STRUCTURAL AND PHYSIOLOGIC DETERMINANTS OF ESTRONE/ESTRADIOL METABOLISM CATALYZED BY HUMAN 17β-HYDROXYSTEROID DEHYDROGENASES TYPES 1 AND 2

APPROVED BY SUPERVISORY COMMITTEE

Committee member Stefan Andersson

Stefan Andersson, Ph.D.

Committee member Michael McPhaul

Michael McPhaul, M.D.

Committee member Perrin White

Perrin White, M.D.

ACKNOWLEDGMENTS

First and foremost, I thank my wife Tonya. She makes everything worth it. I thank my parents, Janis Sherbet and William Sherbet, and my sisters Lisa Singleton and Shelane Sherbet Greenlee. They shaped the person that I am.

I thank my mentor, Richard Auchus, for his careful guidance and enthusiastic support. He sets a standard as a physician-scientist that I aspire to reach.

I thank Mike McPhaul for serving on my committee and for his support through the UT Southwestern Medical Student Summer Research Program and during my year as a Howard Hughes Medical Institute Medical Student Research Fellow. I thank Stefan Anderrson and Perrin White for their support and suggestions and for serving on my committee. I thank the Howard Hughes Medical Institute for funding my fellowship year. I thank Kimberly Loggins for her patient administrative work during my year as a Howard Hughes Fellow.

I thank Gloria Brunschede, Mike Brown, and Joe Goldstein, with whom I began my scientific journey as an awestruck high school student.

Finally, I thank my friends from the Auchus lab: Manisha Gupta, Rick Hall, Naveed Khan, Shruti Krishnan, Jackie Naffin-Olivos, Mahboubeh Papari-Zareei, and Kerri Worthy. They made the last few years so much more fun.

STRUCTURAL AND PHYSIOLOGIC DETERMINANTS OF ESTRONE/ESTRADIOL METABOLISM CATALYZED BY HUMAN 17β-HYDROXYSTEROID DEHYDROGENASES TYPES 1 AND 2

by

DANIEL P. SHERBET

DISSERTATION

Presented to the Faculty of the Medical School

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF MEDICINE WITH DISTINCTION IN RESEARCH

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2006

ABSTRACT

STRUCTURAL AND PHYSIOLOGIC DETERMINANTS OF ESTRONE/ESTRADIOL METABOLISM CATALYZED BY HUMAN 17β-HYDROXYSTEROID DEHYDROGENASES TYPES 1 AND 2

Publication No. 1

DANIEL P. SHERBET

The University of Texas Southwestern Medical Center at Dallas, 2006

Supervising Professor: Richard J. Auchus, M.D., Ph.D.

The 17β-hydroxysteroid dehydrogenases (17β-HSDs) types 1 and 2 interconvert the weak and potent estrogens estrone and 17β-estradiol. In intact cells, each enzyme exhibits a strong directional preference that favors either oxidation (17β-HSD2) or reduction (17β-HSD1). A positively charged arginine (R38) adjacent to the 2′-phosphate stabilizes NADP(H) binding to 17β-HSD1 and favors reduction due to the high cytoplasmic NADPH/NADP+ ratio. In contrast, 17β-HSD2 has a negatively charged glutamate (E116) at the position corresponding to R38 of 17β-HSD1, which presumably repels the 2′-phosphate of NADP(H) and favors oxidation by harnessing the high cytoplasmic NAD+/NADH ratio. Substitution of a negatively charged aspartate, but not

neutral glycine, for R38 of 17β-HSD1 markedly reduces the affinity for NADP(H) and reverses the directional preference to oxidation in intact cells. We hypothesized that E116 confers oxidative preference to 17β-HSD2 and that substitution of either a neutral or a positively charged residue for E116 would reverse the directional preference to favor reduction. Mutations E116G, E116R, and the double mutation E116G+N117R failed to attenuate the >95% oxidative preference of 17β-HSD2 in intact cells. Affinity for all cofactors, as estimated by Km values, were measured for wild-type and mutant 17β-HSD2 enzymes in yeast microsomes. For wild-type 17β-HSD2, affinity for NAD(H) is nearly 1000-fold greater than for NADP(H), and the mutant enzymes retain high affinity for NAD(H) yet only slightly better affinity for NADP(H). We conclude that the directional preference of 17β-HSD1 is principally governed by electrostatic interactions between R38 and the 2′-phosphate of NADP(H), but that the oxidative preference of 17β-HSD2 is not solely due to E116 in the cofactor-binding domain. These data suggest that the directional preference of 17β-HSD2 is controlled by other aspects of its cofactor-binding domain, such as the size of the cofactor-binding pocket.

TABLE OF CONTENTS

PRIOR PUBLICATIONS & PRESENTATIONS

Sherbet, D.P. and Auchus, R.J. (2005) Human 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2) retains strict oxidative preference despite mutagenesis and chimerism that favor reduction. P2-296, 87th Annual Endocrine Society Meeting, San Diego. Abstract.

Sherbet, D.P. and Auchus, R.J. (2005) Distinct structural determinants govern the directional preference for human 17β-hydroxysteroid dehydrogenases types 1 and 2 in intact cells. 2005 Meeting of Medical Fellows, Howard Hughes Medical Institute, Chevy Chase, MD. Abstract and Oral Presentation.

Sherbet, D.P., and Auchus, R.J (2005) Structural determinants of 17β-hydroxysteroid dehydrogenase directionality. 43rd Annual UT Southwestern Medical Student Research Forum. Abstract and Oral Presentation.

Sherbet, D.P., Tiosano, D., Kwist, K.M., Hochberg, Z., and Auchus, R.J. (2003) CYP17 mutation E305G causes isolated 17,20-lyase deficiency by selectively altering substrate binding. *J Biol Chem 278*, 48563-48569.

Sherbet, D.P., and Auchus, R.J (2003) Biochemical characterization of E305G, a CYP17 mutation causing isolated 17,20-lyase deficiency. 41st Annual UT Southwestern Medical Student Research Forum. Abstract.

Sherbet, D.P., Tiosano, D., Koren, I., Hochberg, Z., and Auchus, R.J. (2002) CYP17 mutation E305G is devoid of Δ^5 lyase activity but has increased Δ^4 lyase activity: Genetic proof that the Δ^4 pathway is insufficient for complete formation of the male phenotype. OR62-5, 84th Annual Endocrine Society Meeting, San Francisco. Abstract and Oral Presentation.

Sherbet, D.P., and Auchus, R.J (2002) Mutation E305G in the active site of CYP17 causes isolated 17,20-lyase deficiency by a novel mechanism. 40th Annual UT Southwestern Medical Student Research Forum. Abstract and Oral Presentation.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

μg – Microgram

μM – Micromolar

11β-HSD – 11β-Hydroxysteroid dehydrogenase

17β-HSD – 17β-Hydroxysteroid dehydrogenase

AKR – Aldo-keto reductase

cDNA – Complementary deoxyribonucleic acid

ER – Endoplasmic reticulum

G6PDH – Glucose-6-phosphate dehydrogenase

H6PDH – Hexose-6-phosphate dehydrogenase

HEK-293 – Human embryonic kidney 293

HSD – Hydroxysteroid dehydrogenase

MR – Mineralocorticoid receptor

NAD+ – Nicotinamide adenine dinucleotide, oxidized form

NADH – Nicotinamide adenine dinucleotide, reduced form

NADP+ – Nicotinamide adenine dinucleotide phosphate, oxidized form

NADPH – Nicotinamide adenine dinucleotide phosphate, reduced form

PCOS – Polycystic ovary syndrome

PCR – Polymerase chain reaction

SCOR – Short-chain oxidoreductase

TLC – Thin-layer chromatography

WT – Wild-type

CHAPTER ONE
Introduction

Steroid hormones are responsible for a diverse repertoire of physiologic functions, including reproduction, growth, and metabolic homeostasis. Classic steroidogenic tissues such as the adrenal cortex, testis, and ovary synthesize a variety of steroids, which circulate in the bloodstream and act on target tissues. The response of a particular peripheral tissue to this steroid milieu, however, is determined by several factors. In order to respond to a specific class of steroid hormones, the cognate steroid receptors must be expressed by a particular tissue. As important as receptor content, however, is the ability of a tissue or cell to metabolize internalized steroids. An example of such an action is the kidney, which prevents transactivation of the mineralocorticoid receptor (MR) by metabolizing cortisol (a potent MR agonist) to the inactive steroid cortisone (a weak MR agonist). This reaction is catalyzed by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), a member of the hydroxysteroid dehydrogenase (HSD) class of enzymes. The various HSDs catalyze the activation or inactivation of specific steroids by catalyzing an oxidation or reduction reaction at discrete positions on the steroid nucleus. Thus, by interconverting weak and potent glucocorticoids, androgens, and estrogens, HSDs serve as crucial regulators of intracellular concentrations of active steroid hormones.

A number of diseases have been linked to deficiency of specific HSDs. Apparent mineralocorticoid excess is a disorder that presents with hypertension and hypokalemia in childhood, resulting from a deficiency of 11β-HSD2 in the kidney (1). Lack of 11β-

HSD2 activity results in a failure to inactivate cortisol to cortisone in the kidney and persistent activation of the MR independent of plasma volume. Similarly, the autosomal recessive, sex-limited disorder known as 17β-HSD3 deficiency results from a lack of 17β-HSD3 activity and a corresponding failure to convert androstenedione to testosterone (2). This failure to produce testosterone leads to male pseudohermaphroditism.

17β-HSDs types 1 and 2 are members of the short-chain oxidoreductase (SCOR) family of oxidoreductases (3). 17β-HSD1 utilizes NADPH as its preferred cofactor in intact cells, which enables the reduction of estrone to estradiol in the ovary and placenta. 17β-HSD2, in contrast, utilizes NAD+ as its preferred cofactor and thus functions in the oxidative direction in vivo, inactivating both estradiol and testosterone to the less potent 17-ketosteroids estrone and androstenedione, respectively, in peripheral tissues (4). These reactions appear to be unidirectional in intact cells because it is difficult to convincingly demonstrate metabolism in the "reverse" direction using conventional radiochemical assays. The observation that the 17β-HSDs appear to be unidirectional catalysts when expressed in intact cells contrasts with the bidirectional nature of the enzymes when purified and studied in the test tube (5). Such purified preparations typically catalyze reactions in either the oxidative or the reductive direction dependent upon reaction conditions utilized, including pH and the relative concentrations of all substrates—particularly cofactor—included in the reaction.

Recent work using a double-isotope scrambling assay has shown that the reactions catalyzed by 17β-HSDs types 1 and 2 are actually bidirectional in intact cells and achieve functional equilibria. Furthermore, the rates of the "reverse" reactions are two to three orders of magnitude faster than rates inferred from single-isotope assays and

equal to the rate of the "forward" reactions (6). For example, 17β-HSD1 reduces estrone to estradiol in intact HEK-293 cells, but the reaction proceeds to an equilibrium steroid distribution of 92% estradiol and 8% estrone, at which point the rates of conversion of estrone to estradiol and of estradiol to estrone are equivalent (6). Thus, both 17β-HSD isoforms catalyze rapid interconversion of their respective steroid pairs but appear to function in only one direction in vivo because they establish an equilibrium that strongly favors either the oxidized or reduced form of their substrate steroid pairs. This bidirectional model has important physiological consequences beyond just clarifying our understanding of 17β-HSD chemistry. For example, even small amounts of active 17-hydroxysteroids produced by the "backwards" reaction of an "oxidative" 17β-HSD could significantly alter gene expression due to the exquisitely high affinity of potent 17β-hydroxysteroids for their cognate receptors (6).

Due to the high cellular concentration of cofactor relative to steroid, the directional preference of a 17β-HSD is directly related to the affinity of the enzyme for a particular nicotinamide cofactor pair (5). For example, the large cytoplasmic [NADPH]:[NADP+] gradient drives 17β-HSD1 in the reductive direction because the NADPH/NADP+ cofactor pair binds strongly to 17β-HSD1. Conversely, 17β-HSD2 exhibits a much weaker affinity for the NADPH/NADP+ cofactor pair, instead preferring NAD+/NADH. Since cellular metabolism maintains a high [NAD+]:[NADH] gradient, the oxidized form of the cofactor drives steroid oxidation by 17β-HSD2. The central importance of cofactor to the directional preference of HSDs leads to the hypothesis that alterations in cofactor availability can alter 17β-HSD equilibrium set-points and directional preference. Cofactor availability may be alterable through several

mechanisms, including metabolic changes that alter cofactor concentrations and gradients, as well as structural changes in 17β-HSD cofactor binding sites that could lead to changes in cofactor affinity (5).

Insight into the structural mechanisms regulating cofactor affinity and therefore directional preference is suggested by the alignment of the cofactor binding domains of a number of SCOR-type HSDs shown in Figure 1.

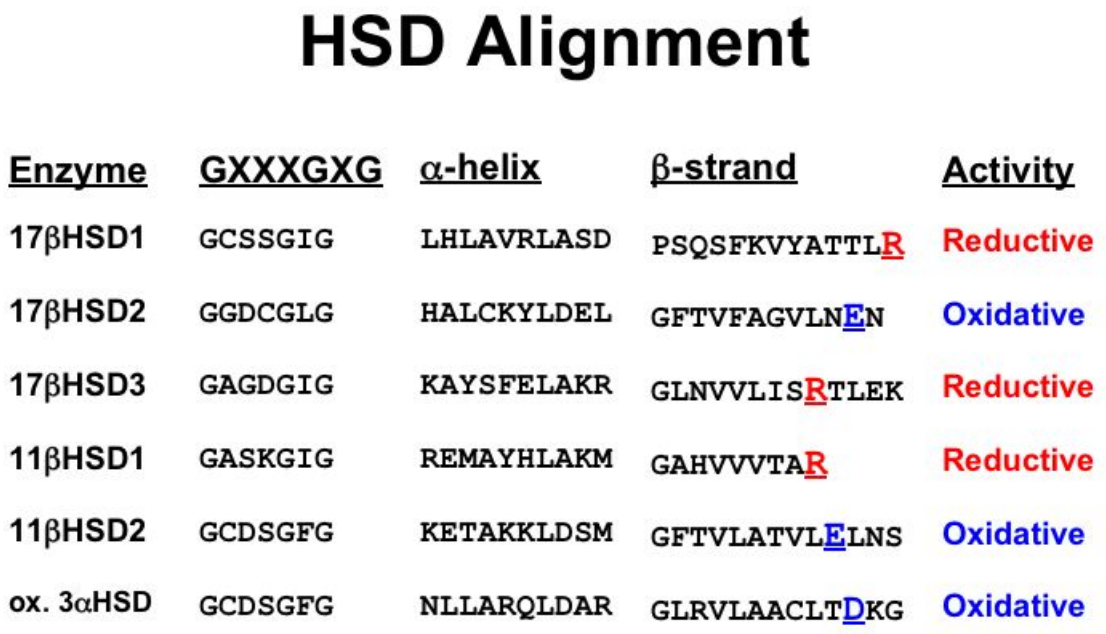

Figure 1. An alignment of the glycine-rich cofactor-binding region of a number of SCOR-type HSDs. Reductive HSDs tend to have a positively charged arginine (underlined, in red) residue at the C-terminal end of the first β-strand following the GXXXGXG motif, while oxidative HSDs have a negatively charged glutamate or aspartate in the corresponding position (underlined, in blue).

A positively charged arginine (R38 in 17β-HSD1) lies at the C-terminal end of the first beta strand following the glycine-rich cofactor-binding region of the reductive HSDs 17β-HSD1, 17β-HSD3, and 11β-HSD1. In the analogous position for oxidative

HSDs lies a negatively charged amino acid—either glutamic acid (17β-HSD2 and 11β-HSD2) or aspartic acid (oxidative 3α-HSD). Figure 2 shows the structure of the cofactor binding region for 17β-HSD1, with bound NADPH in yellow and R38 highlighted in blue.

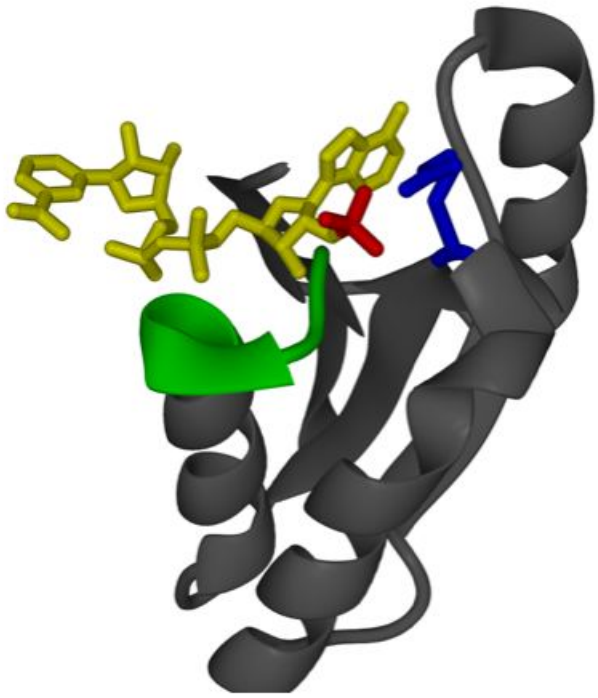

Figure 2. Cofactor-binding site of 17β-HSD1, showing NADP(H) in yellow, the 2'-phosphate of NADP(H) in red, and arginine 38 of 17β-HSD1 in blue (from reference 5) .

This diagram shows that R38 is in close approximation to the negatively charged 2'-phosphate of NADP(H). Thus, a positive charge in this position (as occurs with the reductive HSDs) is expected to stabilize the 2'-phosphate of NADP(H) and result in a high affinity for NADP(H) and the corresponding reductive directional preference that is observed in intact cells. Conversely, a negative charge in this position, as is common to

the oxidative enzymes, would repel the 2'-phosphate of NADP(H) and disfavor NADP(H) binding, while allowing NAD(H) to bind with high affinity and thus conferring an oxidative directional preference in intact cells. These observations have been confirmed in part by studies in which leucine 37 of 17β-HSD1 was mutated to aspartic acid to introduce a negative charge into the cofactor-binding region adjacent to the 2'-phosphate that distinguishes NADP(H) from NAD(H) (7). This mutation disfavors NADPH binding and allows NAD+ preferential access, as occurs naturally for the wild-type enzyme 17β-HSD2. This mutation converts 17β-HSD1 to an "oxidative" enzyme in vivo, inverting the equilibrium distribution to 95% estrone and 5% estradiol; however, the intrinsic rates of both reactions at functional equilibrium remain rapid (6).

As mentioned above, intermediate metabolism, which maintains normal cofactor gradients, should also affect HSD directional preference. Recent studies demonstrate that NADPH depletion (i.e. glucose deprivation) reduces the magnitude of the equilibrium estradiol/estrone ratio established by 17β-HSD1 (8). Thus, the equilibrium distribution established by 17β-HSD1, and likely other HSDs as well, is not fixed but rather is modifiable by factors both intrinsic and extrinsic to the enzyme. An understanding of these factors is crucial to understanding HSD physiology, in particular the mechanisms modulating target cell hormone concentrations in health and disease.

Mutations that modify cofactor usage and thus alter equilibrium steroid distributions are likely to be rare. However, it is more likely that physiological conditions that alter equilibrium distributions can cause human disease. For example, polycystic ovary syndrome (PCOS), which afflicts 5% of reproductive-aged women, is characterized by an overproduction of testosterone and dihydrotestosterone, yet the

relative contributions of the various enzymes responsible for this androgen excess remain unknown. The human HSD enzyme that efficiently converts androstenedione to testosterone is 17β-HSD3, but this isoform is expressed only in the testis. One hypothesis is that in PCOS the equilibrium steroid distribution of 17β-HSD2, which normally produces androstenedione, has been altered so that it now "produces" testosterone. Since only a small change in the androstenedione/testosterone ratio is necessary to yield an androgen excess state, large shifts in the equilibrium distribution are not required.

Finally, the recent identification of an alternate pathway to dihydrotestosterone that does not use testosterone as an intermediate hints further at the importance of HSD directional preference in steroid physiology. In this "backdoor" pathway, 5α-reduced C_{21} steroids are 3α-reduced by 3α-HSD enzymes, cleaved to 5α-reduced C_{19} steroids directly, and converted to dihydrotestosterone by oxidative 3α-HSDs in target tissues (9). The 3α-HSD isoforms responsible for these conversions are not known, and the fine regulation of these transformations also rests on the principles of HSD directional preference.

CHAPTER TWO
Materials and Methods

Site-directed Mutagenesis

Sequential PCR using overlapping mutagenic oligonucleotides (10) was used to introduce mutations R38K, R38G, and R38D into 17β-HSD1, and mutations E116G, E116D, E116G+N117R (double mutation), and E116R into 17β-HSD2. The oligonucleotide primer sequences used for these PCRs are listed in Table 1.

Primer	Sequence (5'-3')
pPGKS2	GTAGAGATAACGTCGATGACTTCCC
tPGKAS1	GCAACACCTGGCAATTCCTTACCTTCC
17β2S1Bg	AGATCTATGAGCACTTTCTTCTCGG
17β2AS1Eco	GAATTCCTAGGTGGCCTTTTTCTTG
17β1R38KAS	TTTCAGGTCCTTCAACGTGGCATACAC
17β1R38KS	GCCACGTTGAAGGACCTGAAAACACAG
17β1R38GAS	TTTCAGGTCACCCAACGTGGCATACAC
17β1R38GS	GCCACGTTGGGTGACCTGAAAACACAG
17β1R38DAS	TTTCAGGTCATCCAACGTGGCATACAC
17β1R38DS	GCCACGTTGGATGACCTGAAAACACAG
17β2E116DAS	TGGGCCATTGTCATTCAAAACTCCGGC
17β2E116DS	AGTTTTGAATGACAATGGCCCAGGAGC
17β2E116GAS	TGGGCCATTTCCATTCAAAACTCCGGC
17β2E116GS	AGTTTTGAATGGAAATGGCCCAGGAGC
17β2E116RAS	TGGGCCATTTCTATTCAAAACTCCGG
17β2E116RS	AGTTTTGAATAGAAATGGCCCAGGAGC
17β2EG,N117RAS	TCCTGGGCCTCTTCCATTCAAAACTCC
17β2EG,N117RS	TTGAATGGAAGAGGCCCAGGAGCTGAG

Table 1. Primer sequences used for site-directed mutagenesis. All primers are listed in 5'-3' orientation.

Oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA). Primer pairings for the initial and final sets of reactions, along with reaction conditions, are detailed in Table 2.

Mutation	Reaction 1 Primers	Reaction 2 Primers	Reaction 3 Primers
17β-HSD1 R38K	pPGKS2 + 17β1R38KAS	17β1R38KS + tPGKAS1	pPGKS2 + tPGKAS1
17β-HSD1 R38G	pPGKS2 + 17β1R38GAS	17β1R38GS + tPGKAS1	pPGKS2 + tPGKAS1
17β-HSD1 R38D	pPGKS2 + 17β1R38DAS	17β1R38DS + tPGKAS1	pPGKS2 + tPGKAS1
17β-HSD2 E116D	pPGKS2 + 17β2E116DAS	17β2E116DS + tPGKAS1	pPGKS2 + tPGKAS1
17β-HSD2 E116G	pPGKS2 + 17β2E116GAS	17β2E116GS + tPGKAS1	pPGKS2 + tPGKAS1
17β-HSD2 E116R	17β2S1Bgl + 17β2E116RAS	17β2E116RS + 17β2AS1Eco	17β2S1Bgl + 17β2AS1Eco
17β-HSD2 E116G+N117R	17β2S1Bgl + 17β2EG,N117RAS	17β2EG,N117RS + 17β2AS1Eco	17β2S1Bgl + 17β2AS1Eco

Table 2. PCR primer pairings used for 17β-HSD mutagenesis. Reactions were performed in 50 μL reaction volumes using 50 pmol of each primer, 200 μM dNTPs, and 2.5 U of Mercury brand True Fidelity polymerase (Continental Lab Products, San Diego, CA) in manufacturer's buffer. For reactions 1 and 2 for each mutation, 10 ng of template (V10-17βHSD1 for the 17β-HSD1 mutations, V10-17βHSD2 for the 17β-HSD2 single mutations, and pcDNA3-17βHSD2E116G for the 17β-HSD2 E116G+N117R double mutation) was used. Reaction 3 for each mutation utilized as template 1 μL of a 1:10 dilution in water of the PCR product from each of reactions 1 and 2. Cycling conditions for each PCR were as follows: 94 C for 3 min, followed by 26 cycles at 50 C for 30 s, 72 C for 1.5 min, and 94 C for 1 min, followed by a final annealing/extension cycle at 50 C for 30 s and 72 C for 4 min.

Final PCR products were gel-purified using the QIAEX II gel extraction kit (QIAEX Inc., Valencia, CA), digested with B*gl*II and *Eco*RI, and ligated into the *Bam*HI/*Eco*RI sites of the pcDNA3 mammalian expression vector (Invitrogen). Wild-type and mutant 17β-HSD2 cDNAs were digested with *Bgl*II and *Eco*RI and cloned into the *Bgl*II/*Eco*RI site of the yeast expression vector V10 (11). The resulting constructs were sequenced to confirm that only the intended mutation was introduced into the cDNA.

Chimeras

Chimeric cDNAs encoding an enzyme containing the first 80 amino acids of 11β-HSD2 joined to the N-terminal 308 amino acids of 17β-HSD2 were created by PCR utilizing primers with sequences that overlap both 17β-HSD2 cDNA and 11β-HSD2 cDNA (Table 3).

Primer	Sequence (5'-3')
T7	TAATACGACTCACTATAGGG
SP6	TACGATTTAGGTGACACTATAG
11β2Nterm17β2AS	TGCCTTCTGATCCACCGGCAGGCGCTGC
17β2CtermS	CAGCGCCTGCCGGTGGATCAGAAGGCAG
11β2S1Bgl	CAGATCTATGGAGCGCTGGCCTTGG

Table 3. PCR Primer sequences used for chimera creation.

These primers were utilized in the combinations detailed in Table 4 to create final cDNA products containing the N-terminus of 11β-HSD2 joined to the C-terminus of either wild-type 17β-HSD2 (11β2-17β2 WT), 17β-HSD2 E116G (11β2-17β2 E116G), or 17β-HSD2 E116G+N117R (11β2-17β2 E116G+N117R). These cDNA products were digested with *Bgl*II and *Eco*RI restriction enzymes and cloned into the *Bam*HI/*Eco*RI site

of pcDNA3. The resulting constructs were sequenced to confirm that chimerization was accomplished at the correct sequence points and that no inadvertent mutations were introduced.

Chimera	Reaction 1	Reaction 2	Reaction 3
11β2-17β2 WT	T7 + 11β2Nterm17β2AS	17β2CtermS + SP6	11β2S1Bgl + 17β2AS1Eco
11β2-17β2 E116G	T7 + 11β2Nterm17β2AS	17β2CtermS + SP6	11β2S1Bgl + 17β2AS1Eco
11β2-17β2 E116G+N117R	T7 + 11β2Nterm17β2AS	17β2CtermS + SP6	11β2S1Bgl + 17β2AS1Eco

Table 4. PCR pairings used for chimera creation. Reaction conditions and cycling parameters are as described in Table 2, with the inclusion of 3% DMSO for reactions 1 and 2 for each chimera. For reaction 1 for all three chimeras, 10 ng of 11β-HSD2 in pcDNA3 was used as template. For reaction 2, 10 ng of wild-type (WT) 17β-HSD2 in pcDNA3 was used as template, 10 ng of 17β-HSD2 E116G in pcDNA3 was used as template for the E116G chimera, and 10 ng of 17β-HSD2 E116G+N117R was used as template for the E116G+N117R chimera. Reaction 3 for each chimera used as template 1 μL of a 1:10 dilution in water of each of reactions 1 and 2 for that same chimera.

Steroid Metabolism in Transfected HEK-293 cells

HEK-293 cells were cultured and transfected as described previously (12). Briefly, cells were seeded into 6-well plates at ~60% confluency and transfected the next day with 1 μg of the pcDNA3 plasmid containing the appropriate cDNA. Cotransfections of expression vectors for 17β-HSD2 and mouse H6PDH (kindly gifted to us by Dr. Paul Stewart) were performed using 1 μg of each plasmid. The next day, the medium in each well was exchanged with 2 ml of complete medium containing the appropriate ^{3}H-labeled steroid (~200,000 cpm) along with unlabeled steroid to a final steroid concentration of 0.1 μM. Cells were incubated with this media at 37 C, and 0.6

mL aliquots were removed at 2, 4, and 8 hours. Media aliquots were extracted with a 1:1 mixture of ethyl acetate:isooctane, mixed with 10 nmol each of cold estrone and estradiol, dried under nitrogen, and subjected to thin-layer chromatography (TLC) as described previously. TLC plates were placed in an iodine chamber to allow visualization of estrone and estradiol spots, which were excised and quantitated with liquid scintillation counting as described (16).

17β-HSD2 Yeast Expression and Microsome Kinetics

The W303B strain of *S. cerevisiae* was transformed with V10 plasmid containing either wild-type 17β-HSD2 or the appropriate 17β-HSD2 mutation, as described (15). Microsomes were isolated as described previously (12). Microsome incubations were performed at 30 C in 50 mM potassium phosphate buffer (pH 7.4) with varying concentrations of microsomes, steroid, and nicotinamide cofactors. Reactions were stopped by extracting with 1:1 ethyl acetate:isooctane, subjected to TLC, and visualized/quantitated as described as described above for cell culture extracts. Km values were determined by assuming Michaelis-Menten kinetics and fitting data to Lineweaver-Burk plots.

17β-HSD1 Expression, Purification, and Kinetics

To express 17β-HSD1 and 17β-HSD1 mutation R38D in *E. coli*, the cDNA for wild-type 17β-HSD1 was digested with *Bam*HI and *Eco*RI, and then the approximately ~800 base pair (bp) fragment was cloned into the *Bam*HI/*Eco*RI site of bacterial expression vector pLW01 (13) to create pLW01-17β1Bam/Eco. Next, the cDNAs for 17β-HSD1 wild-type and 17β-HSD1 R38D, and plasmid pLW01-17β1Bam/Eco were digested with both *Bam*HI and *Nco*I. The ~180 bp *Bam*HI/*Nco*I fragments of the 17β-

HSD1 wild-type and 17β-HSD1 R38D cDNAs were cloned into the *Bam*HI/*Nco*I-digested pLW01-17β1Bam/Eco, creating pLW01-17βHSD1 and pLW01-17βHSD1R38D. These plasmids were transformed into *E. coli* strain C41(DE3) [Avidis, Saint-Beauzire, France]. Bacteria were then grown to an OD_{600} of ~1 in 100 ml of Terrific Broth supplemented with ampicillin, after which plasmid expression was induced with isopropyl-β-D-thiogalactopyranoside (Fisher) overnight at 24 C. Cells were collected by centrifugation and resuspended in 1.5 ml of 1.5 M sorbitol, 50 mM Tris•HCl, 1 mM EDTA pH 8.0. This suspension was incubated with 0.6 mg/ml lysozyme for 30 min at 4 C, followed by cell breakage with sonication. Cellular debris was removed by centrifugation, and glycerol was added to the supernatant to a concentration of 50%. Samples were heated at 67 C for 2 hours, followed by centrifugation at ~18,000 X g for 30 min to remove precipitate. The supernatant was diluted with 2.25 mL of 20 mM potassium phosphate, 1 mM EDTA, and shaken with 0.5 ml of Reactive Red agarose beads (Sigma) overnight at 4 C. Beads were collected by centrifugation and washed twice with 20 mM potassium phosphate, 1 mM EDTA, 20% glycerol, pH 7.4 [buffer KEG] containing 200 mM KCl, followed by one wash with buffer KEG. Beads were then washed three times with buffer KEG containing 1 mM NAD+ to elute 17β-HSD. The wash was concentrated and buffer was exchanged for KEG using a Centricon ultrafiltration unit (Millipore Corporation, Bedford, MA). Assays to estimate the apparent Km values for the resulting purified enzymes were performed in 50 mM KPi, pH 7.4 containing purified enzyme, 40 μM estrone (~100,000 cpm/reaction of [^{3}H]-estrone), and either 2-250 μM NAD or 0.2-400 μM NADP+. Apparent Km values were calculated by fitting data to Lineweaver-Burk plots.

CHAPTER THREE
Results

17β-HSD1

Transiently transfected HEK-293 cells expressing 17β-HSD1 in which the arginine at amino acid position 38 is substituted with lysine achieve an equilibrium distribution that lies overwhelmingly towards estradiol, similar to wild-type 17β-HSD1 (Figure 3, red line).

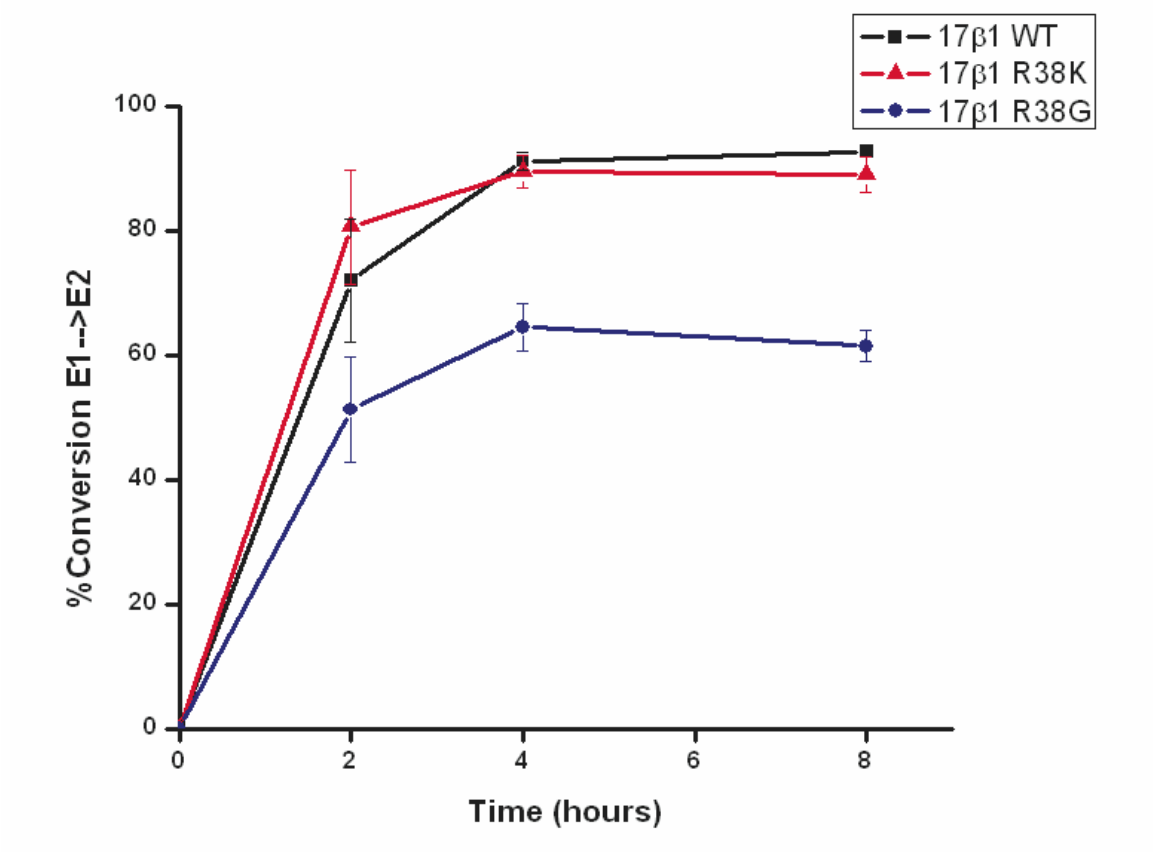

Figure 3. Cultured HEK-293 cells were transfected with expression vectors containing the cDNAs for either 17β-HSD1 WT or mutations R38K or R38G. Cells were then incubated with radiolabeled estrone, and medium was removed at 2, 4, and 8 hours for measurement of estradiol/estrone content. The results for 17β-HSD1 WT are plotted with black squares, R38K with red triangles, and R38G with blue circles. Each data point represents the mean +/- S.D. of at least 3 independent experiments (some error bars are unseen because they lie within the data points).

When neutral glycine is substituted for the positively charged arginine at position 38 (R38G), 17β-HSD1 establishes an equilibrium in intact cells that still lies predominately in the reductive direction, but less prominently (equilibrium distribution 60% estradiol, 40% estrone) than either wild-type 17β-HSD1 or mutation R38K (Figure 3, blue line). Figure 4 shows the results obtained by substituting negatively charged aspartate for arginine (R38D), thereby completely reversing the positive charge at position 38.

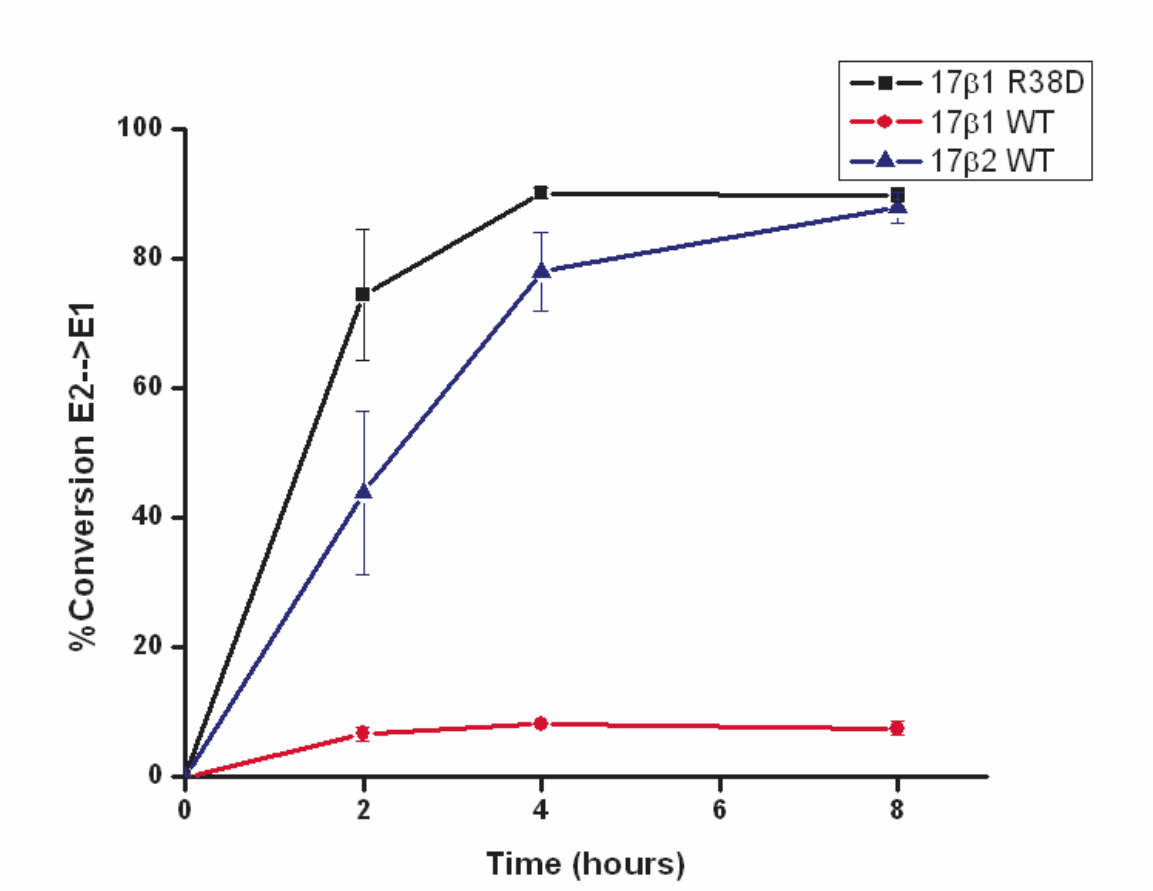

Figure 4. Cultured HEK-293 cells were transfected with expression vectors containing the cDNAs for either 17β-HSD1 mutation R38D, 17β-HSD1 WT, or 17β-HSD2 WT. Cells were then incubated with radiolabeled estradiol, and medium was removed at 2, 4, and 8 hours for measurement of estradiol/estrone content. The results for 17β-HSD1 R38D are plotted with black squares, 17β-HSD1 WT with red circles, and 17β-HSD2 WT with blue triangles. Each data point represents the mean +/- S.D. of at least 3 independent experiments (some error bars are unseen because they lie within the data points).

The resulting enzyme now exhibits an oxidative directional preference, with an equilibrium distribution approaching 90% estrone and 10% estradiol. This distribution is similar to that of wild-type 17β-HSD2 and starkly different from that seen with wild-type 17β-HSD1 (Figure 4).

In order to confirm that the basis for this shift in directional preference is due to a change in cofactor affinity, we expressed and purified both wild-type 17β-HSD1 and 17β-HSD1 R38D in *E. coli*. A sample gel showing various steps in the purification process is shown in Figure 5.

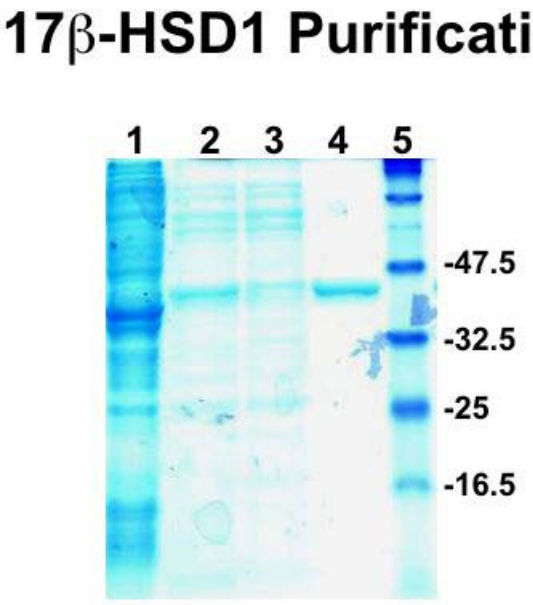

Figure 5 Coomassie-blue-stained polyacrylamide gel showing various fractions during the purification of 17β-HSD1. Lane 1, crude fraction. Lane 2, after heat treatment in 50% glycerol. Lane 4, unbound to Reactive Red agarose beads. Lane 4, after elution from Reactive Red Agarose beads, Lane 5, Molecular size marker

Pilot experiments with purified wild-type and R38D 17β-HSD1 demonstrate that the relative affinity of wild-type 17β-HSD1 for NADP+ is 20-fold greater than the affinity for NAD+ (Table 5). In contrast, mutation R38D exhibits a 3-fold higher affinity for

NAD+ over NADP+, demonstrating that a change in cofactor affinity is responsible for the change in directional preference.

	WT	***R38D***
NAD+	~ 10 μM *	~30 mM
NADP+	~0.5 μM	~100 mM

Table 5. Approximate apparent Km values for NAD(P) for 17β-HSD1 WT and R38D. The value for NAD+ for WT 17β-HSD1 is from reference 7. The data are from a single experiment.

17β-HSD2

Intact cells expressing 17β-HSD2 in which the glutamate at amino acid position 116 is substituted with aspartate (E116D) establish an equilibrium distribution similar to that seen with WT 17β-HSD2 (Figure 6, black squares and blue triangles, respectively).

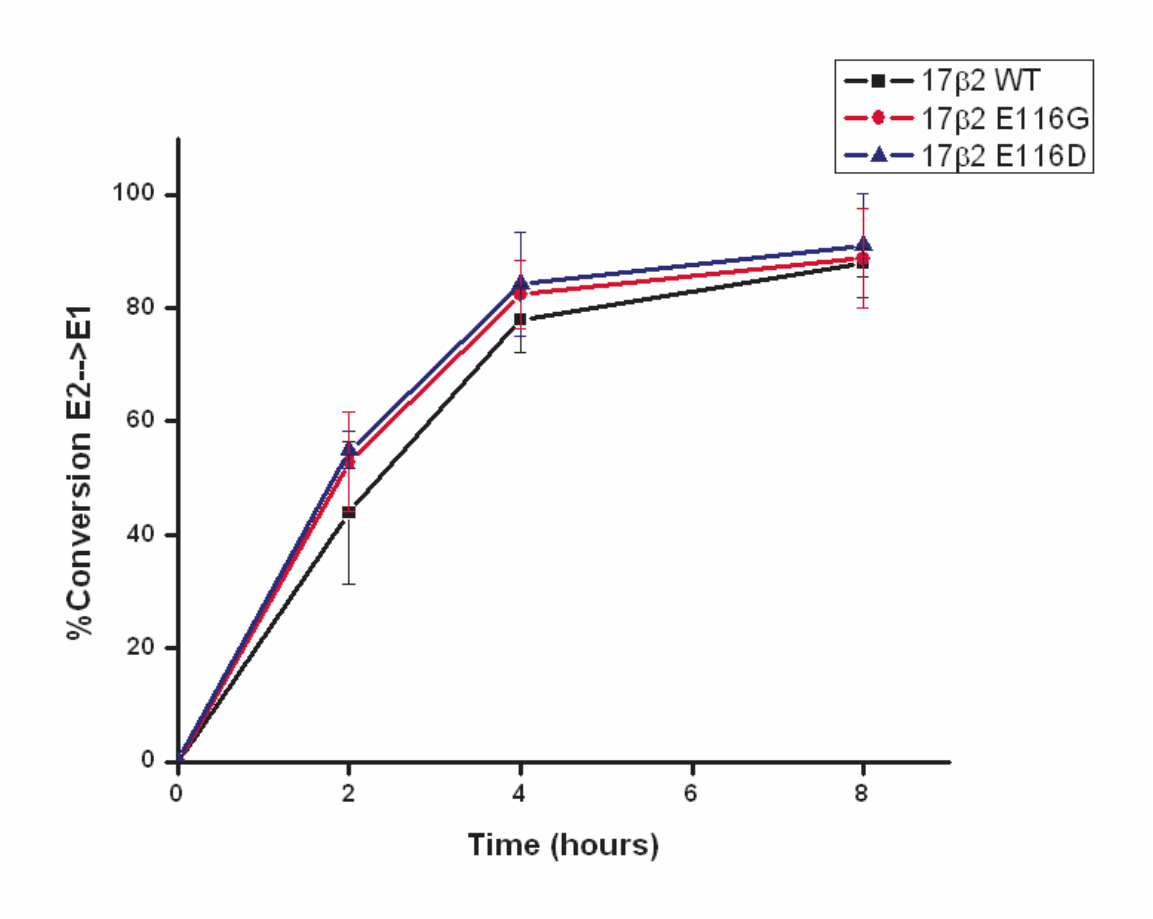

Figure 6. Cultured HEK-293 cells were transfected with expression vectors containing the cDNAs for either 17β-HSD2 WT, 17β-HSD2 E116D, or 17β-HSD2 E116G. Cells were then incubated with radiolabeled estradiol, and medium was removed at 2, 4, and 8 hours for measurement of estradiol/estrone content. The results for 17β-HSD2 WT are plotted with black squares, 17β-HSD2 E116G with red circles, and 17β-HSD2 E116D with blue triangles. Each data point represents the mean +/- S.D. of at least 3 independent experiments.

Contrary to our predictions, when E116 is completely neutralized by substituting glycine (E116G), the resultant mutant enzyme remains strongly oxidative in directional preference (Figure 6, red circles). A double mutation created by mutating N117 to a

positively charged arginine in the cDNA for E116G (E116G+N117R) also exhibited a strongly oxidative directional preference when introduced into cultured cells incubated with radiolabeled estradiol (Figure 7, blue triangles). Even when E116 was directly mutated to a positively charged arginine (E116R), the resulting enzyme maintained a strongly oxidative directional preference when expressed in HEK-293 cells.

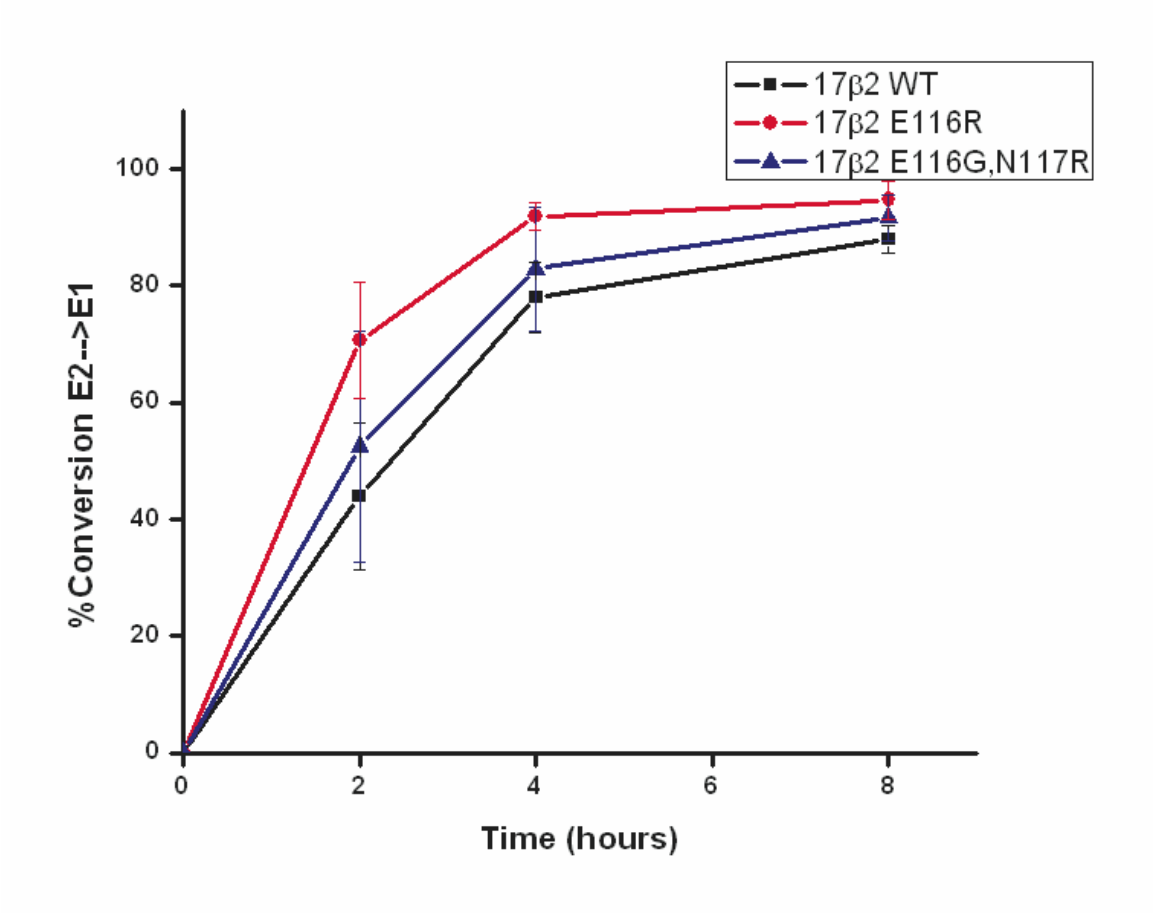

Figure 7. Cultured HEK-293 cells were transfected with expression vectors containing the cDNAs for either 17β-HSD2 WT, 17β-HSD2 E116R, or 17β-HSD2 E116G+N117R. Cells were then incubated with radiolabeled estradiol, and medium was removed at 2, 4, and 8 hours for measurement of estradiol/estrone content. The results for 17β-HSD2 WT are plotted with black squares, 17β-HSD2 E116R with red circles, and 17β-HSD2 E116G+N117R with blue triangles. Each data point represents the mean +/- S.D. of at least 3 independent experiments.

These results demonstrate that neither neutralization of the negatively charged glutamate in the cofactor-binding domain, nor addition of an arginine at or near this residue is sufficient to confer a reductive preference for 17β-HSD2 in intact cells.

One possible explanation for the failure of the aforementioned mutations to reverse the directional preference of 17β-HSD2 is the subcellular location of this enzyme. A KKYK motif in the N-terminus of 17β-HSD2 is predicted to orient its catalytic domain towards the lumen of the endoplasmic reticulum (ER). The ER lumen, in contrast to the cytoplasm, is a strongly oxidative environment with independent regulation of cofactor gradients. Only one HSD with a reductive directional preference is known to face the ER lumen: 11β-HSD1. The NADPH cofactor for 11β-HSD1 does not come from the cytoplasmic pool, but rather is generated by the activity of the intralumenal enzyme hexose-6-phosphate dehydrogenase (H6PDH) [14]. We considered the possibility that our HEK-293 cells are deficient in H6PDH. In order to ascertain the influence of H6PDH on both wild-type and E116G 17β-HSD2, H6PDH was expressed in HEK-293 cells along with either wild-type 17β-HSD2 or mutation E116G.

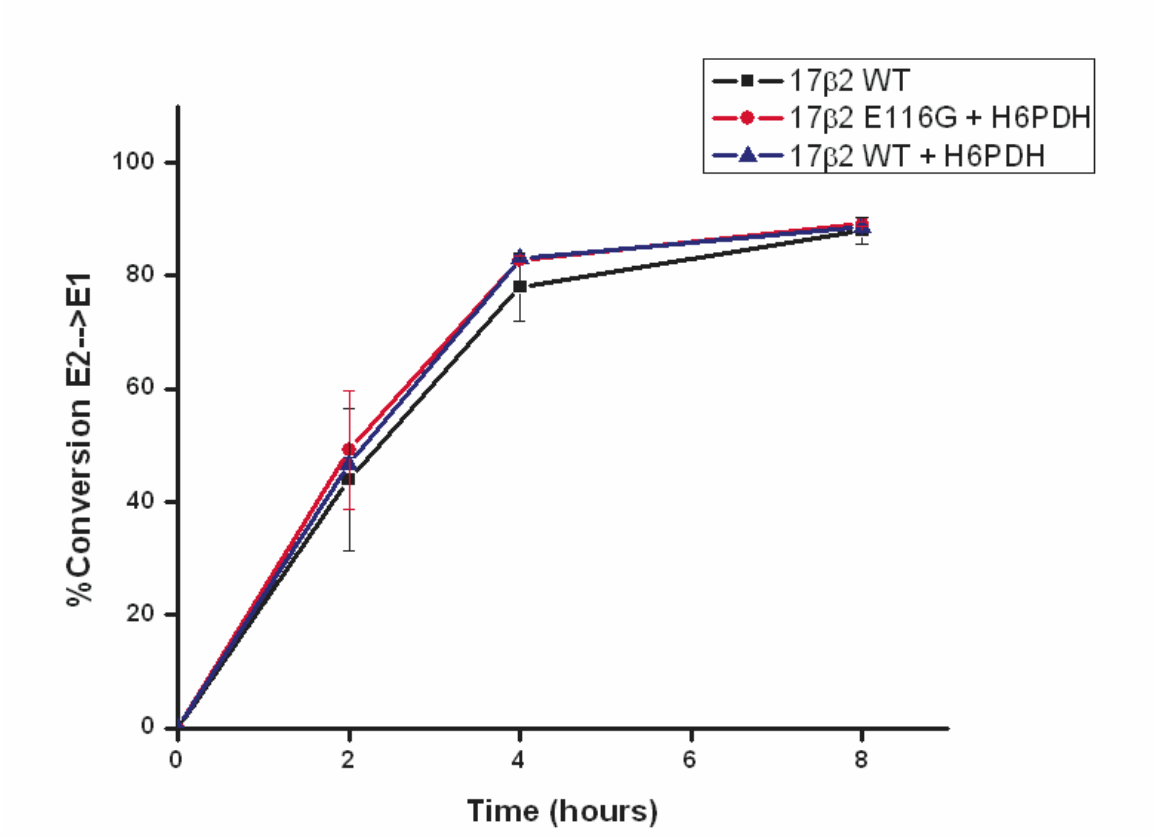

Figure 8. Cultured HEK-293 cells were transfected with expression vectors containing the cDNAs for either 17β-HSD2 WT, 17β-HSD2 WT + H6PDH, or 17β-HSD2 E116G + H6PDH. Cells were then incubated with radiolabeled estradiol, and medium was removed at 2, 4, and 8 hours for measurement of estradiol/estrone content. The results for 17β-HSD2 WT are plotted with black squares, 17β-HSD2 WT + H6PDH with red circles, and 17β-HSD2 E116G + H6PDH with blue triangles. Each data point represents the mean +/- S.D. of at least 3 independent experiments.

Figure 8 shows that expression of H6PDH had no effect on the directional preference of either enzyme, with both maintaining a strongly oxidative directional preference.

Finally, we attempted to reengineer 17β-HSD2 to face the cytoplasm by constructing chimeras with 11β-HSD2, an oxidative ER-bound HSD that faces the cytoplasm. The site of chimerization was chosen to be residue 80, because both 17β-HSD2 and 11β-HSD2 have identical lengths in this region and share proline and valine at residues 79-80, 8 residues N-terminal to the GXXXGXG motif. Chimerization of 17β-HSD2 by replacing its N-terminal 80 amino acids with the N-terminus of 11β-HSD2

(which directs the active site of 11β-HSD2 towards the cytoplasm) also had no effect on the oxidative directional preference of either wild-type, E116G, or E116G+N117R 17β-HSD2 (Figure 9). Thus, we were unable to alter the strong oxidative preference of 17β-HSD2 despite mutagenesis of the cofactor-binding region, coexpression of H6PDH, or chimerism.

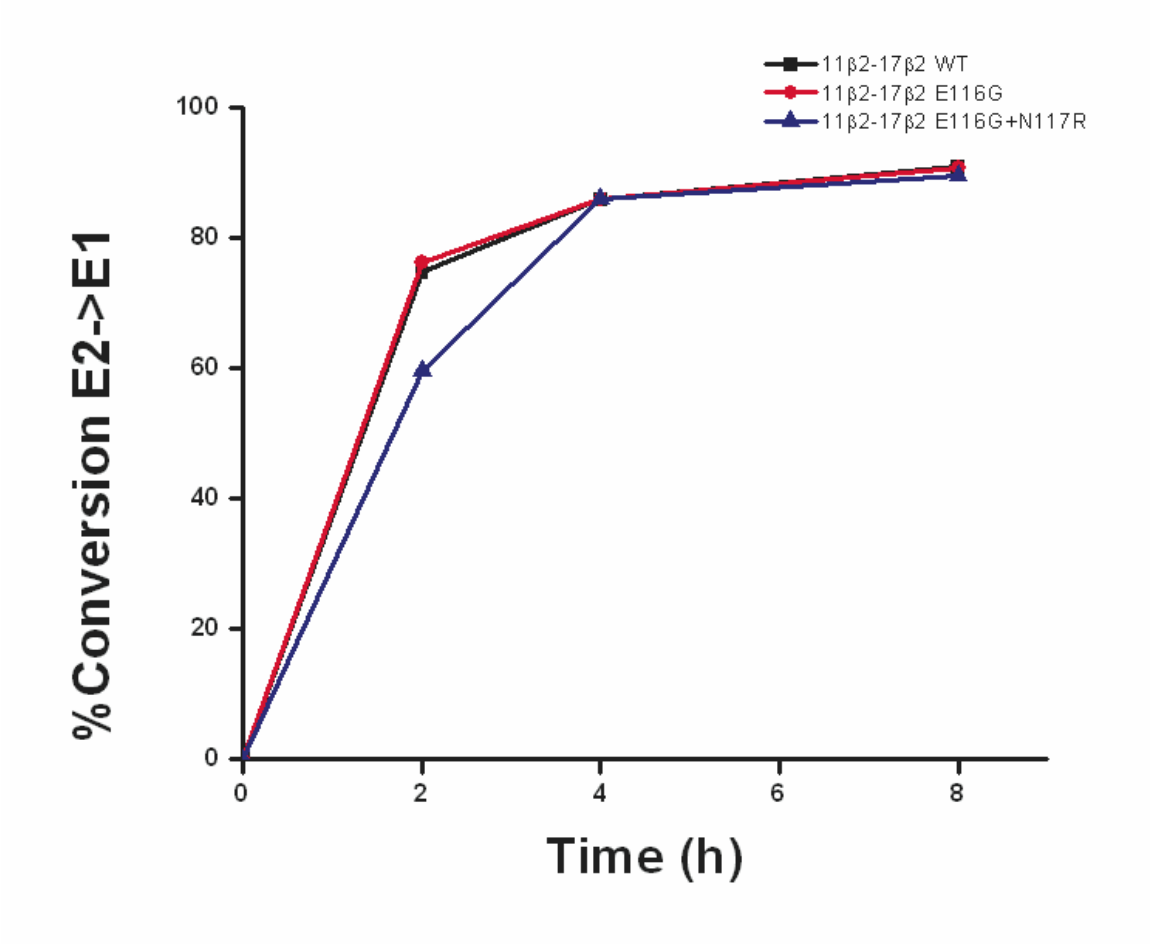

Figure 9. Cultured HEK-293 cells were transfected with expression vectors containing cDNAs for 11β-HSD2-17β-HSD2 chimeras constructed by joining the first 80 amino acids of 11β-HSD2 to the C-terminal 308 amino acids of either WT 17β-HSD2 (black squares), mutation E116G (red circles), or mutation E116G+N117R (blue triangles). Cells were then incubated with radiolabeled estradiol, and medium was removed at 2, 4, and 8 hours for measurement of estradiol/estrone content. The data are from a single experiment.

To understand the kinetic basis for the results of experiments with 17β-HSD2 and the various 17β-HSD2 mutations in intact cells, both wild-type 17β-HSD2 and mutations

E116G and E116R were expressed in yeast, microsomes were isolated, and kinetic experiments were performed to determine the apparent Km values of cofactors for each enzyme. The results are shown in Table 6.

	WT	***E116G***	***E116R***
NAD+	25.2 ± 11.6 μM	26.6 ± 5 μM	59.8 ± 12 μM
NADH	0.98 ± 0.3 μM	3.7 ± 1.5 μM	2.2 ± 0.77 μM
NADP+	12.2 ± 2.7 mM	6.2 ± 3.7 mM	7.2 ± 5.6 mM
NADPH	5.7 ± 4.7 mM	34.7 ± 0.3 mM	1.82 ± 0.4 mM

Table 6. Apparent Km values measured in microsomes isolated from yeast expressing either 17β-HSD2 WT, 17β-HSD2 E116G, or 17β-HSD2 E116R. Data shown are the mean ± S.D. (N=2 for WT with NAD, E116G with NADPH, and E116R with NADP, NADH, and NADPH. N≥3 for all other values)

The apparent Km values for both 17β-HSD2 WT, 17β-HSD2 E116G, and 17β-HSD2 E116R fall in the micromolar range for the NAD(H) cofactor pair. In contrast, apparent Km values for the NADP(H) cofactor pair falls in the millimolar range for all three enzymes. Thus, site-directed mutagenesis of the cofactor-binding domain of 17β-HSD2 failed to significantly increase the affinity for NADP(H) as predicted. These mutations also had little effect on the affinity of 17β-HSD2 for NAD(H).

CHAPTER FOUR
Discussion

As described above, previous studies have demonstrated the importance of cofactor availability on the directional preference of 17β-HSD1 and AKR1C9 (8,16). Thus, alteration of the NADPH:NADP+ gradient in intact cells can decrease and even reverse the reductive directional preference of 17β-HSD1. Similarly, we hypothesized that alteration of cofactor binding by changing relative affinities of the 17β-HSDs for specific cofactors would provide another mechanism by which directional preference can be modulated. We therefore introduced a variety of mutations into the cofactor-binding domains of both 17β-HSD1 and 17β-HSD2. These mutations were designed to alter the charge of crucial residues in both enzymes in an effort to alter the relative affinity of each 17β-HSD for its preferred cofactor.

Previous studies with mutation L37D of 17β-HSD1 demonstrated that it was possible to alter the directional preference of 17β-HSD1 by introducing a negative charge into the cofactor-binding domain (6,7). This mutation markedly decreased the affinity of 17β-HSD1 for NADPH, thereby allowing 17β-HSD1 to utilize NAD+ to drive oxidation of estradiol. R38 of 17β-HSD1 lies adjacent to the 2'-phosphate of bound NADP(H), and the x-ray structure of 17β-HSD1 with bound NADP+ suggests that the positively charged guanidinium stabilizes binding of the 2'-phosphate of NADP(H). A negative charge adjacent to R38 presumably would repel the 2'-phosphate, and is the basis for the decreased affinity of the L37D mutation for NADP(H) (7).

It is unclear from studies of L37D, however, whether simple neutralization of the positively charged arginine of 17β-HSD1 is sufficient to disfavor NADP(H) binding and

reverse the reductive directional preference of the enzyme. To answer this question and to further elucidate the critical residues for cofactor binding, we utilized a PCR-based site-directed mutagenesis to create three 17β-HSD1 mutations: R38K, R38G, and R38D. The cDNAs for these mutations were transfected into HEK-293 cells, incubated with [^{3}H]-estrone, and allowed to reach equilibrium. The R38K mutation displayed a strong reductive preference very similar to wild-type 17β-HSD1 (Figure 3). When R38 was neutralized by substituting glycine, the resulting mutant enzyme retained a reductive directional preference, but with a significantly decreased magnitude compared to wild-type 17β-HSD1 (Figure 3). These two results reinforce the importance of the positively charged arginine for maximizing reductive directional preference, presumably through the stabilizing interaction of arginine with the 2'-phosphate of NADP(H).

When R38 was replaced with negatively charged aspartate, thereby completely reversing the charge at position 38, a reversal of directional preference to oxidation was observed in intact cells (Figure 4). The R38D mutation exhibited an oxidative directional preference similar to that of wild-type 17β-HSD2, in contrast to 17β-HSD1 (Figure 4). This result confirms the conclusion drawn from previous experiments with the L37D mutation, demonstrating that 17β-HSD1 cannot maintain a reductive directional preference with a negative charge at the binding site for the 2'-phosphate of NADP(H). To confirm that these mutations changed the directional preference of 17β-HSD1 by altering relative binding affinity for the nicotinamide cofactors, 17β-HSD1 and the 17β-HSD1 mutations were expressed and purified from *E. coli*. Preliminary data from kinetic studies with these purified 17β-HSD1 enzymes indicates that the R38D mutation exhibits Km values in the millimolar range for both NAD+ and NADP+ but has a threefold higher

affinity for NAD+ over NADP+ (Table 5). In contrast, the Km values for recombinant wild-type 17β-HSD1 are 11.8 μM and 0.6 μM for NAD+ and NADP+, respectively (7).

The data from all three 17β-HSD1 mutations together suggest several rules governing the reductive preference of 17β-HSD1. First, a negative charge is prohibited in the binding site for the 2'-phosphate of NADP(H). Second, neutral residues in this position result in an enzyme that exhibits a reductive directional preference, albeit significantly decreased. Third, a positive charge in this region is necessary to stabilize the 2'-phosphate of NADP(H) and thus maximize reductive preference. These results and conclusions are concordant with those obtained with AKR1C9, another reductive HSD of the structurally and functionally distinct AKR family of HSDs (16). For AKR1C9, R276 forms a salt bridge with the 2'-phosphate of NADP(H), analogous to R38 of 17β-HSD1. the wild-type enzyme reduces dihydrotestosterone 98% to androstanediol at equilibrium in HEK-293 cells. Mutation R276G retains a slightly lower reductive preference that is readily attenuated by NADPH depletion. Mutation R276E, however, demonstrates a strong oxidative directional preference under all conditions.

Based upon the above results and those from AKR1C9, the basis of the maximal directional preference of the reductive HSDs is primarily determined by the absence of a negative charge in the cofactor-binding region, whereas the magnitude of this preference is maximized by presence of a positive charge. These results suggested that the structural basis of the directional preference for oxidative HSDs is driven by a negatively charged amino acid (E116 for 17β-HSD2) in the same region. We hypothesized that E116 of 17β-HSD2 alone confers an oxidative directional preference upon the enzyme, and that either neutralization of E116 or substitution of a positive charge for E116 would be

sufficient to enhance NADP(H) binding and to reverse the directional preference to favor reduction.

When the17β-HSD2 mutation E116D was introduced into HEK-293 cells, the resulting cells efficiently oxidized estradiol to estrone, demonstrating a strong oxidative directional preference similar to wild-type 17β-HSD2 (Figure 6). This result supported our hypothesis that a negative charge in the cofactor binding domain of an HSD maintains an oxidative directional preference. Unexpectedly, neutralization of this negative charge, achieved by introducing mutation E116G, did not attenuate the strong oxidative preference HEK-293 cells. This result suggests that E116, though well conserved among oxidative HSDs, is not required for an oxidative directional preference. Furthermore, introduction of a positive charge into the nicotinamide cofactor binding site also failed to alter the directional preference of 17β-HSD2, as evidenced by both the direct E116R mutation and the double mutation E116G+N117R (Figure 7). This result demonstrates that the oxidative directional preference of 17β-HSD2 persists even in the face of a positive charge, which we predicted would stabilize NADP(H) binding much like it does for the reductive enzymes.

Unlike 17β-HSD1 and a number of other SCOR-type enzymes, a KKYK motif in the N-terminus of 17β-HSD2 is predicted to orient its active site towards the lumen of the ER (17). The ER lumen is a strongly oxidizing environment; furthermore, cofactor gradients and their homeostatic mechanisms in the ER lumen are poorly understood and are different from those in the cytoplasm. Whereas glucose-6-phosophate dehydrogenase (G6PDH) is the principal NADPH-regenerating enzyme in the cytoplasm, hexose-6-phophate dehydrogenase (H6PDH) is an NADPH-generating enzyme located within the

lumen of the ER (18). H6PDH catalyzes the first two steps of the pentose phosphate pathway using glucose-6-phosphate as a substrate and generating NADPH from NADP+. H6PDH is essential to maintain the reductive activity of the enzyme 11β-HSD1, which, like 17β-HSD2, is also located in the ER membrane with its active site oriented towards the lumen (19).

Low levels of H6PDH have been found in HEK-293 cells, raising the possibility that these cells are deficient in the ability to generate sufficient NADPH within the ER lumen to allow our 17β-HSD2 mutations to catalyze ketosteroid reduction (20). To explore this possibility, mouse H6PDH was expressed in HEK-293 cells along with 17β-HSD2 and the 17β-HSD2 mutations. Expression of H6PDH produced no change in the oxidative directional preference of either wild-type 17β-HSD2 or the E116G mutation (Figure 8). An identical result was obtained when the double mutation E116G+N117R was expressed along with H6PDH, further reinforcing this result. Since we could not measure and/or alter cofactor availability and cofactor gradients within the ER lumen, we attempted to orient the active site of 17β-HSD2 towards the cytoplasm, where cofactor gradients are better understood.

To reorient the active site of 17β-HSD2 towards the cytoplasm, we created a chimeric enzyme containing the N-terminus of 11β-HSD2 joined to the C-terminus of 17β-HSD2. 11β-HSD2 is an oxidative HSD that catalyzes the conversion of cortisol to cortisone and is located in the ER membrane with its active site directed towards the cytoplasm. The N-terminus of 11β-HSD2 lacks the ER retention signal found in 17β-HSD2 (21). The cDNA segment encoding the 80 N-terminal amino acids of 11β-HSD2 was substituted for the segment encoding the N-terminus of wild-type 17β-HSD2 and

that of mutations E116G and E116G+N117R. When expression vectors containing the cDNAs for these chimeric enzymes were transfected into HEK-293 cells, the chimeric enzymes showed excellent activity and retained strong oxidative directional preferences (Figure 9).

Our inability to alter the directional preference of 17β-HSD2 despite mutating at or around E116 suggests, in contrast to reductive 17β-HSD1, that this residue is not the sole determinant of the oxidative directional preference of 17β-HSD2. Other factors must therefore contribute to maintaining the oxidative directional preference of 17β-HSD2. We investigated the possibility that the orientation of the active site of 17β-HSD2 towards the lumen of the ER might govern the oxidative directional preference by limiting NADPH availability. The strong oxidative directional preference in the face of coexpression of H6PDH as well as chimerism to favor cytoplasmic orientation suggests that the orientation of the active site of 17β-HSD2 towards the ER lumen also is not a primary determinant of directional preference in intact cells.

Our hypothesis regarding the impact of mutations at E116 on the directional preference of 17β-HSD2 hinged upon the ability of such mutations to alter cofactor affinity for 17β-HSD2. Since the above results showed that these mutations had no impact on the directional preference of 17β-HSD2 in intact cells, we directly measured the affinities of wild-type 17β-HSD2 and the17β-HSD2 mutations for the nicotinamide cofactors. Both wild-type and mutant 17β-HSD2 enzymes were expressed in yeast, from which microsomes were isolated and used for kinetic studies. The results of these studies are shown in Table 6. Wild-type 17β-HSD2 exhibits an affinity for NAD(H) in the micromolar range, 1000-fold higher than is millimolar affinity for NADP(H). These

relative affinities are only slightly attenuated for both the E116G and the E116R mutations. Our results support the hypothesis that a change in cofactor affinity is necessary to change the directional preference in intact cells, but our mutations were insufficient to substantially alter these affinities.

We conclude that additional structural features beyond E116 preclude high-affinity NADP(H) binding by 17β-HSD2 and render the oxidative directional preference of this enzyme resistant to alteration. One potential explanation which has yet to be explored is that the cofactor binding pocket of 17β-HSD2 is not large enough to accommodate the 2'-phosphate of 17β-HSD2.

Implications and Future Directions

Taken together, the results for 17β-HSD1 and 17β-HSD2 validate the importance of cofactor affinity in determining directional preference of HSDs. Further studies will be necessary to extend this work to other HSDs beyond 17β-HSD1 and 17β-HSD2. We predict that the directional preference of other reductive HSDs, like 17β-HSD1, is governed by the absence of a negative charge and the presence of a conserved positive charge in the cofactor-binding site. Studies with AKR1C9, mentioned above, support this model for the AKR enzymes. Future studies will target the positive charge of other SCOR-type HSDs, such as 17β-HSD3. Definitive kinetics with 17β-HSD1 and our 17β-HSD1 mutations will be performed to accurately measure the affinity of mutations R38G and R38D for NAD(H) and NADP(H). Furthermore, we have obtained crystals of wild-type 17β-HSD1 with bound cofactor, and we intend to obtain similar crystals for our

17β-HSD1 mutations, in order to study the structural basis for this change in cofactor affinity.

Our results with 17β-HSD2 suggest that the basis for the directional preference of oxidative HSDs is more complicated than that for reductive HSDs. Further studies with 17β-HSD2 are needed to understand the basis for the invariant oxidative directional preference of the enzyme. Our challenges with 17β-HSD2, however, are two-fold. First, since point mutations were unable to alter either cofactor binding or directional preference, other properties of the cofactor binding site of 17β-HSD2 are responsible for the poor affinity for NADP(H). Second, the putative orientation of 17β-HSD2 towards the ER lumen makes it difficult to understand what cofactor gradients it is exposed to.

The above challenges posed by 17β-HSD2 will be targeted in two ways. First, computer models of 17β-HSD2 will be constructed to identify other residues that impede NADP(H) binding and which can be targeted with site-directed mutagenesis. Second, protease protection assays and immunofluorescence studies can definitively demonstrate the orientation of both wild-type 17β-HSD2 and the 17β-HSD2 chimeras in the ER membrane. For this purpose we have obtained 17β-HSD2 antibodies and have created constructs encoding both wild-type and chimeric 17β-HSD2 enzymes containing a C-terminal tetrahistidine tag that acts as an epitope for commercially available antibodies.

We will also use site-directed mutagenesis to target the cofactor-binding region of other oxdidative HSDs. Preliminary studies in which D63 (analogous to E116 of 17β-HSD2) of RODH is mutated to glycine demonstrate that this enzyme also resists reversal of oxidative directional preference in the face of mutagenesis (22). Several other HSDs are inserted into the ER membrane, including 11β-HSD1, the oxidative 3α-HSD known

as RODH (oriented to the cytoplasm), and 11β-HSD2 (oriented to the cytoplasm). We plan to create chimeras to reorient these HSDs within the ER membrane to elucidate the effect of lumenal and cytoplasmic cofactor gradients on directional preference of HSDs.

Finally, we believe that the types of experiments and ideas developed in this thesis will provide a comprehensive understanding of the biochemical principles that govern the directional preferences, and thus the physiologic functions, of enzymes in the important and diverse family of hydroxysteroid dehydrogenases.

BIBLIOGRAPHY

1. **White PC** 2001 11β-hydroxysteroid dehydrogenase and its role in the syndrome of apparent mineralocorticoid excess. Am J Med Sci 322:308-315.
2. **Andersson S, Geissler WM, Wu L, Davis DL, Grumbach MM, New MI, Schwarz HP, Blethen SL, Mendonca BB, Bloise W, Witchel SF, Cutler GB Jr, Griffin JE, Wilson JD, Russell DW.** 1996 Molecular genetics and pathophysiology of 17β-hydroxysteroid dehydrogenase 3 deficiency. J Clin Endocrinol Metab 81:130-6.
3. **Duax WL, Thomas J, Pletnev V, Addlagatta A, Huether R, Habegger L, Weeks CM.** et al. 2005 Determining structure and function of steroid dehydrogenase enzymes by sequence analysis, homology modeling, and rational mutational analysis. Ann N Y Acad Sci. 1061:135-48.
4. **Andersson S, Moghrabi N** Physiology and molecular genetics of 17β-hydroxysteroid dehydrogenases. Steroids 62:143-7.
5. **Agarwal AK, Auchus RJ** 2005 Minireview: cellular redox state regulates hydroxysteroid dehydrogenase activity and intracellular hormone potency. Endocrinology. 146:2531-8.
6. **Khan N, Sharma KK, Andersson S, Auchus RJ.** 2004 Human 17β-hydroxysteroid dehydrogenases types 1, 2, and 3 catalyze bi-directional equilibrium reactions, rather than unidirectional metabolism, in HEK-293 cells. Arch Biochem Biophys. 429:50-9.
7. **Huang YW, Pineau I, Chang HJ, Azzi A, Bellemare V, Laberge S, Lin SX**. 2001 Critical residues for the specificity of cofactors and substrates in human

estrogenic 17β-hydroxysteroid dehydrogenase 1: variants designed from the three-dimensional structure of the enzyme. Mol Endocrinol. 15:2010-20.

8. **Papari-Zareei M and Auchus RJ**, unpublished observations.
9. **Auchus RJ.** 2004 The backdoor pathway to dihydrotestosterone. Trends Endocrinol Metab. 15:432-8.
10. **Gupta MK, Geller DH, Auchus RJ.** 2001 Pitfalls in characterizing P450c17 mutations associated with isolated 17,20-lyase deficiency. J Clin Endocrinol Metab. 86:4416-23.
11. **Pompon D, Louerat B, Bronine A, Urban P.** 1996 Yeast expression of animal and plant P450s in optimized redox environments. Methods Enzymol. 272:51-64.
12. **Sherbet DP, Tiosano D, Kwist KM, Hochberg Z, Auchus RJ.** 2003 CYP17 mutation E305G causes isolated 17,20-lyase deficiency by selectively altering substrate binding. J Biol Chem. 278:48563-9.
13. **Bridges A, Gruenke L, Chang YT, Vakser IA, Loew G, Waskell L.** 1998 Identification of the binding site on cytochrome P450 2B4 for cytochrome b5 and cytochrome P450 reductase. J Biol Chem. 273:17036-49.
14. **Draper N, Walker EA, Bujalska IJ, Tomlinson JW, Chalder SM, Arlt W, Lavery GG, Bedenko O, Ray DW, Laing I, Malunowicz E, White PC, Hewison M, Mason PJ, Connell JM, Shackleton CH, Stewart PM.** 2003 Mutations in the genes encoding 11β-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency. Nat Genet. 34:434-9.
15. **Auchus RJ, Lee TC, Miller WL.** 1998 Cytochrome b5 augments the 17,20-lyase

activity of human P450c17 without direct electron transfer. J Biol Chem. 273:3158-65.

16. **Papari-Zareei M, Brandmaier A, Auchus RJ.** 2006 Arginine 276 controls the directional preference of AKR1C9 (rat liver 3α-hydroxysteroid dehydrogenase) in human embryonic kidney 293 cells. Endocrinology. 147:1591-7.
17. **Wu L, Einstein M, Geissler WM, Chan HK, Elliston KO, Andersson S.** 1993 Expression cloning and characterization of human 17β-hydroxysteroid dehydrogenase type 2, a microsomal enzyme possessing 20α-hydroxysteroid dehydrogenase activity. J Biol Chem. 268:12964-9.
18. **Hewitt KN, Walker EA, Stewart PM.** 2005 Minireview: hexose-6-phosphate dehydrogenase and redox control of 11β-hydroxysteroid dehydrogenase type 1 activity. Endocrinology. 146:2539-43.
19. **Bujalska IJ, Draper N, Michailidou Z, Tomlinson JW, White PC, Chapman KE, Walker EA, Stewart PM.** 2005 Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11β-hydroxysteroid dehydrogenase type 1. J Mol Endocrinol. 34:675-84.
20. **Atanasov AG, Nashev LG, Schweizer RA, Frick C, Odermatt A.** 2004 Hexose-6-phosphate dehydrogenase determines the reaction direction of 11β-hydroxysteroid dehydrogenase type 1 as an oxoreductase. FEBS Lett. 571:129-33.
21. **Odermatt A, Arnold P, Stauffer A, Frey BM, Frey FJ.** 1999 The N-terminal anchor sequences of 11β-hydroxysteroid dehydrogenases determine their orientation in the endoplasmic reticulum membrane. J Biol Chem. 274:28762-70.
22. **Sherbet DP and Auchus RJ,** unpublished observations.

VITAE

Daniel Sherbet was born in Dallas, Texas. He grew up in the Dallas suburbs of Duncanville and DeSoto, and graduated from DeSoto High School as Valedictorian in 1997. He attended the University of Texas at Austin, where he was a Terry and a Crawford Scholar and was inducted into Phi Beta Kappa. At the University of Texas, he completed a Senior Honors Thesis under the guidance of Professor Steve Clarke. He graduated with honors with a Bachelor of Science in Biology in 2001.

Daniel matriculated at the University of Texas Southwestern Medical School in 2001, where he did research with Professor Richard Auchus. He presented posters and/or oral presentations at the annual UT Southwestern Medical Student Research Forum in 2002, 2003, and 2005 and at the 2002 and 2005 Endocrine Society annual meeting. He was awarded a 2004-2005 Howard Hughes Medical Institute Medical Student Research Fellowship, and a 2006 Endocrine Society Medical Student Achievement Award.

He will begin his postgraduate training in Internal Medicine at the University of Texas Southwestern Medical School in July 2006. He is married to Tonya Hart Sherbet, and they live in Dallas, Texas.

Contact Information:
1311 Brunner Ave
Dallas, Texas 75224
(214) 943-9363
danielsherbet@yahoo.com