomes [15].

```
     250          253 254                          261
...R Gly-Gln-Phe-Tyr-Tyr-Ile-Ser-Asp-Asp-Thr-Pro-His-Gln-Ser-Tyr-

                                              273
Asp-Asn-Leu-Asn-Tyr-Thr-Leu-Ser-Lys E F G L R L D S R W S F P L S

                                                                 320
L M Y W I G F L L E I V S F L L R P I Y T Y R P P F N R H I V T...
```

Fig. 4. This tryptic peptide in human placental 3β-HSD/isomerase was identified independently by 3β-HSD-directed [4, 5] and by isomerase-site-directed [6] affinity radiolabeling steroids. The radiolabeled His^{261} identified in these studies and the Tyr^{253}, Tyr^{254} residues found to be mutated in the gene for the type II enzyme in 3β-HSD-deficient patients [2] are labeled. Amino acids in the tryptic peptide are represented by three-letter abbreviations in boldface type. The other residues in this partial sequence of the known primary structure [11] are represented by single-letter amino acid abbreviations that are not in boldface type.

formed. Finally, enzyme assays that are specific for and distinguish between the 3β-HSD and isomerase activities were used to determine which of the two activities was modified by each mutation.

In our affinity radiolabeling studies using the 3β-HSD-site-directed steroid, 2α-bromo[2′-^{14}C]acetoxyprogesterone (2α-[^{14}C]BAP), and the isomerase-site-directed secosteroid, 5,10-secoestr-4-yne-3,10,17-trione, the tryptic peptide shown in Fig. 4 was covalently modified at His^{261} and was protected from modification by co-incubation with the 3β-HSD substrate, pregnenolone [4] or with the isomerase substrate, 5-androstene-3,17-dione [6]. Moreover, diethyl pyrocarbonate, a reagent that modifies His residues, inactivated the 3β-HSD activity in absence but not in the presence of the 3β-HSD substrate, pregnenolone [4].

In the analogous tryptic peptide of the type II (adrenal) enzyme, the codon for Tyr^{253} or Tyr^{254} was found to be mutated (Y253N or Y254D) in the human type II gene of two patients with 3β-HSD/isomerase deficiency [2]. The amino acid sequence of the human type I tryptic peptide shown in Fig. 4 is homologous [2] to the corresponding human type II tryptic peptide (except for the substitution of Ile^{270} in type II for Thr^{270} in type I). The independent identification of amino acids in the same tryptic peptide by affinity labeling (His^{261}) and by sequencing the type II gene in 3β-HSD-deficient patients (Tyr^{253}, Tyr^{254}) represents an unprecedented consensus of data that guided our choice of these residues for site-directed mutagenesis.

Mutagenesis studies have shown that a tyrosine residue has a catalytic function in a steroid Δ^5-isomerase. Using the Y14F mutant enzyme, Tyr^{14} was shown to participate in the isomerase reaction mechanism of 3-oxo-Δ^5-steroid isomerase from *Pseudomonas testosteroni*. With this conservative replacement strategy, both residues are aromatic, but Phe lacks the hydroxyl group necessary to act as a general acid in the isomerase reaction mechanism [16]. This assignment for Tyr^{14} was recently supported by heteronuclear multidimensional NMR spectroscopy of the bacterial isomerase [17]. Based on these studies, we chose to substitute Phe for Tyr in the human type I Y253F and Y253,254F mutant enzymes.

A His residue participates in the reaction mechanism of human placental 17β-hydroxysteroid dehydrogenase [18] and in bacterial lactate dehydrogenase [19], malate dehydrogenase [20] and fumarate reductase [21]. Choosing a substitution for His^{261} was not as straightforward as it was for Tyr^{253} because there is not a completely suitable, alternative amino acid residue. Arg was selected because His and Arg are both basic amino acids, Arg is unlikely to participate in hydride ion transfer, and Arg can function as a binding residue [18, 21]. Arg is more positively charged than His at pH 7.4, and this difference was exploited in the studies that compared the activation of isomerase by NAD^+ versus NADH. Chou–Fasman and Garnier–Osguthorpe–Robson analysis of the H261R and wild-type enzymes using the Protylze program (Scientific and Educational Software, State Line, PA) predicted the same structure for the two proteins. When Gln, Leu or Asn was substituted for His^{261}, the Protylze program predicted a change in secondary structure, thus eliminating these mutation options.

The H261R mutant enzyme has no 3β-HSD activity but exhibits V_{max} values for isomerase substrate utilization (Table 1, Fig. 2B) and for isomerase activation by NADH (Table 3, Fig. 3B) that are similar to those of the wild-type enzyme. Because the isomerase activity is intact on the normally bifunctional, single protein, the H261R mutation did not inactivate the enzyme by inducing an inappropriate change in the secondary structure of the enzyme. Hence, this kinetic data suggests that His^{261} might participate in either the substrate binding or catalysis of the 3β-HSD activity. The ability of NADH to activate the isomerase activity of H261R in a concentration-dependent manner shows that the mutation did not simply shift the enzyme from the 3β-HSD to the isomerase conformation. Moreover, the dramatic decrease in the ability of NAD^+ to activate the isomerase activity of H261R (Table 3, Fig. 3A) may

have been due to the introduction of positively charged Arg[261] in the active site. Our data is consistent with Arg repelling the positively charged pyrimidine ring of NAD^+ but allowing uncharged NADH to activate isomerase. This selective activation of isomerase provides additional evidence that the H261R mutant is a functional enzyme without disruption of the protein structure. In addition, Arg^+ could have prevented NAD^+ from acting as the hydride ion acceptor from the 3β-hydroxysteroid substrate so that the 3β-HSD activity of the H261R mutant enzyme was abolished. These data are consistent with our model for the sequential reaction mechanism in which the coenzyme used in the 3β-HSD reaction induces a conformational change in the enzyme that activates isomerase [7].

Kinetic analyses of substrate utilization by the purified Y253F mutant, microsomal Y253,254F mutant, and wild type enzymes suggest that these Tyr residues play a role in the isomerase activity. The 7.0-fold decrease in the V_{max} for the isomerase activity of Y253F and the complete loss of isomerase activity with the Y253,Y254F mutant suggests that Tyr[253] or Tyr[254] can function as a critical residue, as Tyr[14] does in the bacterial isomerase [16].

The diminished V_{max} of NADH (Table 3) that was observed with the Y253F mutant compared to wild-type reflects the reduced catalytic ability of the mutant enzyme to convert 5-androstene-3,17-dione to androstenedione (Table 1). Since the NADH/NAD^+ ratios (Table 3) measured for the activation of Y253F isomerase and of wild-type isomerase are similar, the Y253F mutation did not modify the ability of the coenzymes to interact with the enzyme. In addition, the kinetic constants of NAD^+ as the cofactor for 3β-HSD were very similar in the Y253F and wild-type enzymes (Table 2).

These observations link His[261] with the 3β-HSD activity and link Tyr[253], Tyr[254] with the isomerase activity. Our use of specific, targeted mutations have allowed us to address the original question regarding the organization of the catalytic centers of the two enzyme activities. Although the tertiary structure of 3β-HSD/isomerase is not known, the proximity of these three residues in the primary structure is most consistent with catalysis at overlapping but distinct regions of the bifunctional enzyme protein.

REFERENCES

1. Thomas J. L., Myers R. P. and Strickler R. C., Human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5 → 4-ene-isomerase: purification from mitochondria and kinetic profiles, biophysical characterization of the purified mitochondrial and microsomal enzymes. *J. Steroid Biochem.* **33** (1989) 209–217.
2. Simard J., Durocher F., Mebarke F., Turgeon C., Sanchez R., Labrie Y., Couet J., Trudel C., Rheaume E., Morel Y., Luu-The V. and Labrie F., Molecular biology and genetics of the 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase gene family. *J. Endocr.* **150** (1996) S189–S207.
3. Luu-The V., Takahashi M. and Labrie F., Purification of microsomal and mitochondrial 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4 isomerase from human placenta. *Ann. N. Y. Acad. Sci.* **595** (1990) 386–388.
4. Thomas J. L., Nash W. E., Myers R. P., Crankshaw M. W. and Strickler R. C., Affinity radiolabeling identifies peptides and amino acids associated with substrate binding in human placental 3β-hydroxy-Δ^5-steroid dehydrogenase. *J. Biol. Chem.* **268** (1993) 18507–18512.
5. Thomas J. L., Nash W. E., Crankshaw M. W. and Strickler R. C., Affinity labeling in the presence of the reduced diphosphopyridine nucleotide NADH identifies peptides associated with the activities of human placental 3β-hydroxy-Δ^5-steroid dehydrogenase/isomerase. *J. Soc. Gynecol. Invest.* **1** (1994) 155–163.
6. Thomas J. L., Evans B. W. and Strickler R. C., Affinity radiolabeling identifies peptides associated with the isomerase activity of human type I (placental) 3β-hydroxysteroid dehydrogenase/isomerase. *Biochemistry* **36** (1997) 9029–9034.
7. Thomas J. L., Frieden C., Nash W. E. and Strickler R. C., An NADH-induced conformational change that mediates the sequential 3β-hydroxysteroid dehydrogenase/isomerase activities is supported by affinity labeling and the time-dependent activation of isomerase. *J. Biol. Chem.* **270** (1995) 21003–21008.
8. Nash W. E., Mercer R. W., Blanco G., Strickler R. C., Mason J. I. and Thomas J. L., Over-expression of human type I (placental) 3β-hydroxy-5-ene-steroid dehydrogenase/isomerase in insect cells infected with recombinant baculovirus. *J. Steroid Biochem. Molec. Biol.* **50** (1994) 235–240.
9. Weiner M. P., Costa G. L., Schoettlin W., Cline J., Mathur E. and Bauer J. C., Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. *Gene* **151** (1994) 119–123.
10. Chau Y. M., Crawford P. A., Woodson K. G., Polish J. A., Olson L. M. and Sadovsky Y., Role of steroidogenic-factor 1 in basal and 3′,5′-cyclic adenosine monophosphate-mediated regulation of cytochrome P450 side-chain cleavage enzyme in the mouse. *Biol. Reproduction* **57** (1997) 765–771.
11. Luu-The V., Lachance Y., Labrie C., Leblanc G., Thomas J. L., Strickler R. C. and Labrie F., Full length cDNA structure and deduced amino acid sequence of human 3β-hydroxy-5-ene steroid dehydrogenase. *Mol. Endocrinol.* **3** (1989) 1310–1312.
12. Bradford M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72** (1976) 248–254.
13. Thomas J. L., Myers R. P., Rosik L. O. and Strickler R. C., Affinity alkylation of human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5 → 4-ene-isomerase by 2α-bromoacetoxyprogesterone: evidence for separate dehydrogenase and isomerase sites on one protein. *J. Steroid Biochem.* **36** (1990) 117–123.
14. Segel, I. H., *Enzyme Kinetics*. John Wiley, New York, 1975, pp. 47, 211.
15. Thomas J. L., Myers R. P. and Strickler R. C., Analysis of coenzyme binding by human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5 → 4-ene-isomerase using 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, an affinity labeling cofactor analog. *J. Steroid Biochem. Molec. Biol.* **39** (1991) 471–477.
16. Kuliopulos A., Talalay P. and Mildvan A. S., Combined effects of two mutations of catalytic residues on the ketosteroid isomerase reaction. *Biochemistry* **29** (1990) 10271–10280.
17. Wu Z. R., Ebrahimian S., Zawrotny M. E., Thornburg L. D., Perez-Alvardo G. C., Brothers P., Pollack R. M. and Summers M. F., Solution structure of 3-oxo-Δ^5-steroid isomerase. *Science* **276** (1997) 415–418.
18. Labrie F., Luu-The V., Lin S.-X., Labrie C., Simard J., Breton R. and Belanger A., The key role of 17β-hydroxysteroid dehydrogenase in sex steroid biology. *Steroids* **62** (1997) 148–158.
19. Clarke A. R., Wilks H. M., Barstow D. A., Atkinson T., Chia W. N. and Holbrooke J. J., An investigation of the contribution made by the carboxylate group of an active site histidine–as-

partate couple to binding and catalysis in lactate dehydrogenase. *Biochemistry* **27** (1988) 1617–1622.

20. Hall M. D., Levitt D. G. and Banaszak L. J., Crystal structure of Escherichia coli malate dehydrogenase. A complex of apoenzyme and citrate at 1.87 A resolution. *J. Molec. Biol.* **226** (1992) 867–882.
21. Schroder I., Gunsalus R. P., Ackrell B. A., Cochran B. and Cecchini G., Identification of active site residues of Escherichia coli fumarate reductase by site-directed mutagenesis. *J. Biol. Chem.* **266** (1991) 13572–13579.
22. Thomas J. L., Berko E. A., Faustino A., Myers R. P. and Strickler R. C., Human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5 $\rightarrow$ 4-ene-isomerase: purification from microsomes, substrate kinetics and inhibition by product steroids. *J. Steroid Biochem.* **31** (1988) 785–793.