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Structure/function of human type 1 3 β -hydroxysteroid dehydrogenase: an intrasubunit disulfide bond in the Rossmann-fold domain and a Cys residue in the active site are critical for substrate and coenzyme utilization

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Abstract

The human type 1 (placenta, breast tumors) and type 2 (gonads, adrenals) isoforms of 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD) are key enzymes in steroidogenic pathways leading to the production of all active steroid hormones. Kinetic analyses of purified 3 β -HSD1 show that the Michaelis–Menten constants (K_m) for substrates and cofactor are decreased dramatically (3- to 8-fold) by the addition of β -mercaptoethanol (BME), which suggest that a disulfide bond may be critical to ligand utilization. Western immunoblots and SDS-PAGE of purified 3 β -HSD1 in the presence or absence of BME showed a lack of intersubunit disulfide bonds in the dimeric enzyme. The Rossmann-fold domain of 3 β -HSD1 contains two Cys residues, Cys72 and Cys111, which are capable of forming an intrasubunit disulfide bond based on their proximity in our structural model. Our structural model also predicts that Cys83 may affect the orientation of substrate and cofactor. To test these predictions, the C72S, C72F, C111S, C111A, C83S and C83A mutants of 3 β -HSD1 were produced, expressed, and purified. BME failed to diminish the K_m values of substrate and cofactor for C72S, C72F, C111S and C111A but produced a 2.5 decrease in K_m values for C83A ligands similar to wild-type 3 β -HSD. Thus, our results support the presence of an intrasubunit disulfide bond between Cys72 and Cys111 that participates in the tertiary structure of the Rossmann-fold domain. Although C83S had no enzyme activity, the C83A mutant enzyme exhibited 2- to 5-fold higher K_m values for substrate and cofactor but had similar K_{cat} values compared to wild-type 3 β -HSD. These data characterize the roles of Cys residues in 3 β -HSD and validate the predictions of our structural model.

Keywords

3 β -hydroxysteroid dehydrogenase; short-chain oxidoreductase; structure-based mutagenesis; structure-function relationship

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Introduction

The human type 1 (placenta, mammary gland, breast tumors, prostate, prostate tumors) and type 2 (gonads, adrenals) isoforms of 3 β -hydroxysteroid dehydrogenase (EC 1.1.1.145)/steroid Δ^5 - Δ^4 -isomerase (EC 5.3.3.1) (3 β -HSD/isomerase) are encoded by two distinct genes which are expressed in a tissue-specific pattern [1]. 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 [2]. 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 [3,4]. In addition to placenta and other human peripheral tissues, type 1 3 β -HSD/isomerase (3 β -HSD1) enzyme is selectively expressed in breast tumors [5], 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 (3 β -HSD2) is 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 3 β -HSD1 that can help control the timing of labor and slow the growth of hormone-sensitive tumors without compromising steroidogenesis in the adrenal enzyme.

In an early report [6], the Michaelis-Menten kinetic constants of substrates (DHEA, pregnenolone) for microsomal 3 β -HSD1 from human placenta, were obtained in buffer containing β -mercaptoethanol (BME), and the K_m values for these substrates were 0.2-0.4 μ M, 10-fold lower than the K_m values of 2-4 μ M reported by other investigators for microsomal [7] or purified 3 β -HSD1 [2] prepared from human placenta in the absence of BME. An elegant study using microsomal human placental 3 β -HSD in the presence of H₂O₂ or BME showed that the enzyme utilized substrates with dramatically lower K_m values when Cys residues were in the reduced form due to BME [8]. However, there has been no identification of disulfide bonds in human 3 β -HSD that may explain these results. The Rossmann-fold domain of 3 β -HSD1, a member of the short-chain oxidoreductase (SCOR) family of enzymes [9], contains two Cys residues, C72 and C111, that are capable of forming an intrasubunit disulfide bond based on their proximity in our structural model. To test this prediction, the C72S, C72F, C111S and C111A mutants of 3 β -HSD1 were produced, expressed, purified and characterized.

In addition, Cys83 is positioned in our structural model of human 3 β -HSD so that it may interact with cofactor in the active site. To determine if Cys83 of human 3 β -HSD is critical to the function of the enzyme and to test the effect of BME on this function, the C83S and C83A mutants were produced, expressed, purified and characterized.

2. Methods and materials

2.1 Chemicals

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

2.1 Bioinformatics/Computational Biochemistry/Graphics

As described previously [10], a three dimensional model of human 3 β -HSD1 has been developed based upon X-ray structures of two related enzymes: the ternary complex of *E. coli* UDP-galactose 4-epimerase (UDPGE) with an NAD cofactor and substrate (PDB AC: 1NAH) [11] and the ternary complex of human 17 β -hydroxysteroid dehydrogenase (17 β -

HSD1) with NADP and androstenedione (PDB AC: 1QYX) [12]. Amino acid sequence alignments were performed using CLUSTAL W (1.81) multiple sequence alignment [13]. The three-dimensional graphics of the enzyme were created using ICM Browser Pro (MolSoft L.L.C., La Jolla, CA) and Pymol (DeLano Scientific, San Carlos, CA).

2.2. Site-directed mutagenesis

Using the Advantage cDNA PCR kit (BD Biosciences Clontech, Palo Alto, CA) and pGEM-3 β HSD1 as template [14], double-stranded PCR-based mutagenesis was performed with the primers in Table 1 to create the cDNA encoding the C72S, C72F (C72A could be not created due to the codon differences), C111S, C111A, C83S and C83A mutants of 3 β -HSD1. 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). 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).

2.3. Expression and purification of the mutant and wild-type enzymes

The mutant 3 β -HSD1 cDNA was introduced into baculovirus as previously described [14]. Recombinant baculovirus was added to 1.5×10^9 Sf9 cells (1L) at a multiplicity of infection of 10 for expression of each mutant enzyme. The expressed mutant and wild-type enzymes were separated by SDS-polyacrylamide (12%) gel electrophoresis in presence or absence of BME (10 mM), probed with our anti-3 β -HSD1 polyclonal antibody and detected using the ECL western blotting system with antirabbit, peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Each expressed enzyme was purified from the 100,000 g pellet of the Sf9 cells (2 L) by our published method [2] using Igepal CO 720 (Rhodia, Inc., Cranbury, NJ) instead of the discontinued Emulgen 913 detergent (Kao Corp, Tokyo). Each expressed, purified mutant and wild-type enzyme produced a single major protein band (42.0 kDa) on SDS-polyacrylamide (12%) gel electrophoresis that co-migrated with the human wild-type 1 control enzyme in presence or absence of BME (10 mM). Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard [15].

2.4. Kinetic 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 (3-100 μ M) plus NAD⁺ (0.2 mM) and purified enzyme (0.04 mg) at 27°C in 0.02 M potassium phosphate, pH 7.4, in presence or absence of BME (10 mM). The slope of the initial linear increase in absorbance at 340 nm per min (due to NADH production) was used to determine 3 β -HSD1 activity. Kinetic constants for the isomerase substrate were determined at 27°C in incubations of 5-androstene-3,17-dione (20-100 μ M), with or without NADH (0.05 mM) and purified enzyme (0.01-0.04 mg) in 0.02 M potassium phosphate buffer, pH 7.4. Isomerase activity was measured by the initial absorbance increase at 241 nm (due to androstenedione formation) as a function of time. Blank assays (zero-enzyme, zero-substrate) assured that specific isomerase activity was measured as opposed to non-enzymatic, "spontaneous" isomerization [16]. In addition, isomerase incubations without added coenzyme (NADH) were used to measure any basal (zero-coenzyme) isomerase activity in the mutants, and this basal activity was subtracted as a blank. Changes in absorbance were measured with a Varian (Sugar Land, TX) Cary 300 recording spectrophotometer. The Michaelis-Menten constants (K_m , V_{max}) were calculated from Lineweaver-Burke (1/S vs. 1/V) plots and verified by Hanes-Woolf (S vs. S/V) plots. The

k_{cat} values (min^{-1}) were calculated from the V_{max} values (nmol/min/mg) and represent the maximal turnover rate ($\text{nmol product formed/min/nmol enzyme dimer}$).

Kinetic constants for the 3β -HSD1 cofactor were determined for the purified mutant and wild-type enzymes in incubations containing NAD^+ (10-200 μM), DHEA (100 μM) and purified enzyme (0.04 mg) in 0.02 M potassium phosphate, pH 7.4, in presence or absence of BME (10 mM) at 27°C using the spectrophotometric assay at 340 nm. Kinetic constants for the isomerase cofactor as an allosteric activator were determined in incubations of NADH (0-50 μM), 5-androstene-3,17-dione (100 μM) and purified enzyme (0.01-0.04 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.

Our duplicate determinations of the kinetic constants used highly purified mutant and wild-type enzymes to produce very reliable results with little variation between the values. The standard deviations reported for the mean values in the kinetic tables show that the variation between duplicates is $\leq 6\%$ in most cases and a maximum of 9% for the low-activity mutants. With such low variation in the duplicate kinetic determinations, differences of 2- to 8-fold between the kinetic constants of mutant and wild-type enzymes or in the presence or absence of BME are interpreted to represent real differences.

3. Results

3.1. Predictions of the functions of Cys residues by the structural model

Predictions of the functions of Cys72, Cys111 and Cys83 are based on our structural model of human 3β -HSD, which has 51% homology of the Rossmann-fold domain (amino acids 1-160) [8] and 40% identity of the key fingerprint residues that interact with bound substrate and cofactor compared to the crystallographic structure of *E. coli* UDP-galactose-4-epimerase [11]. Site-directed mutagenesis has confirmed the predicted function of the catalytic residues and key substrate and cofactor binding residues [8,17]. In the current study, our structural model of human 3β -HSD1 predicts an intrasubunit disulfide bond between Cys72 and Cys111 based on a distance of approximately 2.03 angstroms ($\text{\AA}$) between the S_γ atoms (Fig. 1). As shown in Fig. 2, the model predicts hydrogen-bonding of the sulfhydryl group of Cys83 with the 3'-hydroxyl group (2.80 $\text{\AA}$ distance) and possible interaction with the 2'-hydroxyl (4.6 $\text{\AA}$) on the nicotinamide ribose of the bound NAD^+ . There also is a hydrophobic interaction between Cys83 and carbon 3 (C3) of the NAD^+ ribose (3.19 $\text{\AA}$).

3.2. Site-Directed Mutagenesis, Expression and Purification of the Mutant Enzymes

The cDNA encoding the C72S, C72F, C111S, C111A, C83S and C83A mutants of 3β -HSD1 were produced by double-stranded, PCR-based mutagenesis and inserted into baculovirus. These amino acids were predicted to be critical residues by our modeled tertiary structure for human 3β -HSD (Fig. 1). 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]. In addition, the protein bands in Figures 3 and 4 show that BME did not break intersubunit disulfide links in the wild-type or mutant forms of 3β -HSD to produce protein bands of smaller size.

3.3 Kinetic Analyses of the Mutant Enzymes

The Michaelis-Menten kinetic values measured for the utilization of substrates and cofactors by purified C72S, C72F, C111S, C111A and C83A are summarized in Table 2 and Table 3. C83S has no measurable 3β -HSD or isomerase activity and is not included in the kinetic tables. Although BME produced a 2- to 8-fold decrease in the K_m values measured for the substrate and cofactors of the wild-type 3β -HSD and isomerase activities, BME failed to diminish the

K_m values of the substrates and cofactors for C72S, C72F, C111S and C111A. In contrast, the K_m values of the substrates and cofactor of C83A were diminished 2.5-fold by BME, similar to the effect measured with the wild-type enzyme. Thus, as predicted by our structural model, the presence of an intrasubunit disulfide bond between Cys72 and Cys111 is supported.

Cys83 is adjacent to the 2',3'-hydroxyl groups of the nicotinamide ribose of NAD⁺ in the 3 β -HSD active site according to our structural model (Fig. 1). Substitution of this Cys residue with a Ser (C83S) completely abolishes 3 β -HSD and isomerase activities, but substitution of Cys83 with an Ala (C83A) produces a fully active enzyme with elevated K_m values (2- to 5-fold) for both substrates and cofactor (Tables 2 and 3). Especially interesting is the ability of the isomerase activity of C83A to be fully functional without allosteric activation by NADH (Table 2). The basal isomerase activity of wild-type 3 β -HSD is only 5% of the maximal NADH-activated level of 536 nmol/min/mg [18], while the basal isomerase activity of C83A is already maximal at 570 nmol/min/mg in the absence of NADH.

4. Discussion

Ever since the kinetics of human 3 β -HSD1 were determined, widely different apparent K_m values have been reported for substrates of the enzyme from different laboratories. In enzyme preparations that utilized BME [6-8], the K_m values of substrate steroids were 10-fold lower than those reported by laboratories which avoided the use of BME [2,16,19], which reduces disulfide bonds. The dilemma has finally been resolved by the current study on the effects of BME on wild-type human 3 β -HSD1 and the 3 β -HSD1 mutants- C72S, C72F, C111S, C111A, C83S and C83A. In addition, the critical roles of these Cys residues in the function of human 3 β -HSD have been elucidated.

Although Ser is a conservative substitution for Cys in terms of the bulk of the amino acid side chain, our structural model predicts that the region of the Rossmann-fold occupied by Cys72 and Cys111 is very hydrophobic. The high polarity of Ser in the C72S mutant produced very low enzyme activities, and C111S had unusually high activities for both 3 β -HSD and isomerase (K_{cat} values in Tables 2 and 3). This suggests that the C72S and C111S mutants disrupted the conformation of the Rossmann-fold domain in that region (Fig. 1). In the western blot for C72S in the presence or absence of BME (Fig. 3), the protein band appeared as a duplex and to run slightly lower than the other 3 β -HSD proteins on the gel. This suggests that the protein conformation of C72S has been substantially altered by substitution of Cys with polar Ser at this position. In contrast, substitution of Cys72 and Cys111 with hydrophobic residues to produce the C72F and C111A mutants yields enzymes with catalytic activities (K_{cat} values) similar to the wild-type enzyme, suggesting that the mutant protein conformation in this region remained intact.

There are no intersubunit disulfide bonds in the enzyme dimer according to our SDS-PAGE and western blot analysis of pure enzyme with/without BME (Figures 3 and 4). However, our kinetic analyses of purified wild-type 3 β -HSD1 show that the Michaelis-Menten constants (K_m) for substrates and cofactors are decreased 3-8 fold by the addition of BME, which suggests that the intrasubunit disulfide bond is critical to ligand utilization. Our structural model for human 3 β -HSD predicts that Cys72 and Cys111 are aligned properly in the tertiary structure of the enzyme monomer for a disulfide link to form. The addition of BME did not decrease the K_m values of the substrates and cofactors for C72S, C72F, C111S and C111A. However, the K_m values of the substrates and cofactor of C83A were diminished 2.5-fold by BME. The lack of an intrasubunit disulfide link in the C72S, C72F, C111S and C111A mutant enzymes must have prevented the dramatic lowering of the K_m values for substrates and cofactors by BME that was observed for the wild-type enzyme. Conversely, C83A responded to treatment by

BME with a shift in kinetic values similar to that of wild-type 3β -HSD, most likely because the intact Cys72-Cys111 intrasubunit disulfide bond in C83A was reduced by the reagent.

Although most members of the short-chain oxidoreductase (SCOR) family of enzymes lack disulfide bonds, there are reports of an intrasubunit disulfide bond in the Rossmann-fold domain in the crystal structures of two other members of the SCOR family. The first is 3-oxoacyl-(acyl carrier protein) reductase from *Thermotoga maritima* (PDB 1O5I), a predicted 3-Beta ACP reductase. The disulfide bridge occurs between C37-C54 [20]. The second example is human L-xylulose reductase (PDB 1PR9) and that disulfide bridge is between C138-C150 [21]. Our demonstration of an intrasubunit disulfide bond between C72-C111 in human 3β -HSD represents the first assignment of a functional role for a disulfide bond in the SCOR family as a mechanism for enhancing the affinity of the enzyme for substrates and cofactors.

The Cys83 residue is positioned in our structural model (Figures 1 and 2) so that it could influence the orientation of enzyme-bound cofactor. The C83S mutation in the active site (Fig. 1) abolishes the 3β -HSD and isomerase activities of the enzyme, but the C83A mutation produces fully active enzyme with elevated K_m values for ligands. The presence of a polar Ser in the Cys83 location may block the hydrophobic interaction observed with the carbon 3 (C3) of the nicotinamide ribose of NAD^+ (Fig. 2), thus blocking the binding of cofactor and abolishing catalytic activity. The γ oxygen on the serine would also create repulsive electrostatic environment around the 3'-hydroxyl group of the ribose NAD^+ . The presence of the hydrophobic Ala in C83A would not interfere with this hydrophobic interaction or produce electrostatic repulsion but should abolish hydrogen-bonding of the sulfhydryl group of Cys83 with the 3'-hydroxyl group on the nicotinamide ribose of the bound NAD^+ , which is reflected by the dramatic increases in the cofactor and substrate K_m values. Another member of the SCOR family, UDP-galactose 4-epimerase from *Trypanosoma brucei*, has been crystallized that has a similarly positioned Cys99 residue within the NAD^+ binding pocket, which may contribute to the stability of the substrate through van der Waals forces [22].

In purified human placental 3β -HSD/isomerase (3β -HSD1), NADH is required as an allosteric activator of the isomerase activity [18]. The C83A mutant of 3β -HSD1 exhibits fully active isomerase in the absence of NADH (Table 3), which suggests that Cys83 is a key residue involved in the NADH-induced conformational change that activates isomerase. The addition of BME to incubations of C83A produce effects similar to those observed with wild-type 3β -HSD1: a 2.5-fold decrease in the K_m values of substrates and cofactors, which are most likely due to reduction of the Cys72-Cys111 intrasubunit disulfide bond by BME in the C83A mutant.

This evaluation of the roles of key Cys residues in human 3β -HSD1 has explained the reasons for the historically divergent values of Michaelis-Menten constants that have been reported in the literature for this enzyme [2,6-8]. The importance of an intersubunit disulfide linkage to utilization of substrate and cofactor has been characterized, and the role of a Cys residue in the orientation of cofactor by a member of the SCOR family of enzymes has been elucidated. Enhanced understanding of the structure and function of human 3β -HSD may lead to the development of specific inhibitors of the type 1 isoform in breast tumors to produce new approaches to the treatment of hormone-sensitive cancers.

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Fig. 1. The structural model of human 3 β -HSD1 shows the predicted intrasubunit disulfide bond between Cys72 and Cys111. The predicted distance between the S γ atoms is 2.03 Å. The position of the Cys83 residue relative to bound NAD⁺ and substrate, DHEA, are shown as well. The protein backbone (green), carbon (yellow), sulfur (gold), oxygen (red) and nitrogen (blue) atoms are indicated.

Fig. 2.
The predicted interactions of Cys83 with the nicotinamide ribose of bound NAD⁺ are shown. The carbon (green), sulfur (orange), oxygen (red) and nitrogen (blue) atoms are indicated. C3 is carbon 3.

Fig. 3.

Western immunoblots of the expressed mutant and wild-type enzymes in the presence or absence of 10 mM β -mercaptoethanol (BME). The Sf9 cell homogenate (4.0 μ g) containing H232A, S322A, N100A or N100S plus the purified control wild-type 3 β -HSD1 (0.05 μ g) were separated by SDS-polyacrylamide (7.5%) gel electrophoresis. The 42.0 kDa band of the enzyme monomer was detected using our anti-3 β -HSD antibody.

Fig. 4. SDS-Polyacrylamide (7.5%) gel electrophoresis of the purified mutant and wild-type enzymes in the presence or absence of 10 mM β -mercaptoethanol (BME). Each lane was over-loaded with 4.0 μ g of purified protein, and the bands were visualized by Coomassie Blue staining. The Amersham protein molecular weight marker was run to determine the size of protein bands.

Table 1
Oligonucleotide primers used for site-directed mutagenesis.

Mutation	Direction	Nucleotide sequence of primer ¹
C111A	Forward	5'- GAGGCCGCTGTCCAAGCTAGTGTG -3'
	Reverse	5'- TTGGACAGCGGCCTCTAACAGGAGC -3'
C111S	Forward	5'- GAGGCC <u>TCT</u> GTCCAAGCTAGTGTG -3'
	Reverse	5'- TTGGACAGAGGCCTCTAACAGGAG -3'
C83A	Forward	5'- CACCGCCGCTATCATTGATGTCTTCG -3'
	Reverse	5'- AATGATAGCGGCGGTGTGGATGATGAC -3'
C83S	Forward	5'- CACCGCCT <u>CT</u> ATCATTGATGTCTTC -3'
	Reverse	5'- AATGATAGAGGCGGTGTGGATGATG -3'
C72F	Forward	5'- AGAGCC <u>TTC</u> CAGGACGTCTCGGTCATC -3'
	Reverse	5'- GTCCTGGAAGGCTCTCTTCAGGAATG -3'
C72S	Forward	5'- AGAGCCAGCCAGGACGTCTCGGTCATCATC -3'
	Reverse	5'- GTCCTGGCTGGCTCTCTTCAGGAATG -3'

¹ The mutated codons are underlined.

Table 2 Substrate kinetics for the 3β-HSD and isomerase activities of the purified mutant and wild-type enzymes.

Purified Enzyme	K _m μM	3β-HSD ¹ k _{cat} min ⁻¹	k _{cat} /K _m min ⁻¹ μM ⁻¹	K _m μM	Isomerase ² k _{cat} min ⁻¹	k _{cat} /K _m min ⁻¹ μM ⁻¹
C72S (+ BME)	2.8 ± 0.2	1.2 ± 0.1	0.43 ± 0.03	19.2 ± 1.0	2.9 ± 0.1	0.15 ± 0.01
C72F	4.4 ± 0.3	1.3 ± 0.1	0.23 ± 0.01	26.8 ± 1.3	2.9 ± 0.1	0.11 ± 0.01
C72F (+ BME)	12.7 ± 0.5	3.2 ± 0.1	0.25 ± 0.01	62.0 ± 1.9	67.7 ± 2.4	1.09 ± 0.03
C111S	11.5 ± 0.6	3.0 ± 0.2	0.26 ± 0.01	67.0 ± 2.7	65.9 ± 2.6	0.98 ± 0.04
C111S (+ BME)	18.1 ± 0.5	5.2 ± 0.2	0.29 ± 0.01	78.5 ± 2.7	78.0 ± 2.3	0.99 ± 0.03
C111A	16.8 ± 0.7	5.2 ± 0.2	0.31 ± 0.01	65.9 ± 2.6	75.3 ± 3.0	1.14 ± 0.05
C111A (+ BME)	26.8 ± 1.6	1.9 ± 0.1	0.07 ± 0.004	32.0 ± 1.9	24.2 ± 1.5	0.76 ± 0.05
C83A	23.7 ± 1.3	1.8 ± 0.1	0.08 ± 0.004	30.8 ± 1.7	23.5 ± 1.3	0.76 ± 0.04
C83A (+ BME)	17.1 ± 1.0	3.6 ± 0.2	0.21 ± 0.01	70.9 ± 2.5	112.6 ± 3.4	1.59 ± 0.06
3β-HSD1	7.5 ± 0.3	2.8 ± 0.1	0.16 ± 0.01	28.1 ± 1.0	69.5 ± 2.4	2.47 ± 0.09
3β-HSD1 (+ BME)	3.7 ± 0.2	3.3 ± 0.2	0.89 ± 0.04	27.9 ± 1.1	50.2 ± 2.0	1.80 ± 0.08
	0.7 ± 0.04	2.9 ± 0.1	4.14 ± 0.20	5.9 ± 0.3	53.7 ± 2.7	9.10 ± 0.4

¹ Kinetic constants for the 3β-HSD substrate (DHEA) were determined in incubations containing NAD⁺ (100 μM), dehydroepiandrosterone (2–100 μM) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4, in presence or absence of BME (10 mM). Each value represents the mean of duplicate determinations ± standard deviation.

² Kinetic constants for the isomerase substrate (5-androstene-3,17-dione) were determined in incubations of NADH (50 μM), 5-androstene-3,17-dione (16.67–100 μM) and purified enzyme (0.02 mg) in 0.02 M potassium phosphate buffer, pH 7.4, in presence or absence of BME (10 mM).

Table 3
Cofactor kinetics for the 3β-HSD and isomerase activities of the purified mutant and wild-type enzymes.

Purified Enzyme	K_m μM	$NAD\ 3\beta\text{-HSD}^1$ k_{cat} min^{-1}	k_{cat}/K_m $\text{min}^{-1}\ \mu M^{-1}$	K_m μM	k_{cat} min^{-1}	$NADH\ Isomerase^2$ k_{cat}/K_m $\text{min}^{-1}\ \mu M^{-1}$
C72S	15.3 ± 0.8	1.3 ± 0.1	0.08 ± 0.005	4.8 ± 0.4	2.3 ± 0.2	0.48 ± 0.04
C72S (+ BME)	19.4 ± 1.2	1.2 ± 0.1	0.06 ± 0.004	4.7 ± 0.3	1.6 ± 0.1	0.34 ± 0.03
C72F	46.8 ± 2.8	2.6 ± 0.2	0.06 ± 0.004	1.5 ± 0.1	37.4 ± 2.2	24.9 ± 1.5
C72F (+ BME)	47.6 ± 2.4	2.5 ± 0.2	0.05 ± 0.004	1.6 ± 0.1	37.3 ± 2.2	23.3 ± 1.4
C111S	19.0 ± 1.0	5.2 ± 0.3	0.27 ± 0.01	0.7 ± 0.03	79.4 ± 4.0	113 ± 5.7
C111S (+ BME)	16.6 ± 0.8	5.2 ± 0.3	0.31 ± 0.01	0.7 ± 0.03	95.8 ± 4.8	137 ± 6.8
C111A	25.5 ± 1.8	2.1 ± 0.1	0.08 ± 0.006	3.5 ± 0.2	18.2 ± 1.0	5.2 ± 0.3
C111A (+ BME)	22.7 ± 1.6	1.9 ± 0.1	0.08 ± 0.006	4.6 ± 0.3	18.1 ± 1.0	7.9 ± 0.5
C83A	73.2 ± 3.7	3.8 ± 0.2	0.05 ± 0.003	N.S.	N.S. (570 ± 17) ³	
C83A (+ BME)	29.9 ± 1.8	2.4 ± 0.1	0.08 ± 0.005	N.S.	N.S. (368 ± 15) ³	
3β-HSD1	34.1 ± 1.7	3.5 ± 0.2	0.10 ± 0.005	4.6 ± 0.2	45.0 ± 1.8	9.8 ± 0.4
3β-HSD1 (+ BME)	12.0 ± 0.6	2.9 ± 0.1	0.24 ± 0.012	0.6 ± 0.03	30.0 ± 1.5	50.0 ± 2.8

¹ Kinetic constants for the 3β-HSD cofactor were determined in incubations containing NAD⁺ (13-100 μM), DHEA (100 μM) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4, in presence or absence of BME (10 mM). Each value represents the mean of duplicate determinations ± standard deviation.

² Kinetic constants for the isomerase cofactor were determined in incubations of NADH (2-50 μM), 5-androstene-3,17-dione (50 μM) and purified enzyme (0.02 mg) in 0.02 M potassium phosphate buffer, pH 7.4, in presence or absence of BME (10 mM).

³ N.S.- NADH not required to stimulate isomerase; (nmol/min/mg- basal isomerase activity without NADH).