Human oestrogenic 17β-hydroxysteroid dehydrogenase specificity: enzyme regulation through an NADPH-dependent substrate inhibition towards the highly specific oestrone reduction

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Human oestrogenic 17β-hydroxysteroid dehydrogenase (17β-HSD1) catalyses the final step in the biosynthesis of all active oestrogens. Here we report the steady-state kinetics for 17β-HSD1 at 37 °C and pH 7.5, using a homogeneous enzyme preparation with oestrone, dehydroepiandrosterone (DHEA) or dihydrotestosterone (DHT) as substrate and NADP(H) as the cofactor. Kinetic studies made over a wide range of oestrone concentrations (10 nM–10 μM) revealed a typical substrate-inhibition phenomenon. Data analysis using the substrate-inhibition equation $v = V \cdot [s]/\{K_m + [s](1 + [s]/K_i)\}$ gave a K_m of 0.07 ± 0.01 μM, a k_{cat} (for the dimer) of 1.5 ± 0.1 s^{-1}, a specificity of 21 $\mu M^{-1} \cdot s^{-1}$ and a K_i of 1.3 μM. When NADH was used instead of NADPH, substrate inhibition was no longer observed and the kinetic constants were significantly modified to 0.42 ± 0.07 μM for the K_m, 0.8 ± 0.04 s^{-1} for the k_{cat} and 1.9 $\mu M^{-1} \cdot s^{-1}$ for the specificity. The modification of an amino acid in the cofactor-binding site (Leu36Asp) eliminated the substrate inhibition observed in the presence of NADPH, confirming the NADPH-dependence of the phenomenon. The possible formation of an enzyme–$NADP^+$–oestrone dead-end complex during the substrate-inhibition process is supported by the competitive inhibition of oestradiol oxidation by oestrone. Kinetic studies performed with either DHEA ($K_m = 24 \pm 4$ μM; $k_{cat} = 0.47 \pm 0.06$ s^{-1}; specificity = 0.002 $\mu M^{-1} \cdot s^{-1}$) or DHT ($K_m = 26 \pm 6$ μM; $k_{cat} = 0.2 \pm 0.02$ s^{-1}; specificity = 0.0008 $\mu M^{-1} \cdot s^{-1}$) in the presence of NADP(H) resulted in low specificities and no substrate inhibition. Taken together, our results demonstrate that the high specificity of 17β-HSD1 towards oestrone is coupled with an NADPH-dependent substrate inhibition, suggesting that both the specificity and the enzyme control are provided for the cognate substrate.

Key words: dead-end complex, enzyme kinetics, oestradiol biosynthesis, steroid dehydrogenase.

INTRODUCTION

Oestradiol, the most potent oestrogen, is implicated in several physiological processes, such as pregnancy, lipid balance and bone density. Despite its important role in human physiology, oestradiol is also implicated in human pathologies as it acts as a mitogenic factor stimulating the proliferation of hormone-sensitive breast-cancer cells [1]. According to previous reports [2,3], the high levels of oestradiol found within breast-cancer cells are generated by the activity of human type-1 oestrogenic 17β-hydroxysteroid dehydrogenase (17β-HSD1, EC 1.1.1.62), an enzyme that is expressed in breast-cancer cells. Considering that breast cancer is the most common cancer among women in the Western world [4], and that it was estimated that during the year 2000 there would be more than 41 000 deaths from breast cancer in the U.S.A. alone [5], it is important to develop new therapeutic agents that will aid in the reduction of this form of cancer. In this respect, suppression of 17β-HSD1 activity may promote tumour regression by reducing tumour oestrogen levels [6].

17β-HSD1 has been studied since the late 1950s [7], and over the years it has been demonstrated that the enzyme is expressed in the gonads as well as in several peripheral tissues [8]. 17β-HSD1 catalyses the reversible 17β oxido-reduction of steroids *in vitro* [9] and, although the enzyme prefers C_{18} steroids with an aromatic A-ring [10], it can also, to a much lower extent, catalyse the *in vitro* oxido-reduction of androgens and other steroids [11]. The *in vitro* reaction can use either NAD(H) or NADP(H) as cofactor [7], but with different affinities [12]. Nevertheless, because the enzyme shows high specificity for triphosphate cofactors [12], these latter being predominantly present in their reduced form within cells ([NADPH] $\gg$ [$NADP^+$] [13]), 17β-HSD1 predominantly catalyses the reduction of oestrone into oestradiol in transformed cells [9].

Crystals of 17β-HSD1 exhibiting good diffraction properties were first grown in the early 1990s [14] and the three-dimensional structure of the enzyme was subsequently solved [15]. This membrane-associated protein, a homodimer of 34.5 kDa per subunit [16], possesses a conserved Tyr-Xaa-Xaa-Xaa-Lys sequence at the active site [17] and is a member of the short-chain dehydrogenase/reductase (SDR) superfamily. The binding site is composed of two clefts: one which binds the cofactor and the other one which binds the substrate. The first segment, βA–βF, forms a typical Rossmann fold which binds NAD(P)(H) cofactors, and the second segment, βD–βG, partially belonging to the Rossmann fold, governs steroid binding [15]. The three-dimensional structure of the complex of 17β-HSD1 with oestradiol highlights the high complementarity that exists between oestradiol and the active site [18]. Recent studies showing the complex formed between the enzyme and dehydroepiandrosterone (DHEA) revealed the contribution made by Leu-149,

Abbreviations used: 17β-HSD1, human type-1 oestrogenic 17β-hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; SDR, short-chain dehydrogenase/reductase.

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present in the active site, to the substrate recognition and C_{18}/C_{19} discrimination achieved by 17β-HSD1 [19]. From a mechanistic point of view, it has previously been established that the chemical transformation is not a rate-limiting step in the overall reaction and that the reaction could proceed via a random mechanism [20]. Recent reports have demonstrated the profound impact that the nature of the cofactor can have on 17β-HSD1 activity; for instance, in the presence of NADPH, the enzyme shows a higher specificity towards oestrone than in the presence of NADH [12]. Substrate inhibition has previously been observed for certain dehydrogenases, such as lactate dehydrogenase [21], trimethylamine dehydrogenase [22] and liver alcohol dehydrogenase [23]. Although 17β-HSD1 has been studied extensively for almost 50 years, to our knowledge no such mechanism of substrate inhibition has ever been reported for this enzyme.

EXPERIMENTAL

Materials

Unlabelled oestradiol, oestrone, NADP(H), Tris/HCl, BSA, disodium EDTA, glycerol and PMSF were obtained from Sigma (St Louis, MO, U.S.A). ^{14}C-radiolabelled oestradiol and oestrone were purchased from DuPont-NEN (Boston, MA, U.S.A). Diethyl ether, dichloromethane, toluene and acetone were obtained from Fisher Scientific. Bio-Rad Protein Assay Kit was purchased from Bio-Rad (Montreal, Canada). Silica-coated aluminium TLC plates were purchased from BDH. All other reagents were purchased from Sigma. Restriction endonucleases and modifying enzymes were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and Boehringer Mannheim (Mannheim, Germany). *Taq* DNA polymerase and *Pfu* DNA polymerase were from PE Applied Biosystems (Foster City, CA, U.S.A) and Stratagene Cloning Systems (La Jolla, CA, U.S.A) respectively. *Spodoptera frugiperda* (Sf9) cells, baculovirus expression system (AcMNPv linear-transfection module) and transfer vector pVL1393 were from Invitrogen (Carlsbad, CA, U.S.A.). Transfer vector pFastBac and DH10Bac-competent cells were from Life Technologies (Grand Island, NY, U.S.A.). QuikChange™ site-directed mutagenesis kit was from Stratagene Cloning Systems. All media and supplements used for cell culture were from Life Technologies. The Storm imaging system was from Molecular Dynamics (Sunnyvale, CA, U.S.A). The Aminco-Bowman Series 2 spectrofluorimeter was from Spectronic (Rochester, NY, U.S.A.). Two different software packages were used for kinetic data analysis, ENZFITTER (Biosoft, Cambridge, U.K.) version 1.05 (EGA) and Leonora version 1.0 March 1994 [24].

Site-directed mutagenesis of 17β-HSD1

The 17β-HSD1 Leu36Asp variant was generated via the PCR described by Higuchi et al. [25] and Ho et al. [26]. The 17β-HSD1 cDNA, isolated and characterized in our laboratory, was used as the template for generating the mutants. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit according to the supplier's protocol. Dideoxy nucleotide chain-termination sequencing, based on the method of Sanger and co-workers [27], was performed to verify the sequence integrity of the Leu36Asp variant. PCR fragments were subcloned into the baculovirus transfer vector pVL1393.

Production of the Leu36Asp variant using baculovirus

Generation of the Leu36Asp variant in recombinant baculovirus was achieved using the AcMNPv linear-transfection module. A co-transfection mixture containing 1 μg of linear AcMNPv genomic DNA, 3 μg of purified pVL1393/17β-HSD1 and cationic Insectin™ liposomes was incubated with monolayers of Sf9 cells at 27 °C for 5 days. Plaque assays were performed to select recombinant baculovirus, which were screened visually, then the resultant recombinant baculovirus was purified consecutively three times. PCR was performed to confirm the presence of 17β-HSD1 cDNA and to ensure the purity of each clone of recombinant baculovirus.

Expression of the Leu36Asp variant in Sf9 insect cells

Production of 17β-HSD1 from Sf9 cells was performed as described previously by Breton et al. [28]. Recombinant baculovirus, previously amplified and titred, was used to infect a suspension culture of Sf9 cells (1.8×10^6 cells/ml) at a multiplicity of infection of 10, performed at 27 °C. At 60 h post-infection, the cells were harvested by 10 min centrifugation at 1000 ***g***, washed once with PBS, pelleted and kept at -80 °C for subsequent use.

Determination of protein concentration

Protein concentration for the homogeneous 17β-HSD1 was determined using the Bradford assay. For the Leu36Asp variant, the following procedure was used to determine the protein concentration. The total protein concentration in the cell homogenates was measured by Bradford assay. In order to determine the percentage of protein corresponding to the Leu36Asp variant, SDS/PAGE was run and each band was quantified (ImageQuant software, using a STORM device). The percentage of Leu36Asp variant in the total could then be estimated by dividing the value for the Leu36Asp band by that of the total.

Enzyme purification and steady-state kinetics

17β-HSD1 was purified from fresh human placental tissue as described previously [16]. All reactions were performed at 37.0 ± 0.5 °C, and the reaction mixtures contained 50 mM Tris/HCl, pH 7.5, 0.05 mg/ml BSA and various amounts of ^{14}C-labelled oestrone. The assay concentrations of the cofactors were 500 μM NADH and 20 μM NADPH with the wild-type enzyme, and 1 mM NADH with the Leu36Asp variant. The final content of ethanol for each reaction was standardized to 2%. Reactions were initiated by the addition of the enzyme sample, aliquots were taken and the reaction was stopped by the addition of 3 vol. of diethyl ether on ice. The stopped reaction mixture was chilled using a dry ice/ethanol bath and the aqueous phase was discarded. Diethyl ether was then evaporated and steroids were resuspended in 60 μl of dichloromethane for separation by TLC. The migration solvent system consisted of a 4:1 ratio of toluene/acetone. TLCs were than exposed and quantified using a PhosphorImager (Molecular Dynamics). Initial velocities were measured with less than 10% substrate consumption and were expressed as μmol/min per mg or simply units/mg. Independent experiments, duplicates for the purified native enzyme and quadruplicates for the Leu36Asp variant in cell homogenate, were compiled and treated by the Leonora program or ENZFITTER. Leonora was used for the analysis of the data obtained from the reaction of the native enzyme with NADPH. This program permitted the determination of a K_i value using the substrate-inhibition equation, i.e. $v = V \cdot [s]/\{K_m + [s](1 + [s]/K_i)\}$ [24]. For the data obtained with the native enzyme in the presence of NADH and for the result obtained for the Leu36Asp variant with NADPH, ENZFITTER permitted the analysis of the kinetic parameters utilizing the standard Michaelis–Menten equation.

Kinetic measurements for DHEA and dihydrotestosterone (DHT) were performed using concentrations of steroids between 4 μM and 80 μM. Initial rates were followed and the same experimental conditions as utilized for oestrone were used. Kinetics for the oxidation of oestradiol were studied in the presence and absence of 2 μM oestrone as inhibitor. The reaction mixture contained 100 mM Tris/HCl, pH 7.5, 0.05 mg/ml BSA, 50 μM $NADP^+$, 4.4×10^{-5} mg/ml 17β-HSD1 and various amounts of oestradiol (1–5 μM). The experiments were performed in triplicate, using the fluorescent signal of NADPH at 37.0 ± 0.5 °C. Fluorescence spectroscopy was performed on an Aminco-Bowman Series 2 Luminescence spectrofluorimeter equipped with a temperature-regulated cell compartment. Excitation wavelength was 340 nm with a 4 nm slit, and emission wavelength was 460 nm with an 8 nm slit.

RESULTS

Steady-state kinetics using NADH as the cofactor: no observation of substrate inhibition

Using a saturating concentration of the cofactor (500 μM NADH in reaction; K_m of NADH = 5.7 ± 0.3 μM; Y.-W. Huang and S.-X. Lin, unpublished work) a v versus s plot for oestrone reduction at pH 7.5 and 37 °C was obtained (Figure 1, insert). The double-reciprocal curve (Figure 1) is linear and therefore indicates Michaelis–Menten kinetics. In order to analyse the kinetic parameters precisely, the ENZFITTER program was used. When the data from the v versus s plot were analysed using ENZFITTER, the following kinetic constants for oestrone reduction were obtained: $K_m = 0.42 \pm 0.07$ μM and $k_{cat\ dimer} = 0.8 \pm 0.04$ s^{-1}, thus $k_{cat}/K_m = 1.9$ $\mu M^{-1} \cdot s^{-1}$ (Table 1). The molecular mass of the dimer used to calculate the k_{cat} value was 69 kDa. It should be noted that initial velocities were followed for all experiments described in the Results section.

Steady-state kinetics using NADPH as the cofactor: observation of a substrate-inhibition phenomenon

Using a saturating concentration of NADPH as the cofactor (20 μM NADPH in reaction; K_m of NADPH = 0.9 ± 0.1 μM; Y.-W. Huang and S.-X. Lin, unpublished work) a v versus s plot for oestrone reduction at pH 7.5 and 37 °C was obtained (Figure 2, insert). The insert of Figure 2 shows that the initial velocity increases with oestrone concentration up to 0.2 μM and that it then falls with increasing concentrations of oestrone. Nevertheless, for the very high concentrations of oestrone, the initial velocity tends towards a non-zero value and reaches a plateau near a minimum velocity of 0.4 unit/mg. A Lineweaver–Burk representation is shown in Figure 2. The Lineweaver–Burk plot

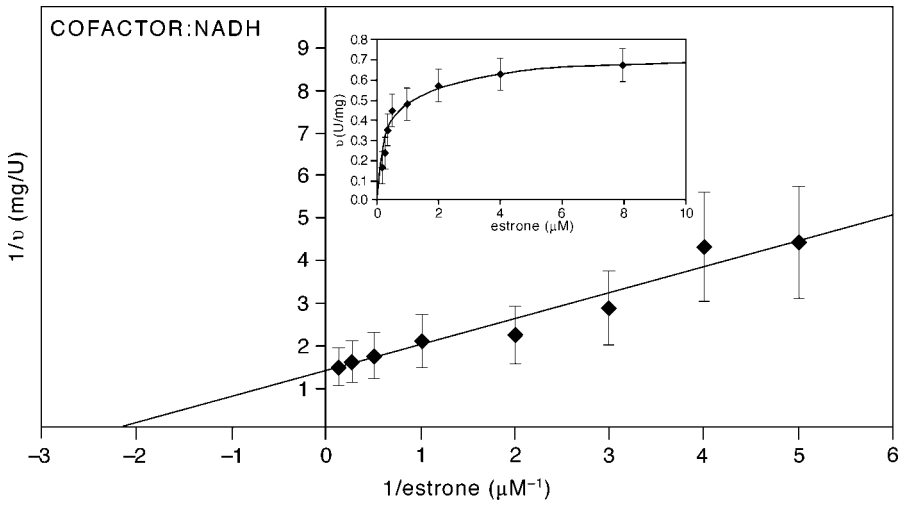

Figure 1 Lineweaver–Burk plot for oestrone reduction catalysed by 17β-HSD1 utilizing NADH as the cofactor

The reaction mixture contained 50 mM Tris/HCl, pH 7.5, 500 μM NADH, 0.05 mg/ml BSA, 2% ethanol and oestrone at 37 °C ($n = 2$). The data were processed using ENZFITTER and the following values were obtained: $K_m = 0.42 \pm 0.07$ μM, $k_{cat} = 0.8 \pm 0.04$ s^{-1}, therefore specificity = 1.9 $\mu M^{-1} \cdot s^{-1}$. The insert shows the v versus s plot for the same reaction. The reaction follows normal Michaelis–Menten kinetics. U, units.

Table 1 Steady-state kinetics for the reduction of oestrone performed by 17β-HSD1 and the Leu36Asp variant form of 17β-HSD1 in the presence of saturating amounts of NADPH or NADH

All reaction mixtures were kept at 37 °C and contained 50 mM Tris/HCl, pH 7.5, 0.05 mg/ml BSA, 2% ethanol and oestrone. Initial rates were followed. The results obtained for the native enzyme with NADH and for the Leu36Asp variant with NADPH came from the analysis performed by the ENZFITTER program. For the particular case of the native enzyme with NADPH, two programs were used: K_m and k_{cat} were obtained utilizing ENZFITTER and K_i was obtained from Leonora. NA, not applicable.

Sample	Cofactor	K_m (μM)	$k_{cat\ dimer}$ (s^{-1})	$k_{cat\ dimer}/K_m$ ($\mu M^{-1} \cdot s^{-1}$)	K_i (μM)
17β-HSD1 (wild-type)	NADH	0.42 ± 0.07	0.8 ± 0.04	1.9	NA
17β-HSD1 (wild-type)	NADPH	0.07 ± 0.01	1.5 ± 0.1	21	1.3
Leu36Asp 17β-HSD1	NADPH	1.0 ± 0.2	0.52 ± 0.04	0.52	NA

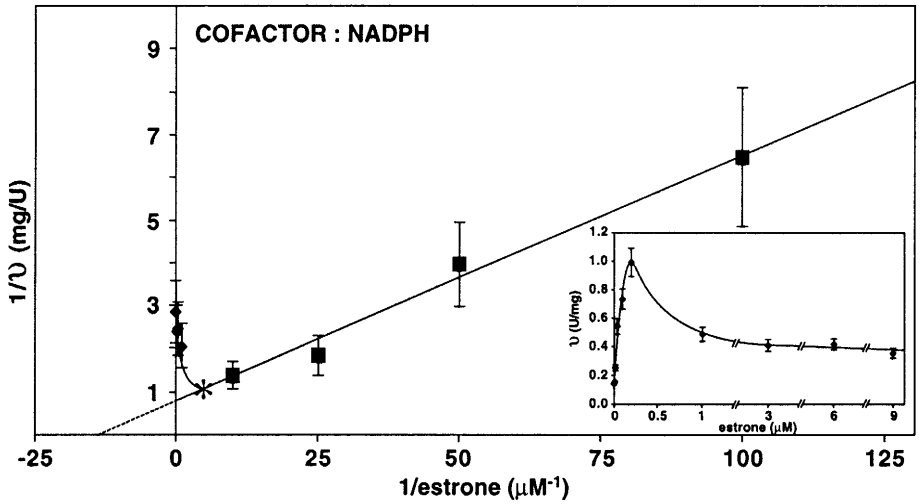

Figure 2 Lineweaver–Burk plot for oestrone reduction catalysed by 17β-HSD1 utilizing NADPH as the cofactor

The reaction mixture contained 50 mM Tris/HCl, pH 7.5, 20 μM NADPH, 0.05 mg/ml BSA, 2% ethanol and oestrone at 37 °C ($n = 2$). The concave shape of the curve no longer reflects standard Michaelis–Menten kinetics but shows substrate inhibition for concentrations of oestrone above 0.2 μM. The asterisk indicates the calculated inflexion point of the curve (see the Appendix). The insert shows the v versus s plot for the same reaction. The data obtained in the ascending part of the curve (< 0.2 μM oestrone) were processed using ENZFITTER and the following values were obtained: $K_m = 0.07 \pm 0.01$ μM, $k_{cat} = 1.5 \pm 0.1$ s^{-1}, therefore specificity = 21 $\mu M^{-1} \cdot s^{-1}$. Encompassing the complete range of concentrations of oestrone and using the substrate-inhibition equation (see the text), the data were further analysed with the program Leonora and the following values were obtained: $K_m = 0.08$ μM, $k_{cat} = 1.6$ s^{-1}, specificity = 20 $\mu M^{-1} \cdot s^{-1}$ and $K_i = 1.3$ μM. U, units.

using NADPH is no longer linear and, in contrast with the linear plot obtained in the presence of NADH, the curve becomes concave. When NADPH was used as the cofactor, different kinetic characteristics were observed for low and high concentrations of oestrone. At low concentrations of oestrone (0.1 μM and less), the reaction was found to obey Michaelis–Menten kinetics. When higher concentrations of oestrone were used (> 1 μM) the enzyme behaved differently and the $1/v$ parameter increased rapidly when the $1/s$ parameter decreased, strongly demonstrating a substrate-inhibition phenomenon. A transition zone between 0.1 μM and 1 μM marked the passage from Michaelis–Menten behaviour to substrate inhibition (Figure 2).

Although not all substrate-inhibiting systems will give strong coupling between their K_i and K_m values, Eszes et al. [21] have mentioned that their K_i, k_{cat} and K_m values were strongly coupled, and consequently it was difficult for them to obtain reliable values for the three parameters using a single equation. Taking this into consideration, the two phenomena (Michaelis–Menten kinetics followed by substrate inhibition) were first separated and analysed independently. As unambiguous Michaelis–Menten behaviour was observed when oestrone concentrations below 0.1 μM were used, this range of concentrations was taken to determine K_m and k_{cat} using ENZFITTER. The following kinetic constants were obtained: $K_m = 0.07 \pm 0.01$ μM, $k_{cat} = 1.5 \pm 0.1$ s^{-1} and $k_{cat}/K_m = 21$ $\mu M^{-1} \cdot s^{-1}$ (Table 1). The k_{cat} obtained from the Michaelis–Menten part of the curve is 1.5 s^{-1} (Table 1) and corresponds to a V_{max} of 1.3 units/mg. Nevertheless, because of the substrate-inhibition phenomenon, this value will never be reached in reality. From the experiments and as can be seen in Figure 2, the minimum value for $1/v$ is near 1.25 mg/unit [or 1/(0.8 unit/mg)]. This experimental value is in good agreement with the theoretical value that can be calculated from eqn (A10) (see the Appendix), namely a real maximal rate (v_{max}) of 0.8 unit/mg can be reached before the onset of the inhibition. When the complete range of data was analysed using the Leonora program [equation $v = V_{max} \cdot [s]/\{K_m + [s](1 + [s]/K_i)\}$ [24], the following kinetic constants were obtained: $K_m = 0.08$ μM, $k_{cat} = 1.6$ s^{-1}, $k_{cat}/K_m = 20$ $\mu M^{-1} \cdot s^{-1}$ and $K_i = 1.3$ μM (Table 1). It should be noted that the analysis of the data obtained for low concentrations of oestrone (by ENZFITTER) gave almost identical values for K_m and k_{cat} to the results obtained from the analysis for the complete range of oestrone concentrations (by Leonora). We therefore observed that the mechanism corresponding to a single equation gives accurate values for the different constants.

The oxidation of oestradiol by $NADP^+$ in the presence and absence of inhibiting amounts of oestrone (2 μM) was studied to determine the type of inhibition. Competitive-inhibition patterns were observed, as shown by the different K_m but similar V_{max} values obtained in the presence ($K_m = 9$ μM, $V_{max} = 6 \times 10^{-4}$ fluorescence units/s) and absence ($K_m = 0.8$ μM, $V_{max} = 8 \times 10^{-4}$ fluorescence units/s) of oestrone.

Kinetic behaviour of the Leu36Asp variant in the presence of a saturating concentration of NADPH: elimination of the substrate-inhibition phenomenon

The kinetic parameters for the Leu36Asp variant were measured in duplicate using cell homogenates containing the over-expressed variant and were found to be as follows: $K_m = 1.0 \pm 0.2$ μM, $k_{cat} = 0.52 \pm 0.04$ s^{-1}, $k_{cat}/K_m = 0.52$ $\mu M^{-1} \cdot s^{-1}$ (Table 1). A control experiment containing cell homogenates but without the over-expressed variant did not show significant activity, confirming that the activity measured in the over-expressed cells is due to the variant (and not to other dehydrogenases). As can be seen, the variant no longer shows substrate inhibition in the presence of NADPH (Figure 3). The specificity observed for the Leu36Asp variant (0.52 $\mu M^{-1} \cdot s^{-1}$) is much lower than the specificity found for the wild-type enzyme in the presence of NADPH (21 $\mu M^{-1} \cdot s^{-1}$), but closer to the specificity found for the wild-type enzyme in the presence of NADH (1.9 $\mu M^{-1} \cdot s^{-1}$; Table 1). An earlier experiment showed that the cofactor concentration

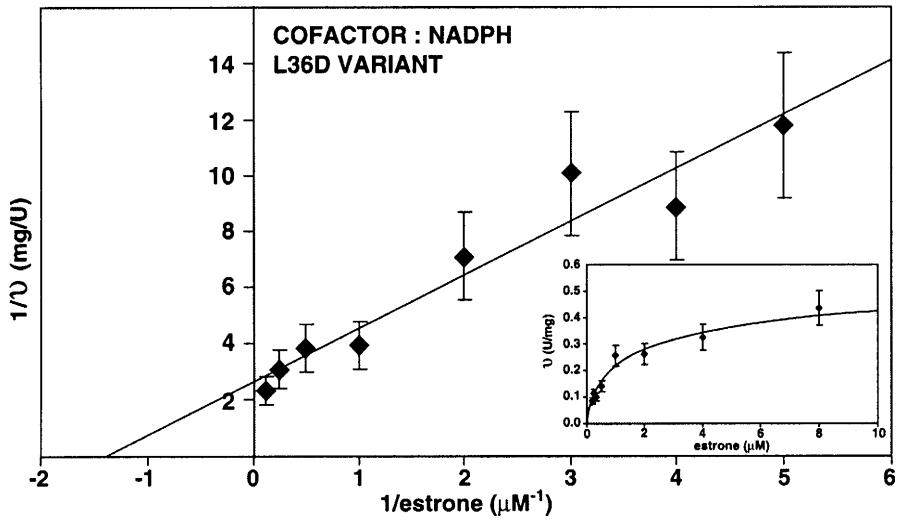

Figure 3 Lineweaver–Burk plot for oestrone reduction catalysed by the Leu36Asp variant in cell homogenate, utilizing NADPH as the cofactor

The reaction mixture contained 50 mM Tris/HCl, pH 7.5, 1 mM NADPH, 0.05 mg/ml BSA, 2% ethanol and oestrone at 37 °C ($n = 4$). The following values were obtained using ENZFITTER: $K_m = 1.0 \pm 0.2$ μM, $k_{cat} = 0.52 \pm 0.04$ s^{-1}, therefore specificity $= 0.52$ $\mu M^{-1} \cdot s^{-1}$. The insert shows the v versus s plot for the same reaction. The reaction follows normal Michaelis–Menten kinetics and, although NADPH was used as the cofactor, substrate inhibition is no longer observed. U, units.

Table 2 Steady-state kinetics for 17β-HSD1 in the presence of DHEA and DHT

All reaction mixtures were kept at 37 °C and contained 50 mM Tris/HCl, pH 7.5, 0.05 mg/ml BSA, 2% ethanol and 4–80 μM of either DHEA or DHT. Initial rates were followed. The results were given by the ENZFITTER program.

Substrate	Cofactor	K_m (μM)	$k_{cat\ dimer}$ (s^{-1})	$k_{cat\ dimer}/K_m$ ($\mu M^{-1} \cdot s^{-1}$)
Oestrone	NADPH	0.07 ± 0.01	1.5 ± 0.1	21
DHT	$NADP^+$	26 ± 6	0.2 ± 0.02	0.0008
DHEA	NADPH	24 ± 4	0.47 ± 0.06	0.002

used was sufficient to saturate the variant enzyme (1 mM NADPH in reaction; K_m of NADPH for the variant $= 238 \pm 30$ μM; Y.-W. Huang and S.-X. Lin, unpublished work).

Steady-state kinetics for DHEA and DHT using NADPH as the cofactor

Using saturating amounts of NADPH as the cofactor, the K_m and k_{cat} for DHEA and DHT were measured at pH 7.5 and 37 °C. For DHEA, a K_m of 24 ± 4 μM, a k_{cat} of 0.47 ± 0.06 s^{-1} and therefore a specificity of 0.002 $\mu M^{-1} \cdot s^{-1}$ were obtained (Table 2). In the case of DHT, a K_m of 26 ± 6 μM, a k_{cat} of 0.20 ± 0.02 s^{-1} and a specificity of 0.0008 $\mu M^{-1} \cdot s^{-1}$ were found (Table 2). For both non-cognate substrates, normal kinetic behaviour was obtained and no substrate inhibition was observed.

DISCUSSION

In addition to the critical importance of NADPH in the high specificity of oestrone reduction, our present results have shown that this cofactor is a prerequisite for substrate inhibition of 17β-HSD1, a mechanism of enzyme regulation. Oestrone seems to impose a double constraint on 17β-HSD1 that is not observed for the other ligands: the enzyme recognizes oestrone with the highest specificity while being subjected to a control by substrate inhibition. Our results also confirm and provide further evidence that NADH and NADPH, despite their close chemical resemblance, are not necessarily interchangeable. NADPH is generated in the pentose phosphate pathway and is generally used in reductive biosynthetic reactions, whereas NADH on the other hand is generated by the Krebs cycle and participates in using the free energy of metabolite oxidation to synthesize ATP (oxidative phosphorylation) [13]. Furthermore, the intracellular ratio of $[NADP^+]/[NADPH]$ is near 0.01, but the intracellular ratio of $[NAD^+]/[NADH]$ is near 1000 [13], indicating that NADPH is much more likely than NADH to be used as the cofactor for reductive reactions *in vivo*. This is especially true for 17β-HSD1, which shows a clear preference for NADPH [12] (K_m of NADPH $= 0.9 \pm 0.1$ μM and K_m of NADH $= 5.7 \pm 0.3$ μM; Y.-W. Huang and S.-X. Lin, unpublished work). The specificity value obtained in the presence of NADPH is 10 times higher than that observed when using NADH (21 $\mu M^{-1} \cdot s^{-1}$ versus 1.9 $\mu M^{-1}\ s^{-1}$; Table 1).

By comparing the double-reciprocal plots of the kinetics using NADH and NADPH, it becomes clear that the nature of the cofactor is implicated in the substrate-inhibition mechanism. Therefore, the following two aspects are to be considered: on one hand the structure of the cofactor itself and on the other hand the structure of the binding site for the cofactor. Concerning the structure of the cofactor, NADPH differs from NADH due to the presence of the ribose 2′-phosphate. Regarding the structure

of the cofactor's binding site, Mazza et al. [29] reported that 17β-HSD1 appears to be unique among the SDR family because it lacks both the Asp residue at position 36 (Leu-36 in 17β-HSD1) characteristic of the NAD(H)-preferring enzymes, and the basic residue located in the consensus sequence of the dinucleotide binding motif Gly-Xaa-Xaa-Xaa-Gly-Xaa-Gly (which is replaced by Ser-12 in 17β-HSD1). This latter motif forms an ionic interaction with the ribose 2′-phosphate and is characteristic of the NADP(H)-preferring enzymes [29]. Nevertheless, it seems that the preference between NADPH and NADH is not solely governed by the direct interaction with the ribose 2′-phosphate [30]. In order to further validate our conclusion, namely that the substrate inhibition of 17β-HSD1 is induced by NADPH, a single amino acid variation in the binding site for NADPH (Leu-36 into Asp) was performed. It was thought that the negative charge of the aspartic acid residue would repel the extra phosphate group of NADPH, which is also negatively charged. It was also thought that the Leu36Asp variant would show similar kinetic behaviour towards NADPH as that observed for the wild-type enzyme when using NADH. As expected, the variant no longer shows substrate-inhibition behaviour in the presence of NADPH (Figure 3).

The precise mechanism by which this substrate inhibition occurs needs further investigation. A possibility would be the formation of the dead-end enzyme–NADP(H)–oestrone complex, which has been well characterized for some dehydrogenases that use keto-containing substrates [21]. A good indication that such a dead-end complex is formed is the competitive nature of the inhibition. Indeed, when studying the oxidation of 17β-oestradiol by $NADP^+$ in the absence and presence of 2 μM oestrone, the competitive inhibition pattern is observed by spectrofluorescence: the K_m value increased more than 10-fold in the presence of 2 μM oestrone, from 0.8 μM to 9 μM, whereas the V_{max} remained essentially the same within the limits of experimental error (8×10^{-4} and 6×10^{-4} fluorescence units/s, respectively). As the enzyme concentration remained constant for all experiments, the V_{max} values are directly comparable.

When comparing the reduction of oestrone to oestradiol, and the reduction of DHEA to Δ5-androstenediol, we observed a dramatic fall in specificity of 10000-fold (Table 2). This high specificity of 17β-HSD1 towards oestrone is mainly due to the low K_m and can be attributed to a high affinity for the planar shape of oestrogens [18] and to the absence of the C_{19} methyl group [19]. Interestingly, substrate inhibition does not occur with oestradiol nor with the non-cognate substrates (DHT and DHEA) in presence of NADP(H), although reactions could be easily detected with the latter steroids.

Since 17β-HSD1 shows a higher specificity towards oestrone than oestradiol [12] and since initial velocity conditions are satisfied (substrate consumption $\leqslant 10\,\%$), it is unlikely that the substrate-inhibition phenomenon would be caused by misinterpreted product inhibition. In most of the cases, it is difficult to compare previously published K_m values for the reduction of oestrone by 17β-HSD1 with our K_m value, since the possibility of a substrate-inhibition phenomenon was not taken into consideration. This latter fact may explain the discrepancies observed between previously published K_m values for 17β-HSD1 (for example, K_m values of 7 μM [31] and 0.7 μM [11] reported in the past). Nevertheless, the K_m value we obtained for oestrone utilizing NADPH as the cofactor (0.07 ± 0.01 μM) is similar to the K_m reported previously (0.03 ± 0.01 μM) by Jin and Lin [12]. This latter K_m value was obtained by working with concentrations of oestrone below 0.2 μM, and therefore before the onset of substrate inhibition.

Although 17β-HSD1 has been studied for nearly half a century, this report constitutes the first evidence demonstrating that this key enzyme involved in oestradiol biosynthesis is subject to substrate inhibition. It is to be expected that substrate inhibition could be found in other enzymes requiring NADPH as a cofactor. Physiologically, the present mechanism may play a protective role by limiting the effects of an increase in intracellular oestrone levels. Knowing that physiological oestrone levels of 1.5 μM were measured in human placenta [32], it is likely that substrate inhibition ($K_i = 1.3$ μM) takes place *in vivo*. Nevertheless, the real physiological significance of this inhibition needs further investigations. The present results demonstrate the existence of a regulatory mechanism for oestradiol formation by 17β-HSD1 induced by the cofactor NADPH.

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APPENDIX

With the substrate-inhibition equation [33]:

$$v = \frac{V \cdot [s]}{K_m + [s]\left(1 + \frac{[s]}{K_i}\right)} \quad \text{(A1)}$$

the initial velocity can be simplified, thus expressed differently, for the three parts of the curve, namely, the part that obeys Michaelis–Menten kinetics ($< K_m$), the transition region between K_m and K_i and the substrate-inhibition region ($> K_i$).

For the Michaelis–Menten region, at low concentrations of substrate, i.e. when $[s] \ll K_i$, the $[s]/K_i$ term becomes negligible and the substrate-inhibition equation (eqn A1) becomes:

$$v = \frac{V \cdot [s]}{K_m + [s]} \quad \text{(A2)}$$

and therefore Michaelis–Menten kinetics are observed.

For the region of substrate inhibition, at high concentrations of substrate, i.e. when $K_m \ll K_i \ll [s]$, the K_m term in eqn (A1) becomes negligible. Therefore eqn (A1) can be simplified to $v = V \cdot [s]/[s]\{1 + [s]/K_i)\}$. Furthermore, as $K_i \ll [s]$, eqn (A1) can be simplified further to:

$$v = V \cdot \frac{K_i}{[s]} \quad \text{(A3)}$$

The real maximal-velocity value and the substrate concentration at which that velocity is reached: considering eqn (A1), $v = V \cdot [s]/\{K_m + [s](1 + [s]/K_i)\}$, and suggesting that $v' = 1/v$ and $[s'] = 1/[s]$, eqn (A1) can be written in the following form:

$$\frac{1}{v'} = \frac{V \cdot \frac{1}{[s']}}{K_m + \frac{1}{[s']}\left(1 + \frac{1}{K_i[s']}\right)} \quad \text{(A4)}$$

Multiplying the right side by [s′]/[s′]:

$$\frac{1}{v'} = \frac{V}{K_m[s'] + 1 + \frac{1}{K_i[s']}} \quad (A5)$$

Once rearranged:

$$v' = \frac{1}{V} \cdot \left((K_m[s']) + 1 + \frac{1}{K_i[s']} \right) \quad (A6)$$

At the minimum point of the $1/v$ versus $1/[s]$ curve (or $v' \approx [s']$), $dv'/d[s'] = 0$, and therefore

$$\frac{dv'}{d[s']} = \frac{1}{V} \cdot \left(K_m - \frac{1}{K_i}[s']^{-2} \right) = 0 \quad (A7)$$

Thus we have $K_m = 1/K_i[s']^{-2}$; the minimal v' (or maximal velocity) will be reached when

$$[s'] = \frac{1}{\sqrt{(K_m K_i)}} \quad \text{or} \quad [s] = \sqrt{(K_m K_i)} \quad (A8)$$

It should be pointed out that eqn (A8) is consistent with a previously published analysis [24], which describes how the maximal velocity, in the case of substrate inhibition, is reached when $[s]^2 = K_m K_i$. In our case, as $K_m = 0.07\ \mu M$ and $K_i = 1.3\ \mu M$, the value of $[s] = 0.3\ \mu M$. This [s] value is consistent with the observed inflexion point for the curve shown in Figure 2. Replacing the value obtained for [s′] in eqn (A1), we find that

$$v_{max} = \frac{V \cdot \sqrt{(K_m K_i)}}{K_m + \sqrt{(K_m K_i)}[1 + \sqrt{(K_m/K_i)}]} \quad (A9)$$

and therefore:

$$v_{max} = \frac{V \cdot \sqrt{(K_m K_i)}}{2K_m + \sqrt{(K_m K_i)}} = V \cdot \frac{1}{1 + 2\sqrt{(K_m/K_i)}} \quad (A10)$$

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