J. Steroid Biochem. Molec. Biol. Vol. 37, No. 1, pp. 65–70, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

STEREOSPECIFICITY OF HYDROGEN TRANSFER BETWEEN PROGESTERONE AND COFACTOR BY HUMAN PLACENTAL ESTRADIOL-17β DEHYDROGENASE

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(Received 1 February 1990)

Summary—We have previously shown that human placental estradiol-17β dehydrogenase (EC 1.1.1.62; 17β-EDH) catalyzes the conversion of estradiol-17β to estrone and stereospecifically reduces NAD^+ to [4-pro-S]NADH, ([4-B]NADH). Subsequently, this enzyme was found to reduce the ketone function at C-20 of progesterone, and evidence indicates that both activities reside at the same active site. This study was done to further elucidate spatial arrangements of cofactor and the 21-carbon substrate as they bind at the active site. The cofactor, [4B-^{3}H]NADPH, was generated with homogeneous 17β-EDH from term human placenta, utilizing [17α-^{3}H]estradiol-17β and $NADP^+$. The resulting [4B-^{3}H]NADPH was then purified by ion exchange chromatography and was separately incubated (24.4 μM) with a large molar excess of progesterone (150 μM) as substrate in the presence of the enzyme. Following incubation, the steroid reactants and products were extracted, separated by high-performance liquid chromatography and quantitated as to mass and tritium content. Oxidized and reduced cofactor were separated by ion-exchange chromatography and similarly quantitated. In all incubations, equimolar amounts of 20α-hydroxy-4-pregnen-3-one (20α-OHP) and $NADP^+$ were obtained. Radioactivity was stoichiometrically transferred from [4B-^{3}H]NADPH to the steroid product ([^{3}H]20α-OHP). These results further substantiate a single active site for both 17β- and 20α-dehydrogenation enzyme activities. In addition, the enzyme is B-side specific, catalyzing the transfer of the 4B-hydrogen from the dihydronicotinamide moiety of the cofactor, for both C-18 and C-21 steroid substrates. Since the 20α-dehydrogenation by other enzyme sources has always been demonstrated to be an A-side specific reaction, this observation represents an important exception to the Alworth–Bentley rules of enzyme stereospecificity.

INTRODUCTION

Although few steroid dehydrogenases have been purified to homogeneity, estradiol-17β dehydrogenase (17β-EDH, EC 1.1.1.62) from human placenta has been crystallized and studied extensively in our laboratory, from these studies we have proposed a model of the spatial alignment of cofactor and substrate, confirmed the involvement of histidine residues in the catalitic mechanism and presented evidence for inverted substrate binding in the enzyme active site [1–6]. Our interest in this enzyme continues because of its potential regulatory role in placental steroid metabolism during pregnancy and parturition, as well as for our interest in defining the structural and functional similarities among steroid dehydrogenases. Warren *et al.* [7], have previously determined that when [17α-^{3}H]estradiol-17β is used as substrate in the presence of NAD^+ as cofactor, the 17β-EDH transfers the hydrogen to the B-side of the nicotinamide ring, generating [4B-^{3}H]NADH.

The co-identity of the steroid binding sites for the 17β- and the 20α-activities was first suggested by Purdy *et al.* [8]. This proposal was supported by Strickler and coworkers [9] who provided additional evidence based on affinity labeling studies. Their data provided strong arguments that both enzyme activities reside at a single active site. However, utilizing computer modeling, they have suggested that the bound C-21 steroid should be in an inverted orientation with respect to the estrogen substrate [9, 10].

It is important to emphasize that, until now, no conclusive data have been available regarding the stereospecificity of hydrogen transfer when progesterone (C-21 substrate) is reduced by the human placental enzyme (20α-activity).

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Pyridine nucleotide-linked dehydrogenases are known to be stereospecific [11]. According to Alworth [12] and Bentley [13], certain generalizations about the hydrogen transfer with respect to substrate and the C-4 position of the nicotinamide moiety of the cofactor can be made. One of these rules states that when an enzyme reacts with different substrates, the stereospecificity of the hydrogen transfer is the same for each substrate.

The present study was designed to test the proposed theory of inverted C-21 steroid substrate binding with regards to the rules of stereospecificity of hydrogen transfer. We have generated the stereospecifically labeled cofactor, [4B-^{3}H]NADPH, and demonstrated the catalytic transfer of hydrogen to the C-21 substrate by the human placental enzyme.

EXPERIMENTAL

Materials

Pyridine nucleotides, a-ketoglutarate, glutamic acid, dithiothreitol, liver bovine glutamate dehydrogenase, bovine serum albumin (BSA), Coomassie blue R250, and low molecular weight protein standards were purchased from Sigma Chemical Co. (St Louis, Mo.) The Mono Q® Sepharose was from Pharmacia. The Dyematrex Blue A used for the purification of the enzyme was obtained from Amicon. Reagent grade salts, organic acids, analytic and HPLC grade solvents, Scintiverse II, Analtech preparative TLC plates containing fluorescent indicator, Eastman silica gel TLC sheets (No. 6060) and dialysis tubing (Spectro-Por, No. 2) were obtained from Fischer Scientific. Steroid hormones purchases from Steraloids or Sigma, were chromatographically pure. The [^{3}H]$NaBH_4$ (100 mCi/mmol) was obtained from New England Nuclear.

Assay and purification of estradiol-17β dehydrogenase

The homogeneous enzyme was purified from term human placenta as previously reported by Murdock *et al.* [5]. The specific activities of the pooled enzyme preparations used for these experiments were 7.5–8.5 IU/mg as assayed according to Langer and Engel [14]. Enzyme assays were conducted at 25°C. The assay used for 20α-activity was previously described by Strickler *et al.* [9]. Protein concentrations were determined by the method of Bradford [15].

Preparation of [17α-^{3}H]estradiol-17β and [4B-^{3}H]NADPH

The reduction of estrone was described by Roberts and Warren [16]. In order to optimize yield of the labeled [17α-^{3}H]estradiol-17β, the conditions of the reaction were modified as described by Pineda *et al.* [17]. The product yield was 0.16 mmol with an apparent sp. act. of 49.4 mCi/mmol.

The stereospecific reduction of $NADP^+$ was accomplished by a modification of the previously described method [7] using human placental 17β-EDH with [17α-^{3}H]estradiol-17β as substrate. The [4B-^{3}H]NADPH was generated from 0.7 μmol of $NADP^+$ and 1.4 μmol of [17α-^{3}H]estradiol-17β with 0.2 mg of homogeneous placental 17β-EDH in 12 ml of triethanolamine (TEA) buffer, pH 7.8 (10% ethanol). After extraction of the steroids, the pH of the aqueous fraction was immediately adjusted to 7.8. The extracted cofactors were separated and purified on the Mono-Q® ion-exchange column as described by Orr and Blanchard [18] and modified in our laboratory [17]. A delay or failure to adjust the pH allowed increased breakdown products of the labeled reduced cofactor, as manifested by minor peaks of radioactivity which were eluted early in the gradient. The effluent fractions containing [4B-^{3}H]NADPH were pooled, and the cofactor concentration was calculated from the absorbance at 340 nm.

Incubation of 17β-EDH and labeled cofactor with progesterone as substrate

The enzymatic reduction of progesterone (150 μM) was carried out with [4B-^{3}H]NADPH (24.4 μM) in 5 ml of 50 mM K_xPO_4 buffer, pH 7.4 (10% ethanol). The reaction was initiated with the addition of homogeneous 17β-EDH (1.5 μM) and was allowed to proceed for 3 h at 37°C. The reaction was stopped and the steroids were extracted with 5 ml aliquots of diethyl ether:ethylacetate (1:1) until the radioactivity in the aqueous fraction remained unchanged. The organic fractions were pooled, evaporated and resuspended in ethanol. The steroids were separated and quantitated by both TLC and HPLC. The TLC separation was a modification of a method previously described [19]. Aliquots of the extracted steroids were applied to silica gel strips (2 × 7 cm) and developed with benzene:ethanol (9:1). The developed TLC was cut into 0.5 cm sections and counted. In addition, the progesterone and

Table 1. Stereospecific reduction of progesterone to 20α-hydroxyprogesterone (20α-OHP) by human placental estradiol 17β-dehydrogenase with [4B-^{3}H]NADPH as cofactor

	Initial substrates (nmol)	Recovered substrates and products (nmol)	(Total % recovery)[a]	(cpm[b] × 10^{-3})
Progesterone	600	498		0
			94	
[^{3}H]20α-OHP	0	65		829
[4B-^{3}H]NADPH	98	31		1255
			88	
$NADP^+$	0	55		15

[a]The mass of recovered material represent the average of duplicate samples for each separated reaction, and their corresponding sp. act.
[b]Total counts calculated from steroid aliquots recovered from TLC plates and cofactors aliquots eluted from the FPLC as described in Methods.

20α-hydroxy-4-pregnen-3-one (20α-OHP) were separated and quantitated by HPLC [20]. The aqueous fraction was immediately adjusted to pH 7.8 with 1.0 N NaOH, and the oxidized and reduced cofactors were isolated by Mono-Q® ion-exchange chromatography [17].

Incubations of labeled cofactors with glutamate dehydrogenase

The [4B-^{3}H]NADPH (24 μM), diluted to a sp. act. of 32.6 mCi/mM, was incubated at 25°C with α-ketoglutarate (330 μM) in 3 ml of 50 mM K_xPO_4 buffer, pH of 7.0, containing ammonium phosphate (0.5 mM). The reaction was initiated by the addition of 45 mg of glutamate dehydrogenase and was completed in 5 min. The reaction was stopped by placing the samples in a boiling water bath for 3 min. The product, glutamic acid was identified on a Beckman Model 118C amino acid analyzer according to the method of Spackman *et al.* [21] and the mass quantitated by integration of the peak area.

RESULTS

Preparation of the radioactive cofactors

The elution profiles of the oxidized and reduced cofactors eluted from the Mono-Q® column have been previously reported by Pineda *et al.* [17]. The $NADP^+$ eluted at approx. 160 mM KCl and NADPH at 700 mM. Peaks corresponding to the standard NADPH contained 90% of the radioactivity applied to the column. The yield of cofactor from the reaction was 52%, and the sp. act. of the [4B-^{3}H]-NADPH was 44.7 mCi/mmol (1.45 mmol). Non-radioactive NADPH was added to this preparation to increase the stability of the purified, labeled cofactor, yielding a final sp. act. of 32.6 mCi/mmol. The purified reduced cofactor can be stored at −20°C after adjusting the solutions to pH 9.0 with 1 N NaOH. However, only freshly prepared cofactors were employed in the stereospecificity experiments.

Stereospecificity of hydrogen transfer by 17β-EDH

The reaction reached equilibrium after 2 h under the experimental conditions, and the results can be seen in Table 1. The recovery of the steroids and cofactors were 94 and 88%, respectively as determined from measurements of radioactivity. The mass quantitation of steroids and cofactors reported in Table 1 were performed as described in Methods [17]. At the end of the reaction, the 70.3% of the cofactor was oxidized as determined by the disappearance of absorbance at 340 nm. This is in agreement with the observation that all of the water-soluble radioactivity eluted from the Mono Q® column was found only in the

Table 2. Stereospecific reduction of α-ketoglutarate to glutamic acid by glutamate dehydrogenase with [4B-^{3}H]NADPH as cofactor

	Initial substrates (nmol)	Recovered substrates and products (nmol)	(% recovery)[a]	(cpm[b] × 10^{-3})	(nCi/nmol)
α-Ketoglutarate	96	ND	ND	0	—
[^{3}H]Glutamic acid	0	69	72	1850	32.6
[4B-^{3}H]NADPH	73	4	5	143	43.8
$NADP^+$	0	69	95	0	—

[a]The nanomoles of recovered material represent the average of duplicate samples for each component of the reaction and their corresponding sp. act.
[b]Total counts calculated from steroid aliquots of purified substrate and/or products eluted from their respective columns as described in methods.

unreacted [4B-^{3}H]NADPH. The stoichiometric amount of radioactivity that was lost from the cofactor moiety, was generated as [^{3}H]20α-OHP.

To demonstrate that the radioactive label of our enzyme-generated NADPH was at the 4B-position, we utilized the stereospecific catalysis of glutamate dehydrogenase. This enzyme is known to transfer the 4B position hydrogen of the dihydronicotinamide moiety to α-keto-glutarate. When [4B-^{3}H]NADPH was incubated with this enzyme under the conditions previously described, 92.8% of the respective cofactor was oxidized as determined by the decrease in absorbance at 340 nm. All the radioactivity was found in the glutamate peak eluted from the amino acid analyzer. All recoveries were based on radioactivity determinations. No radioactivity was found in the isolated $NADP^+$ fraction (Table 2). In incubations with both 17β-EDH and glutamate dehydrogenase, equimolar amounts of labeled products and unlabeled oxidized cofactors were recovered.

DISCUSSION

Affinity labeling studies of the steroid binding site of the human placental 17β-EDH by Chin and Warren [2] using 16α-bromoacetoxyestradiol 3-methyl ether and Murdock *et al.* [4–6] with both A-ring and D-ring substituted affinity alkylators, have suggested a binding orientation for the C-18 (estrogen) substrates with respect to specific histidine residues at the active site. Subsequent inactivation studies of the enzyme using 16α-bromoacetoxyprogesterone (16α-BAP) prompted Strickler *et al.* [9] and Thomas and Strickler [10] to propose a computer generated model to explain the relative orientations between C-18 and C-21 (progestin) substrates. They attempted to define the minimal spatial volume needed to allow for the oxido-reduction to occur. The model superimposed carbons 17, 20, 24 and the ketone function at C-20 position of 16α-BAP with carbons 13, 17, 16 and the −OH group at C-17 of 16α-bromoacetoxy-estradiol 3-methyl ether. With this inverted alignment between the B faces of the two steroid molecules, their computer-generated model supported the stereospecific catalysis of the C-18 and C-21 substrates at a single binding site. The modeling suggested that when the alignment of either substrate within the enzyme active site is such that the B-face of the estrogen is inverted relative to the B-face of the progestin, this orientation will: (1) accomodate the spatial requirement for binding of both molecules; (2) allow stereospecific oxido-reduction; and (3) explain the affinity radioalkylation of the same amino acid, a single common histidine residue, by the alkylating group on the D-ring of each steroid.

Specific rules have been established by Alworth [12] and Bentley [13] concerning the stereospecificity of enzyme catalysis: "Rule No. 1, a given enzyme will be stereospecific for either A, (4-pro-R) or B, (4-pro-S) paired hydrogens and will, therefore, belong to a single classification; Rule No. 2, when an enzyme can use either NAD^+ or $NADP^+$ (or their reduced forms), the stereospecifically of the reaction is the same with each substrate; Rule No. 3, when an enzyme reacts with a range of substrates, the stereospecificity of the hydrogen transfer is the same for each substrate; Rule No. 4, the stereospecificity of a particular reaction is independent of the source of the enzyme which catalyzes it."

The 17β-EDH from human placenta is B-side specific for the 17β-activity [7]. In the present study, we have synthesized the stereospecifically labeled cofactor [4B-^{3}H]NADPH. In all incubations with the human placental enzyme and progesterone substrate, equimolar amounts of [^{3}H]20α-hydroxyprogesterone ([^{3}H]20α-OHP) and non-labeled oxidized cofactor ($NADP^+$)

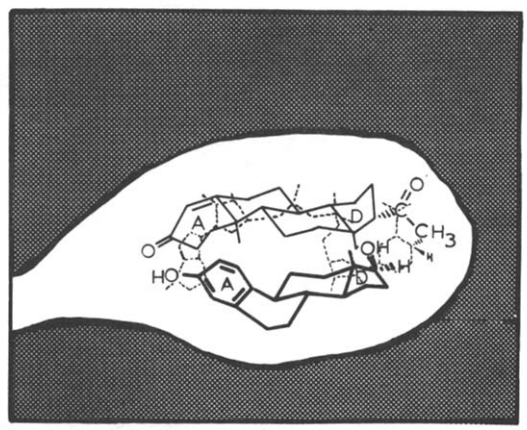

Fig. 1. Schematic representation of the catalytic site of human placental estradiol 17β-dehydrogenase. The site will accomodate both C-18 (bold, solid line) and C-21 (thin, solid line) steroids substrates in such a manner that the progesterone is inverted 180° along the horizontal axis with respect to the natural substrate, estradiol-17β. This orientation of substrates, superimposed on the bound cofactor (dashed-line figure), will accommodate the transfer of hydrogen to and from the B-side of the cofactor's nicotinamide moiety.

were obtained. These results demonstrate that the enzyme catalyzes the transfer of the 4B-hydrogen from the dihydronicotinamide ring of the NADPH, to either C-18 (17β-activity) or C-21 steroids (20α-activity) and supports the theory of the existence of a single active site for 17β- and 20α-HSD activity in the placental enzyme. That the hydrogen transfer was stereospecific was confirmed by the fact that the same labeled cofactor generated by the human enzyme was effectively used by glutamate dehydrogenase, a well known B side enzyme, to generate ^{3}H-labeled glutamate.

We have previously studied the characteristics of the spatial arrangement of cofactor and substrate at the active site of 17β-EDH [3]. Based on those observations, we have postulated that the mechanism of cofactor binding to the active site of this steroid dehydrogenase required the proximation of the adenine moiety of the cofactor to the steroid A-ring, as the ternary complex is generated. For the stereospecific hydrogen transfer to occur, the cofactor pyridine moiety must proximate the D-ring, resulting in an intimate, generally parallel alignment of steroid and cofactor at the enzyme active site during the catalytic event (Fig. 1). Studies by Birktoft and Banaszack [22] and Birktoft *et al.* [23] of several $NAD^+/NADP^+$-dependent dehydrogenases suggest that the cofactor binding domains contain a common structure characterized by nearly parallel beta sheets. The first two are interconnected by a single alpha helix and multiple glycine residues, as well as acidic amino acids residues for ribose binding. Gast and coworkers [24] have found the same characteristic cofactor binding region in the amino terminus of a 17β-EDH cDNA-derived protein. This observation supports the theory of the existence of similarities between mammalian dehydrogenases. The data also favor the proposal by Brenner [25] that dehydrogenases have evolved active sites which adjust the conformation of the nicotinamide cofactor so as to match its reduction potential to that of the substrate.

The coexistence of double enzymatic activity (17β- and 20α-activity) in the presence of unique hydrogen transfer stereospecificity (type B-enzyme), is in accordance with Alworth and Bentley's Rule No. 1, and therefore, belongs to a single classification. This means that the spatial relationship of the cofactor to different substrates on the enzyme active site, is the same in every instance. This model also complies with rules Nos 2 and 3, despite a less favorable and inefficient steric interaction between the C-21 steroid and cofactor as compared with the C-18 counterparts, as demonstrated by the kinetic constants reported by Strickler *et al.* [9]. However, this enzyme does not comply with Rule No. 4. We realized when we started the project that one of the stereospecificity "rules" would have to be broken. Other exceptions to Rule No. 4 have been reported by Groman *et al.* [28] for 17β-activity of 3(17β)-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni.*

Previous studies by Kersey and Wilcox with 20α-HSD from rat ovary [26], Hatano-Sato *et al.* using partially purified 20α-HSD from boar testes [27] and our own studies with homogeneous 20α-HSD from bull testes [17] have demonstrated that 20α-dehydrogenation is *A-side* specific. Neither the rat ovarian nor the bull testicular enzymes have 17β-HSD activity. In the presence of human placental 17β-EDH both the progesterone and estradiol-17β appear to bind at the same active site, and it seems unlikely that the cofactor binding site could rotate 180°. We have demonstrated that with progesterone as substrate, the hydrogen would be transferred from the B-side of the cofactor as is the case with estrone, and thus, Rule No. 4 is broken. If one compares the specificity constants (K_{cat}/K_m) of estradiol-17β and progesterone, as calculated from data presented by Strickler *et al.* [9] it is apparent that the enzyme discriminates greatly between the two substrates and demonstrates a 3.6×10^4-fold preference for estradiol-17β. This observation indicates that its efficiency as a 17β-dehydrogenase is over 30,000 times greater than its efficiency as a 20α-dehydrogenase and leads one to believe that its role as the latter may occur only by circumstance. Alworth and Bentley's Rule No. 4 almost certainly depends on conservation and modification of a single or similar gene(s) through the phylogenetic spectrum. One might conclude that estradiol-17β dehydrogenase came from a different gene precursor tham the "true" 20α-dehydrogenases and that its function as a 20α-dehydrogenase results only from the permissive plasticity of its active site which allows acceptance of progesterone as a much less favorable substrate. The significance of this exception to Rule No. 4 in the case of 17β-EDH from human placenta and its implications in serving a role in metabolic regulation during pregnancy and parturition, will require further study.

Acknowledgements—This research was supported by Research Grant DK-15708 from the National Institutes of Health. The authors wish to thank Dr Ronald Strickler for his advice and comments on the manuscript.

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