Serine 124 completes the Tyr, Lys and Ser triad responsible for the catalysis of human type 1 3β -hydroxysteroid dehydrogenase

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Abstract

Human 3β -hydroxysteroid dehydrogenase/isomerase (3β -HSD) is a key steroidogenic enzyme that catalyzes the first step in the conversion of circulating dehydroepiandrosterone (DHEA), pregnenolone or 17α -hydroxypregnenolone to produce the appropriate, active steroid hormone(s): estradiol, testosterone, progesterone, aldosterone or cortisol respectively. Our mutagenesis studies have identified Tyr154 and Lys158 as catalytic residues for the 3β -HSD reaction. Our three-dimensional homology model of 3β -HSD shows that Tyr154 and Lys158 are oriented near the 3β -hydroxyl group of the bound substrate steroid, and predicts that Ser123 or Ser124 completes a Tyr–Lys–Ser catalytic triad that operates in many other dehydrogenases. The S123A and S124A mutants of human type 1 3β -hydroxysteroid dehydrogenase/isomerase (3β -HSD1) were created by PCR-based mutagenesis, expressed in insect cells using baculovirus and purified to homogeneity. The S124A mutant exhibits no 3β -HSD activity and has a K_m value ($83.6\ \mu\text{M}$) for the isomerase substrate that is threefold greater than that of wild-type 1 isomerase. In contrast, S123A has substantial 3β -HSD activity (DHEA $K_m=11.2\ \mu\text{M}$; $k_{\text{cat}}=0.8\ \text{min}^{-1}$) and utilizes isomerase substrate, 5-androstene-3,17-dione, with a K_m value ($27.6\ \mu\text{M}$) that is almost identical to wild-type. The K_m value ($4.3\ \mu\text{M}$) of S124A for NADH as an allosteric activator of isomerase is similar to that of the wild-type 1 enzyme, indicating that Ser124 is not involved in cofactor binding. S123A utilizes NAD as a cofactor for 3β -HSD and NADH as the activator for isomerase with K_m values that are similar to wild-type. The 3β -HSD activities of S123A and wild-type 3β -HSD increase by 2.7-fold when the pH is raised from 7.4 to the optimal pH 9.7, but S124A exhibits very low residual 3β -HSD activity that is pH-independent.

These kinetic analyses strongly suggest that the Ser124 residue completes the catalytic triad for the 3β -HSD activity. Since there are 29 Ser residues in the primary structure of human 3β -HSD1, our homology model of the catalytic domain has been validated by this accurate prediction. A role for Ser124 in the binding of the isomerase substrate, which is the 3β -HSD product-steroid of the bifunctional enzyme protein, is also suggested. These observations further characterize the structure/function relationships of human 3β -HSD 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.

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Introduction

The human type 1 (placenta, skin, mammary gland, prostate and endometrium) and type 2 (gonads and

adrenals) isoforms of 3β -hydroxysteroid dehydrogenase (EC 1.1.1.145)/steroid Δ^5 – Δ^4 -isomerase (EC 5.3.3.1) (3β -HSD/isomerase) are encoded by two distinct genes which are expressed in a

tissue-specific pattern (Rheaume *et al.* 1991). 3 β -HSD/isomerase catalyzes the conversion of 3 β -hydroxy-5-ene-steroids (dehydroepiandrosterone, pregnenolone) to 3-oxo-4-ene-steroids (androstenedione, progesterone) on a single, dimeric protein containing both enzyme activities (Thomas *et al.* 1989). Androstenedione is converted by placental aromatase and 17 β -hydroxysteroid dehydrogenase 1 (17 β -HSD1) to estradiol, which participates in the cascade of events that initiates labor in humans (Kacsoh 2000, Rainey *et al.* 2002). In addition to placenta and other human peripheral tissues, the type 1 enzyme is selectively expressed in breast tumors (Gingras *et al.* 1999), where it catalyzes the first step in the conversion of circulating dehydroepiandrosterone to estradiol to promote tumor growth. In human adrenals, type 2 3 β -HSD/isomerase is required for the production of cortisol and aldosterone (Rainey *et al.* 2002). Determination of the structure/function relationships of the type 1 enzyme may lead to the development of specific inhibitors of type 1 3 β -HSD/isomerase that can help control the timing of labor and slow the growth of hormone-sensitive tumors without compromising steroidogenesis in the adrenal enzyme.

Mammalian 3 β -HSD/isomerase is a member of the short-chain dehydrogenase/reductase (SDR) family of enzymes. The Rossmann-fold coenzyme domain (β - α - β - α - β) and the Y-X-X-X-K catalytic motif in that domain are highly conserved in the SDR family (Jornvall *et al.* 1995). Many members of the SDR family utilize a catalytic triad of Ser, Tyr and Lys residues to perform the oxidoreductase enzyme reaction (e.g. 17 β -HSD1, UDP-galactose-4-epimerase) (Liu *et al.* 1997). A Tyr residue acts as the catalytic base, the hydroxyl group of Ser binds to the targeted oxo group to stabilize the substrate, and a Lys residue binds to the nicotinamide ribose and lowers the pKa of the phenolic hydroxyl group of the key Tyr to promote the proton transfer (Filling *et al.* 2002). In a few species of 3 β -HSD (macaque, bovine, rat I, II and IV, guinea pig and chicken), Thr124 is positioned to perform the function of Ser124 (Simard *et al.* 1996, Morel *et al.* 1997). Our homology model of the three-dimensional structure of human 3 β -HSD1 has been used to predict key amino acid residues involved in catalysis and coenzyme specificity (Thomas *et al.* 2002, 2003). Our previous mutagenesis study supported roles for Tyr154 and Lys158 in the catalysis of human 3 β -HSD1

(Thomas *et al.* 2002). In the current study, our homology model has predicted a catalytic role for either Ser123 or Ser124. Hence, the S123A and S124A mutants of human 3 β -HSD1 have been produced to evaluate the function of these amino acids in the catalysis of the enzyme. Since there are 29 Ser residues in each monomer of the homodimeric enzyme (Thomas *et al.* 2002), this study also tests the accuracy of our homology model.

Materials and methods

Materials

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

Site-directed mutagenesis

With the Advantage cDNA PCR kit (BD Biosciences Clontech, Palo Alto, CA, USA) and pGEM-3 β HSD1 as template (Thomas *et al.* 1998), double-stranded PCR-based mutagenesis was performed to create the cDNA encoding the S123A and S124A mutant enzymes. The forward and reverse primers used to produce S123A are listed (with the mutant codon in bold italics): 5'-TAC ACC**GCT**AGCATAGAGGTAGC-3' (forward); 5'-TATGCT**AGC**GGTGTAGATGAAGACT-3' (reverse). The primers used to produce S124A were as follows: 5'-CACCAGT**GCC**ATAGAGGTAGCC-3' (forward); 5'-CCTCTAT**GGC**ACTGTGTAGATG-3' (reverse). The presence of the mutated codon and integrity of the entire mutant 3 β -HSD cDNA were verified by automated dideoxynucleotide DNA sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA). Chou-Fasman and Garnier-Osguthorpe-Robson analysis of each mutant enzyme was used to choose amino acid substitutions that produced no apparent changes in the secondary structure of the protein (Protlyze program, Scientific and Educational Software, State Line, PA, USA).

Expression and purification of mutant and wild-type enzymes

The mutant 3 β -HSD cDNA was introduced into baculovirus as previously described (Thomas *et al.* 1998). Recombinant baculovirus was added to 1.5×10^9 Sf9 cells (1 L) at a multiplicity of infection of 10 for expression of each mutant enzyme. The expressed mutant and wild-type enzymes were separated by SDS-polyacrylamide (12%) gel electrophoresis, probed with our anti-3 β -HSD polyclonal antibody and detected using the ECL Western blotting system with antirabbit, peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Each expressed enzyme was purified from the 100 000 g pellet of the Sf9 cells (2 L) by our published method (Thomas *et al.* 1988, 1989, 2002). Each expressed, purified mutant and wild-type enzyme produced a single band (42.0 kDa) on SDS-polyacrylamide (12%) gel electrophoresis that comigrated with the human wild-type 1 control enzyme. Protein concentrations were determined by the Bradford method, using bovine serum albumin as the standard (Bradford 1976).

Kinetics studies

Michaelis–Menten kinetic constants for the 3 β -HSD substrate were determined for the purified mutant and wild-type enzymes in incubations containing dehydroepiandrosterone (DHEA, 2–100 μ M) 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 with time in absorbance at 340 nm per min (due to NADH production) was used to determine 3 β -HSD activity. Kinetic constants for the isomerase substrate were determined at 27 °C in incubations of 5-androstene-3,17-dione (17–150 μ M), NADH (0.05 mM) and purified enzyme (0.01 mg) in 0.02 M potassium phosphate buffer, pH 7.4. Isomerase activity was measured by the initial absorbance increase at 241 nm (due to androstenedione formation) as a function of time. Changes in absorbance were measured with a Varian Cary 219 (Sugar Land, TX, USA) 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–Woelf (S vs S/V) plots (17). The k_{cat} values (min^{-1}) were

calculated from the V_{max} values (nmol/min per mg) and represent the maximal turnover rate (nmol product formed/min per nmol enzyme dimer).

Kinetic constants for the 3 β -HSD cofactor were determined for the purified mutant and wild-type enzymes in incubations containing NAD⁺ (13–100 μ M), dehydroepiandrosterone (100 μ M) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4, at 27 °C, using the spectrophotometric assay at 340 nm. Kinetic constants for the isomerase cofactor were determined in incubations of NADH (2–50 μ M), 5-androstene-3,17-dione (100 μ M) 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.

pH-dependency studies

The effects of pH on the residual 3 β -HSD activities of the Y154F, K158Q, Y269S and K273Q mutants were measured in incubations at 27 °C containing 5 α -androstane-3 β -ol-17-one (100 μ M), NAD⁺ (0.2 mM) and purified enzyme (0.08 mg) in 0.05 sodium phosphate pyrophosphate buffer, pH 7.4 or 9.7, using the 3 β -HSD assay at 340 nm.

Modeling and sequence alignment

The three-dimensional structure of human type 1 3 β -HSD/isomerase was modeled using the crystal structure (Thoden *et al.* 1996) of UDP-galactose 4-epimerase from *Escherichia coli* (Protein Data Bank Accession Code 1A9Z) as template, as described previously (Thomas *et al.* 2003).

Results

Homology modeling targets the 3 β -HSD catalytic domain

We recently reported that 40% of the fingerprint residues of *E. coli* UDP-galactose-4-epimerase, an SDR family member with a known crystallographic structure (Thoden *et al.* 1996), are conserved with respect to human 3 β -HSD1 (Thomas *et al.* 2003). The 3 β -HSD and epimerase catalytic events are closely related in that both use a Tyr-X-X-X-Lys motif at the same position in the primary structure for the oxidation of a hydroxyl group (Gerrattana *et al.* 2001). Similar to the catalytic Tyr149 and Lys153 in UDP-galactose-4-epimerase, our site-directed mutagenesis studies have identified Tyr154

and Lys158 as the catalytic residues for human 3 β -HSD1 (Thomas *et al.* 2002). Another fingerprint residue of UDP-galactose-4-epimerase is the catalytic Ser124, and human 3 β -HSD contains a pair of Ser residues (Ser123 and Ser124) that correspond to this position in the homologous proteins. Thus, our model of the catalytic domain predicts that one of these serine residues, most likely Ser124, may complete the catalytic triad for human 3 β -HSD1 (Fig. 1).

Site-directed mutagenesis, expression and purification of the wild-type and mutant enzymes

The 3 β -HSD1 cDNA mutants (S123A and S124A) were produced by double-stranded, PCR-based mutagenesis and inserted into baculovirus, as described in Methods. As shown by the immunoblot in Fig. 2, the baculovirus system successfully expressed the mutant enzyme proteins as well as the human wild-type 3 β -HSD1 in Sf9 cells. Each expressed enzyme was purified by our published method (Thomas *et al.* 1989) to apparent homogeneity, according to SDS-PAGE (Fig. 2).

Kinetic comparisons of the purified wild-type and mutant enzymes

The Michaelis–Menten kinetic values measured for substrate utilization by purified wild-type 3 β -HSD1 and by the S123A and S124A mutants are summarized in Table 1. The S124A mutant enzyme has no detectable 3 β -HSD activity. However, the S123A mutant exhibits a maximal turnover rate (k_{cat}) that is 24% of the wild-type 3 β -HSD1 activity and a K_m for DHEA that is threefold higher than 3 β -HSD1. S123A utilizes the isomerase substrate steroid with a similar K_m value compared with wild-type 3 β -HSD1, but the S124A mutant utilizes the isomerase substrate much less efficiently.

The cofactor kinetic values in Table 2 reveal that S123A reduces NAD⁺ with a K_m value that is very similar to the K_m of NAD⁺ for wild-type 3 β -HSD1. Although S124A has no detectable 3 β -HSD activity in the cofactor kinetic study, the K_m values measured for NADH as an allosteric activator of isomerase are similar for S124A, S123A and wild-type 3 β -HSD1.

pH-dependency of the wild-type and mutant enzymes

The pH dependency of the 3 β -HSD activities of the S123A and S124A mutants can help determine whether one of these Ser residues is involved in catalysis. If the residual dehydrogenase activity of the mutant enzyme is pH-dependent, the substituted amino acid must not be catalytic because the residual activity has to be due to a different amino acid in the enzyme (Schlegel *et al.* 1998). Although 3 β -HSD activity is undetectable for S124A using DHEA as substrate (Tables 1 and 2), the use of 5 α -androstan-3 β -ol-3-one as substrate enhances residual 3 β -HSD by threefold to detectable levels. This 3 β -hydroxy-5 α -reduced steroid is not a substrate for isomerase activity; therefore, feedback inhibition by a 3-keto-4-ene product steroid (e.g. androstenedione) of 3 β -HSD/isomerase does not limit the 3 β -HSD activity (Milewich *et al.* 1993). As shown in Table 3, the wild-type 3 β -HSD1 and S123A mutant exhibits substantial 3 β -HSD activity at pH 7.4, and that activity is increased by 2.7- to 2.8-fold at the optimal pH 9.7. However, even when 5 α -androstan-3 β -ol-3-one is used as substrate to enhance any residual activity, the S124A mutant enzyme has only trace 3 β -HSD activity at pH 7.4, and this trace activity is not affected when the pH is increased to 9.7 (Table 3).

Discussion

Human 3 β -HSD/isomerase is a unique member of the SDR family. The enzyme sequentially catalyzes the 3 β -HSD activity followed by the isomerase activity on a single, dimeric protein. The coenzyme product of the 3 β -HSD reaction, NADH, induces a conformational change in the enzyme protein that activates the isomerase reaction (Thomas *et al.*

Figure 1 Ribbon structure of the active site of human 3 β -HSD1 based on homology modeling. The primary sequences of 3 β -HSD/isomerase and UDP-galactose-4-epimerase were aligned as described in the text. The NAD⁺ and DHEA structures are included. The catalytic Tyr154 and Lys158 residues (Thomas *et al.* 2002) as well as the potentially catalytic Ser123 and Ser124 residues are shown. Ser124 is shown hydrogen-binding (dotted bonds) to the 3 β -hydroxyl group of DHEA. The Asp36 residue is shown hydrogen-binding (dotted bonds) to the 2',3'-hydroxyl groups of the adenosyl ribose group of NAD (Thomas *et al.* 2003).

Figure 2 Western immunoblots showing the expression of the mutant and wild-type enzymes by baculovirus, and SDS–PAGE of the purified mutant and wild-type enzymes. (A) In the Western blot, the Sf9 cell homogenate (3.0 µg) containing the S123A or S124A enzyme plus the purified control 3β-HSD1 (0.05 µg) was separated by SDS-polyacrylamide (12%) gel electrophoresis. The 42.0 kDa band of the enzyme monomer was detected with our anti-3β-HSD antibody. (B) In SDS–PAGE of the purified mutant enzymes, each lane was overloaded with 2.0 µg purified protein, and the bands were visualized by Coomassie blue staining.

1995). In our previous studies (Thomas *et al.* 1993, 1997), two tryptic peptides associated with the 3β-HSD and isomerase activities were localized in the primary structure of the type 1 enzyme with affinity-radiolabeled steroids. The Gly250 tryptic peptide contains Tyr253 and Asp257, which we have identified as the catalytic residues for the isomerase reaction (Mason *et al.* 1998, Thomas *et al.* 1998, 2003). When this peptide was protected from

affinity alkylation by isomerase substrate or NADH (Thomas *et al.* 1994, 1997), the Glu135 tryptic peptide was identified. The Glu135 peptide contains Tyr154 and Lys158 in a YXXSK motif that is likely to be involved in 3β-HSD catalysis because of its location in the Rossmann-fold domain (Jornvall *et al.* 1995). Figure 3 localizes these catalytic residues in the primary structure of human 3β-HSD1 and shows the positions of

Table 1 Substrate kinetics for the 3β-HSD and isomerase activities of the purified S123A, S124A and wild-type 3β-HSD1 enzymes

	3β-HSD ^a			Isomerase ^b		
	<i>K_m</i> µM	<i>k_{cat}</i> min ⁻¹	<i>k_{cat}/K_m</i> min ⁻¹ µM ⁻¹	<i>K_m</i> µM	<i>k_{cat}</i> min ⁻¹	<i>k_{cat}/K_m</i> min ⁻¹ µM ⁻¹
Purified enzyme						
3β-HSD1	3.7	3.3	0.89	26.8	33.4	1.25
S123A	11.2	0.8	0.07	27.6	8.5	0.31
S124A	N.D.	No activity detected		83.6	2.2	0.03

^aKinetic constants for the 3β-HSD substrate were determined in incubations containing dehydroepiandrosterone (2–100 µM), NAD⁺ (0.1 mM) and purified enzyme (0.03 mg) in 0.02 M postassium phosphate, pH 7.4, 27 °C. *k_{cat}* values (nmol product formed/min per nmol enzyme dimer) were calculated from the *V_{max}* values. N.D., not determined.

^bKinetic constants for the isomerase substrate were determined in incubations of 5-androstene-3,17-dione (15–100 µM) NADH (0.05 mM) and purified enzyme (0.01 mg) in 0.02 M postassium phosphate buffer, pH 7.4, 27 °C. Each *K_m* and *k_{cat}* value represents the mean of triplicate measurements with a standard deviation ≤7% of single mutant enzyme preparations.

Table 2 Coenzyme kinetics for the 3 β -HSD and isomerase activities of the purified S123A, S124A and wild-type 3 β -HSD1 enzymes

	3 β -HSD ^a			Isomerase ^b		
	K_m μM	k_{cat} min^{-1}	k_{cat}/K_m $\text{min}^{-1} \mu\text{M}^{-1}$	K_m μM	k_{cat} min^{-1}	k_{cat}/K_m $\text{min}^{-1} \mu\text{M}^{-1}$
Purified enzyme						
3 β -HSD1	34.1	3.6	0.11	2.4	34.9	14.5
S123A	35.6	0.7	0.02	2.4	7.1	3.0
S124A	N.D.	No activity detected		4.3	2.2	0.5

^aKinetic constants for the 3 β -HSD cofactor were determined in incubations containing NAD⁺ (20–100 μM), dehydroepiandrosterone (100 μM) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4, 27 °C. k_{cat} values (nmol product formed/min per nmol enzyme dimer) were calculated from the V_{max} values. N.D., not determined.

^bKinetic 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. Each K_m and k_{cat} value represents the mean of triplicate measurements with a standard deviation $\leq 6\%$ of single mutant enzyme preparations.

S123 and S124 plus the other 27 Ser residues (underlined).

In agreement with a similar motif (¹⁴⁹Y-X-X-X-K¹⁵³) in UDP-galactose-4-epimerase (Liu *et al.* 1997), the Tyr154 and Lys158 residues appear to be catalytic residues for 3 β -HSD according to the kinetic profiles of the mutant Y154F and K158Q enzymes (Thomas *et al.* 2002). Our homology model of the active site of 3 β -HSD (Fig. 1) localizes these residues in close proximity to the bound A-ring of DHEA and the nicotinamide moiety of NAD⁺. Identification of the third member of the catalytic triad, Ser, would have been difficult before the development of the homology model. There are 29 Ser residues in the 3 β -HSD primary structure (Thomas *et al.* 2002), but the model predicts that only Ser123 or Ser124 is oriented properly to hydrogen bond to the 3 β -hydroxyl group of DHEA and stabilizes the substrate for catalysis by Tyr154 and Lys158.

With DHEA as substrate, the S124A mutant enzyme lacks measurable 3 β -HSD activity, but the S123A mutant retains 24% of control 3 β -HSD activity. The extremely low residual 3 β -HSD activity of the S124A mutant observed with 5 α -androstane-3 β -ol-17-one as substrate was not increased by raising the pH from 7.4 to the optimal pH 9.7. However, the 3 β -HSD activity of the S123A mutant was increased 2.8-fold by this shift in pH, and this pH-dependent increase was mirrored by the wild-type 3 β -HSD activity. The lack of pH dependency of the residual 3 β -HSD activity of S124A strongly suggests that no other amino acid in the enzyme, even Ser123, can perform the catalytic function of Ser124. According to these data, the Ser124 residue appears to complete the catalytic triad for human 3 β -HSD1.

The effects of the mutations on the isomerase activity are also instructive. The S124A mutant utilizes the isomerase substrate steroid with a 3-fold

Table 3 Dependency of the residual 3 β -HSD activities of the purified mutant enzymes on pH

	pH 7.4		pH 9.7		pH 9.7/pH 7.4
	nmol/min per mg ¹	% WT1	nmol/min per mg	% WT1	activity ratio
Purified enzyme					
3 β -HSD1	41.6 $\pm$ 1.2	100	111 $\pm$ 2.1	100	2.7
S123A	9.2 $\pm$ 0.5	22	25.8 $\pm$ 0.4	23	2.8
S124A	2.2 $\pm$ 0.2	5	2.1 $\pm$ 0.3	2	1.0

¹3 β -HSD activities were measured in incubations at 27 °C containing 5 α -androstane-3 β -ol-17-one (100 μM), NAD⁺ (0.2 mM) and purified enzyme (0.08 mg) in 0.05 M sodium phosphate pyrophosphate buffer, pH 7.4 or pH 9.7. WT1 is human wild-type 3 β -HSD1. Values are the means $\pm$ standard deviations of triplicate determinations of single mutant enzyme preparations.

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T G W S C L V T G A G G F L G Q R I I R L L V K E K E L K E I R V L D K A F G P E L R E E

F S K L Q N K T K L T V L E G D I L D E P F L K R A C Q D V S V I I H T A C I I D V F G V

T H R E S I M N V N V K G T Q L L L E A C V Q A S V P V F I Y T S S I E V A G P N S Y K E I
123 124

I Q N G H E E E P L E N T W P A P Y P H S K K L A E K A V L A A N G W N L K N G G T L Y T C
154 158

A L R P M Y I Y G E G S R F L S A S I N E A L N N N G I L S S V G K F S T V N P V Y V G N V

A W A H I L A L R A L Q D P K K A P S I R G N F T Y Y I S D D T P H Q S Y D N L N Y T L
253 257

S K E F G L R L D S R W S F P L S 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 T L S N S V F T F S Y K K A Q R D L A Y K P L Y S W E E A K Q K T V E W V G S L V D R H K

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E T L K S K T Q

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Figure 3 Amino acid sequence of human type 1 3 β -HSD/isomerase. The targeted Ser123 and Ser124 residues are labeled, and all of the 29 Ser residues are underlined. The catalytic Tyr154 and Lys158 residues in the 3 β -HSD domain are labeled (Thomas *et al.* 2002). The critical Tyr253 and Asp257 residues in the isomerase catalytic domain are also noted (Thomas *et al.* 2003).

higher K_m and a 15-fold lower maximal turnover rate (k_{cat}) relative to the wild-type enzyme. This kinetic profile of S124A suggests that Ser124 participates in the alignment of 5-androstene-3,17-dione for the isomerase reaction. These data support our model for sequential activities of the bifunctional enzyme: the 3 β -HSD activity oxidizes DHEA to form 5-androstene-3,17-dione, which remains bound as the substrate steroid of the isomerase activity (Thomas *et al.* 1989, 1995). In contrast, the S123A mutant utilizes the isomerase substrate with the same K_m and with 25% of the maximal isomerase activity of the wild-type enzyme, which is the same decrease in k_{cat} measured for the 3 β -HSD activity of S123A. The kinetic profile of S123A suggests that the removal of this hydroxyl R-group adjacent to the critical Ser124 hinders the proper alignment of DHEA and 5-androstene-3,17-dione in the enzyme active site to some extent, but Ser123 clearly is not a catalytic residue for 3 β -HSD or a key substrate-alignment residue for isomerase.

The coenzyme kinetic profiles suggest that the S123A and S124A mutations alter only substrate utilization by the enzyme. The K_m values of S123A

measured for NAD⁺ as the cofactor for 3 β -HSD activity and for NADH as the allosteric activator of isomerase are almost identical to those of wild-type 3 β -HSD1. Although the complete lack of 3 β -HSD activity by S124A prevented the determination of a K_m value for the utilization of NAD⁺, there was sufficient isomerase activity remaining to measure a K_m value for NADH that was very similar to that of wild-type isomerase. These kinetic data are consistent with Ser124's serving as a critical residue for substrate, but not for coenzyme utilization.

The correct prediction that Ser124 functions with Tyr154 and Lys158 to complete the catalytic triad responsible for the human 3 β -HSD1 activity validates our homology model of the tertiary structure of the enzyme active site. This validation complements our previous study in which the homology model accurately identified Asp36 as the residue responsible for the strict preference of NAD⁺ as cofactor for the 3 β -HSD activity and of NADH as the activator of isomerase instead of NADP⁺ or NADPH respectively (Thomas *et al.* 2003). The homology model can be used to target other key amino acids responsible for subunit interactions of the enzyme dimer, coenzyme

binding, membrane interactions and differences between human 3 β -HSD1 and 3 β -HSD2 to characterize further the structure/function of this key steroidogenic enzyme.

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