SOME KINETIC PROPERTIES OF HUMAN PLACENTAL ESTRADIOL-17β DEHYDROGENASE: PATTERNS OF INHIBITION BY ADENINE NUCLEOTIDES

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INTRODUCTION

While much effort has gone into the study of the regulation of steroid hormone secretion by the hypothalamus and the anterior pituitary, relatively little attention has been given to the microregulation of the enzyme systems involved in the biogenesis and metabolic transformations of steroid hormones. The reasons for this neglect are several: many of the enzymes are membrane-bound, are multienzyme systems and have yielded only reluctantly to efforts to purify and characterize them. Of the soluble enzymes, few have been purified and fewer still crystallized. This is due in part to the fact that Nature in its parsimony only provides those enzymes that act upon substrates at the micromolar to picomolar concentration range in very minute amounts. As a consequence, several thousand-fold purifications are frequently required. The soluble enzymes that have been studied are, for the most part, pyridine nucleotide-dependent dehydrogenases which recommend themselves because of the ease with which their activities may be assayed.

RESULTS AND DISCUSSION

Our laboratory has for some years been interested in one such enzyme (1), the estradiol-17β dehydrogenase (EC 1.1.1.62) of human term placenta which can be prepared in pure form by a relatively simple and straightforward procedure and has recently been crystallized (2). This enzyme catalyzes the reversible interconversion of estradiol-17β and its oxidation product estrone (Fig. 1). It utilizes both nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate as cofactors; the latter is bound more tightly than the former (3). Though its physiological role has not been established, we believe that one of its functions in the placenta is to catalyze the reduction of

*Deceased September 13, 1978.

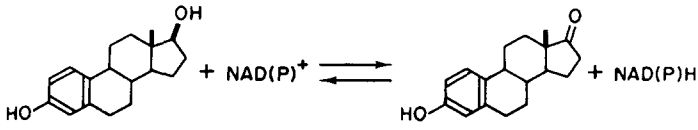

FIG. 1. Catalytic activity of estradiol dehydrogenase of human placenta.

16α-hydroxyestrone to estriol, the principal estrogen of human pregnancy. Though the interconversion of estrone and estradiol is freely reversible, the reduction of 16α-hydroxyestrone is not (Fig. 2); estriol cannot be oxidized to 16α-hydroxyestrone. The inability of the enzyme to catalyze the oxidation of the 17β-hydroxyl group in the presence of 16α-hydroxyl group is shared by the 3(17)β-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni.* It appears that the presence of the 16α-hydroxyl group interferes with the approach of the enzyme to the rear surface of the steroid which is required for the removal of the 17α-hydrogen as a hydride ion.

Estradiol dehydrogenase is a dimeric enzyme of molecular weight 68,000 (4). It is comprised of two subunits indistinguishable in molecular weight as judged by gel electrophoresis in the presence of sodium dodecyl sulfate, but differing in charge. When the native enzyme is subjected to isoelectric focusing, three major and two minor bands of enzyme activity and of protein are observed; in the presence of 8M urea, only three bands are formed. The pattern observed is consistent with the existence of 3 different monomers present in unequal amounts that can interact to form 6 dimers (1). Edman degradation yields a unique amino terminal pentapeptide, a finding consistent with either blocked amino termini, buried amino termini, or subunits with identical amino termini but differing internally in sequence.

As noted above, the enzyme can utilize as cofactor either NAD^+ or $NADP^+$. Certain generalizations can be made about its preferences for steroid substrates. There is absolute specificity for the 17β-configuration of the hydroxyl group in ring D; hydroxyl groups in other parts of the molecule are immune to attack. Steroids with aromatic A ring, B ring or both are preferred to saturated steroids or those with the Δ^4-enone structure. The latter are attacked, but at a very low rate. The enzyme also recognizes differences in the stereochemistry at the two ring junctions but overall it can be described as having a rather broad substrate specificity. The relatively biologically inert

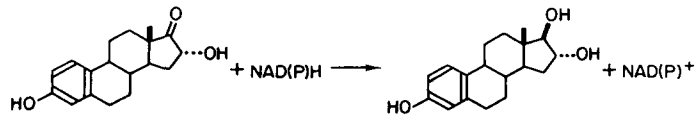

FIG. 2. The irreversible conversion of 16α-hydroxyestrone to estriol by estradiol dehydrogenase.

estradiol-17α is a competitive inhibitor as are the non-steroid compounds, diethylstilbestrol and o,p'-DDD (5).

Pyridine nucleotides that have been modified in the nicotinamide ring can serve as cofactors; certain analogues of NAD^+ that have been modified in the adenine ring are also hydrogen acceptors (6, 7).

The mechanism of the dehydrogenase reaction has been studied by the method of isotope exchange at equilibrium and has been shown to be random when NAD^+ is used as cofactor (8). This means that either steroid or nucleotide may bind to the enzyme independently of the other. When $NADP^+$ is the cofactor, it is bound preferentially and its binding precedes that of the steroids; the latter situation is the more usual one in alcohol dehydrogenase. In neither case, however, is hydride transfer the rate-limiting step. The situation is changed when the 17α-hydrogen atom is replaced by deuterium. There is about a 2-fold isotope effect suggesting that under these circumstances hydride transfer becomes the rate-limiting process (9). The latter conclusion has not been established by detailed kinetic analysis. Hydride transfer is stereospecific; the 17α-hydrogen of the steroid becomes the 4-S-hydrogen of the reduced pyridine nucleotide (10).

In recent studies, we demonstrated that when estradiol dehydrogenase is inactivated irreversibly by treatment with 3-bromoacetoxyestrone (11), the conjugated estrone is capable of interacting with reduced pyridine nucleotides and transferring the hydrogen to the covalently bound molecule of estrone (Fig. 3). We have coined the term "Catalytic Competence" to describe the phenomenon whereby an enzyme covalently linked to a substrate can carry out its normal catalytic function on that substrate. That this is the case demonstrates conclusively that the covalently linked substrate is bound to the enzyme in the same geometrical relation as is the substrate that is non-covalently bound during the normal catalytic process.

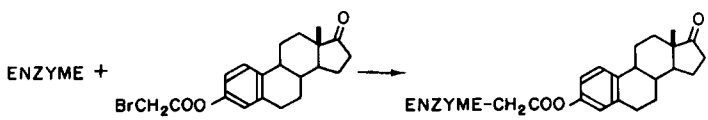

FIG. 3. Affinity labeling of estradiol dehydrogenase with 3-bromoacetoxyestrone.

In experiments with affinity labels based upon substrate-like steroids closely related to 3-bromoacetoxyestrone, we found that pyridine nucleotides decreased the rate of inactivation of the enzyme by the steroid affinity label (1). These observations suggested that the steroid affinity labels might be bound in or near the hydrophobic region of the enzyme that is normally occupied by the adenine moiety of the pyridine nucleotide. We were thus interested in studying compounds that might compete with pyridine nucleotide for this site.

In 1959 Hollander showed that of a series of adenine nucleotides, 2′-adenosine monophosphate was an inhibitor, both of estradiol-17β dehydrogenase and of its transhydrogenase function (12). These results, obtained with crude enzyme preparations, were recently extended by Shaw and Jeffery using purified enzyme who also observed that 5′-ATP is an inhibitor, but that its magnesium complex is less effective than the uncomplexed nucleotide (13).

These observations encouraged us to embark upon a kinetic study of the inhibition of estradiol dehydrogenase by adenine nucleotides. It was found that 5′-AMP, cyclic 3′,5′-AMP, and cyclic 2′,3′-AMP are not appreciably inhibitory. Those nucleotides that are inhibitory are listed in Tables 1 and 2. The compound most active as an inhibitor is 2′-AMP, a substance that does not occur naturally but is, of course, the adenosine moiety of $NADP^+$. As noted above, $NADP^+$ is bound to the enzyme with much greater affinity than is NAD^+. Of the other nucleotides, only 2′,5′-ADP and 5′-ATP are appreciably inhibitory.

TABLE 1. INHIBITOR CONSTANT FOR ADENINE NUCLEOTIDES OF ESTRADIOL DEHYDROGENASE*

Nucleotide	Variable NAD^+	Variable $NADP^+$
2′-AMP	6†	250†
2′,5′-ADP	24	740
5′-ATP	50	2700
5′-ADP	1100	—
5′-ADPR	250	—
3′,5′-ADP	2800	—

*Estradiol-17β: constant.
†μM.

TABLE 2. INHIBITOR CONSTANT FOR ADENINE NUCLEOTIDES OF ESTRADIOL DEHYDROGENASE*

Nucleotide	Variable NADH	Variable NADPH
2′-AMP	4†	130†
2′,5′-ADP	100	550
5′-ATP	94	780
5′-ADP	1800	22000
5′-ADPR	2200	34000
3′,5′-ADP	2600	—

*Estrone: constant.
†μM.

We therefore focused our attention upon 2′-AMP and 5′-ATP. When NAD^+ or NADH is the variable component and steroid constant and saturated, the inhibition is competitive in nature. An example is shown in Figure 4. When the steroid is the variable component, the inhibition is non-competitive (Fig. 5). Estradiol-17β, estrone and 16α-hydroxyestrone gave similar results. As might be expected, the inhibition by 2′-AMP and 5′-ATP is very much less when $NADP^+$ or NADPH are used as cofactors than when NAD^+ or NADH are used.

The fact that neither 5′-AMP, 5′-ADP, nor 5′-ADPR were effective inhibitors, while 5′-ATP and 2′-AMP were effective was somewhat surprising

and suggested that perhaps the two nucleotides were bound to different sites on the enzyme.

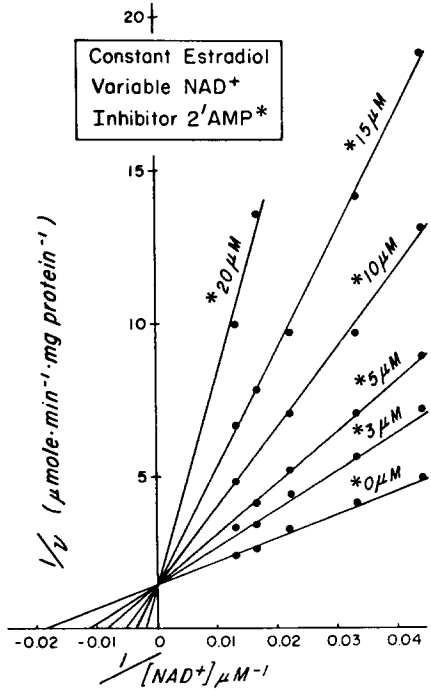

FIG. 4. Competitive inhibition of estradiol dehydrogenase by 2'-AMP at varying concentrations of NAD^+.

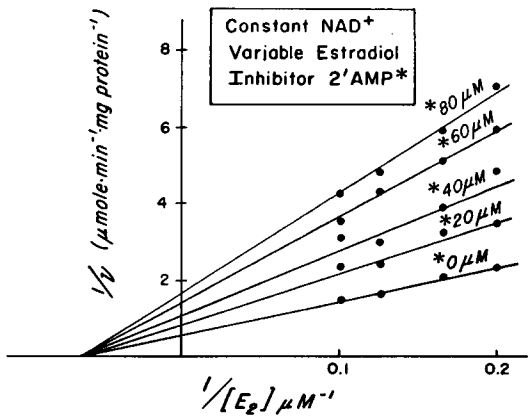

FIG. 5. Non-competitive inhibition of estradiol dehydrogenase by 2'-AMP at varying concentrations of estradiol-17β.

In order to examine this possibility, experiments were performed in which rate measurements were made at constant steroid and pyridine nucleotide concentrations while the relative amounts of 2′-AMP and 5′-ATP were varied. Plots of the reciprocal velocities against concentration of either 2′-AMP or 5′-ATP were parallel at all concentrations tested (Fig. 6). Also, mixtures of 2′-AMP and 5′-ATP were no more inhibitory than was an equal total specific concentration of either inhibitor alone, as shown in Table 3. Competition experiments, however, showed quite clearly that the two nucleotides bind to the same site on the enzyme. These observations tell us that 2′-AMP and 5′-

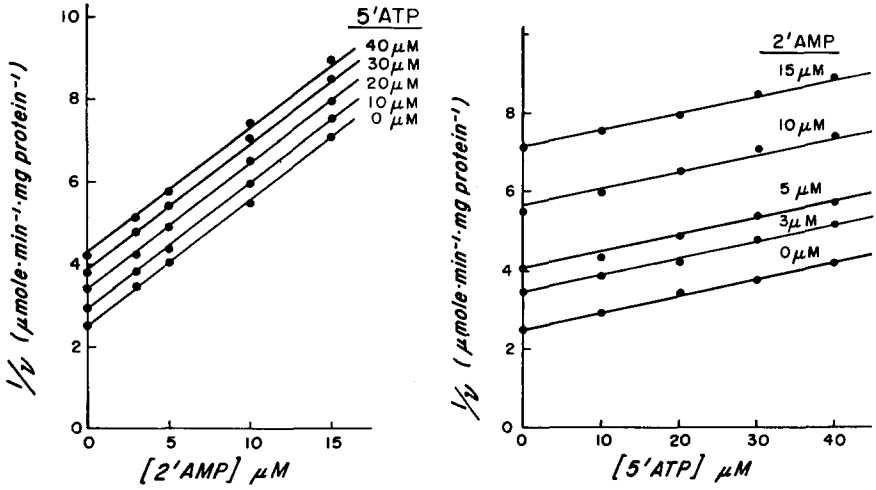

FIG. 6. Inhibition of the estradiol dehydrogenase by mixture of 2′-AMP and 5′-ATP (left) Dixon plot of 1/v versus concentration of 2′-AMP at different fixed concentrations of 5′-ATP, (right) 1/v versus concentration of 5′-ATP at different fixed concentrations of 2′-AMP.

TABLE 3. COOPERATIVE INHIBITION OF ESTRADIOL DEHYDROGENASE BY 2′-AMP AND 5′-ATP

Concentration of inhibitor		Substrate		
2′-AMP	5′-ATP	Estradiol-17β	Estrone	16α-Hydroxyestrone
0	0	1.02*	0.58*	0.31*
$4K_i$	0	0.62	0.35	0.22
0	$4K_i$	0.65	0.36	0.23
$2K_i$	$2K_i$	0.64	0.36	0.21
$8K_i$	0	0.47	0.29	0.14
0	$8K_i$	0.46	0.29	0.15
$4K_i$	$4K_i$	0.48	0.30	0.15

*Velocity, μmole · min^{-1} · mg $protein^{-1}$.

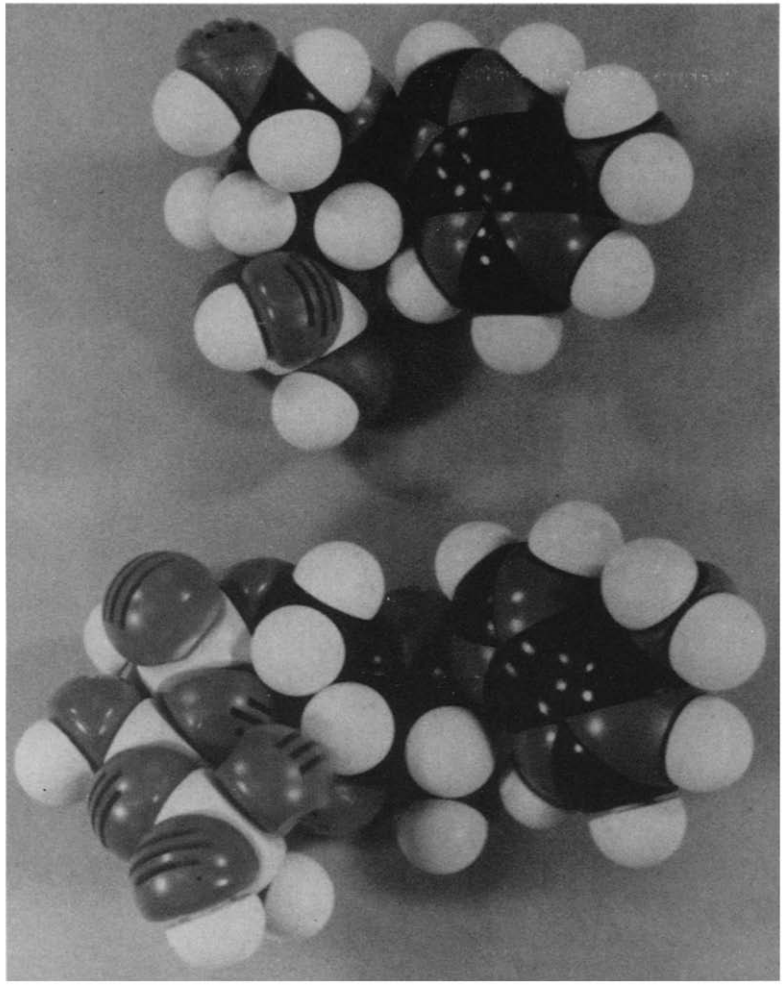

FIG. 7. Comparison of CPK model of 2′-AMP (upper) and 5′-ATP (lower). Terminal phosphate of 5′-ATP swing around the position of phosphate in 2′-AMP.

ATP are mutually exclusive. These results are not obtained if both inhibitors can combine with the dehydrogenase to form a 2'AMP· Enzyme · 5'-ATP complex. An examination of CPK models of the nucleotides suggests that if the charges on the two internal phosphate residues of 5'-ATP are neutralized, perhaps by arginine residues in the protein, then the terminal phosphate can swing around and approximate the position of the phosphate in 2'-AMP. This maneuver does not appear to be possible with either 5'-AMP or 5'-ADP (Fig. 7).

A further kinetic analysis was made of the inhibition of 2'-AMP using several different concentrations of the nucleotide. When double reciprocal plots were made, they appear to fall into two groups. This was made evident when a replot of the slopes of the double reciprocal plots against 2'-AMP concentration was constructed. The replot is parabolic as shown in Figure 8, indicating more than one binding site for the nucleotide. This we believe is the first evidence for cooperativity in this enzyme.

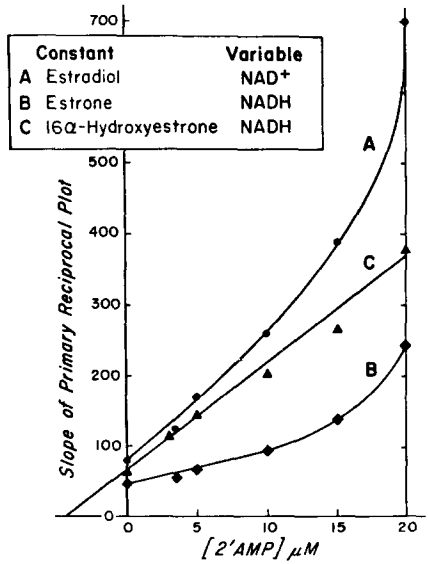

FIG. 8. Replot of slope of Lineweaver-Burk plot versus concentrations of 2'-AMP at varying substrates.

SUMMARY

Estradiol dehydrogenase from human placenta catalyzes the reversible interconversion of estradiol-17β and estrone. In the placenta, it is believed to catalyze the irreversible reduction of 16α-hydroxyestrone to estriol, the major

estrogen in human pregnancy. It can function with either NAD^+ or $NADP^+$ as hydrogen acceptor. The NAD(H)-linked activity of the enzyme is markedly inhibited by 0.5 mM 2′-AMP, and there are no detectable effects of the same concentration of 3′-AMP, 5′-AMP and 3′,5′-cyclic AMP. However, 5′-ATP is a far better inhibitor than any of the other 5′-phosphates of adenosine, such as 5′-AMP, 5′-ADP and 5′-ADPR. Also, the enzyme activity was strongly inhibited by 2′,5′-ADP, but not by 3′,5′-ADP. On the other hand, no inhibitory effects of the nucleotides at a concentration of 0.5 mM on the $NADP^+$-linked activity were observed. The K_m values for NADP(H) are much smaller than those of NAD(H).

The inhibition of estradiol dehydrogenase by 2′-AMP, 2′,5′-ADP, 5′-ATP, 5′-ADP, 5′-ADPR and 3′,5′-ADP appeared to be competitive with respect to NAD(H) or NADP(H) and non-competitive with respect to steroid. The apparent inhibitor constant (K_i) for 2′-AMP calculated from Lineweaver-Burk plots was 4 μM. This inhibitor constant is about one-tenth that of the Michaelis constant for NAD^+ and NADH. Lineweaver-Burk plots were made at several concentrations of 2′-AMP and the slopes plotted against 2′-AMP concentration. The replot was parabolic, indicating more than one binding site for the nucleotide. Similar results were obtained with 5′-ATP, though higher concentrations were required to achieve inhibition than in the case of 2′-AMP.

The 2′,5′-ADP is an inhibitor competitive with NAD(H) though not as potent as 2′-AMP. The results clearly suggest the positive contribution of the 2′-phosphate group of the adenosine moiety of $NADP^+$ for binding the cofactor.

Nicotinamide mononucleotide does not inhibit the NAD(H)- and NADP(H)-linked activities of estradiol dehydrogenase. The failure of NMN to compete with NAD(H) for the cofactor binding site seems to exclude a major role for this portion of the coenzyme in binding to the dehydrogenase; the 2′,5′-ADP moiety of the cofactor is more important for binding than the NMN group. NMN had no effect upon the inhibition by 2′,5′-ADP.

These results indicate a possible role of adenosine nucleotides in regulating the activity of estradiol dehydrogenase.

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