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STUDIES OF THE HUMAN TESTIS. XX—EFFECT OF NADH ON 17α-HYDROXYPREGNENE C17-20 LYASE ACTIVITY

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Summary—A study of the cofactor requirements of C17-20 lyase was carried out using human testis tissue obtained at the time of orchiectomy for treatment of prostatic carcinoma. Washed microsomal fractions were prepared from frozen human testes using a KCl containing buffer. The preparation revealed dose-dependent activity of C17-20 lyase in the presence of either NADPH or commercial or purified NADH. The K_m of NADH for the enzyme was of the order of 10^{-3} M and the K_m of NADPH was determined as 1.6×10^{-5} M. NADH also provided synergistic enhancement of NADPH-mediated lyase activity, and decreased the K_m of NADPH for the lyase but did not change the V_{max} of NADPH-mediated lyase activity. Carbon monoxide inhibited both NADH and NADPH-mediated lyase activities indicating that both activities are catalyzed by cytochrome P-450. Cations including Ca^{2+}, Mg^{2+} and Mn^{2+} were found to inhibit the NADPH-mediated lyase activity but enhanced the lyase activity in the presence of NADH. The results indicate both the presence of NADH-mediated C17-20 lyase activity and the synergistic effect of NADH on NADPH-mediated lyase activity in the human testis.

INTRODUCTION

Steroid 17α-hydroxylase and 17α-hydroxypregnene C17-20 lyase (abbreviated as C17-20 lyase) are monooxygenases which use molecular oxygen and NADPH for their indispensable role in testosterone biosynthesis in the testis. In previous reports [1, 2], we have demonstrated the presence of NADH-mediated 17α-hydroxylase activity in microsomes of the human testis. Since a cytochrome P-450 obtained from porcine testes and purified to a physicochemically single protein has been found to possess oxygenase activities of both 17α-hydroxylase and C17-20 lyase [3] and since NADH has been documented to enhance NADPH-mediated C17-20 lyase in rat testes synergistically [4], the possibility arose that NADH may also catalyze the C17-20 lyase activity in the human testis. The present paper describes the presence of NADH-mediated C17-20 lyase and also the synergistic effect of NADH on NADPH-mediated C17-20 lyase activity in the human testis.

EXPERIMENTAL

Enzyme preparations

Human testes were obtained from patients aged 60–83 years undergoing orchiectomy as treatment for prostatic carcinoma. These patients had received no hormonal therapy prior to surgery. The tissue was stored at −20°C until use. Decapsulated testes were minced and homogenized with a loose fitting Teflon-glass homogenizer in 5 vol of a buffer containing 0.25 M sucrose and 10 mM potassium phosphate (pH 7.4). After centrifugation at 15,000 *g* for 10 min, the supernatant was recentrifuged at 15,000 *g* for 20 min. The resultant supernatant was further centrifuged at 105,000 *g* for 60 min. The pellet was resuspended in a buffer containing 0.15 M KCl, 10 mM potassium phosphate (pH 7.4) and 1 mM EDTA, and sedimentated again at 105,000 *g* for 60 min. The washing was repeated twice. All procedures were performed at 0–4°C. The washed microsomes were stored at −20°C in a mixture of glycerol (20%) and a buffer containing 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose and 1 mM EDTA (80%). Prior to use the washed microsomes were diluted with approx. 20 vol of the same buffer and centrifuged at 15,000 *g* for 10 min. The supernatant obtained was used as the enzyme preparation.

Incubation

Unless otherwise mentioned, the reaction mixture contained substrate [4-^{14}C]17α-hydroxyprogesterone (2×10^5 dpm, 11 μM) dissolved in a drop of propyleneglycol, the enzyme preparation, pyridine nucleotide(s), 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose and 1 mM EDTA. The final volume was adjusted to 3 ml. The enzyme reaction was started by addition of the enzyme preparation and carried out at 37°C for 20 min with constant shaking under a gas phase of 95% O_2 and 5% CO_2. Incu-

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bations were terminated by vigorously shaking the incubation flasks after adding 10 ml of dichloromethane. For the study of the effect of gas phase, the steroid substrate and a pyridine nucleotide solution were prepared in the main chamber of Thumberg type incubation flasks and the enzyme preparation was put in the side arm. Both were prebubbled with Ar gas for 20 s to remove oxygen dissolved in the medium as described by Machino *et al.* [5]. After exchanging the gas phase inside the flasks with O_2–CO or O_2–Ar mixtures, the reaction was started by introducing the enzyme preparation into the main chamber. Other procedures and conditions were identical to those described above.

Extraction and separation of steroids

Before extraction, [1,2-^{3}H]androstenedione (5×10^5 dpm) and [1,2-^{3}H]testosterone (5×10^5 dpm) were added to each incubation flask to allow for correction of losses during the analytical procedures. Non-radioactive androstenedione, 17α-hydroxyprogesterone and testosterone (100 μg each) were also added as carrier steroids and non-radioactive progesterone (100 μg) was added as a chromatographic marker. Steroids were extracted three times with 10 ml of dichloromethane. The combined extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure at 40°C. The extracted steroids were separated by thin layer chromatography using Silica gel G and GF254, (4:1, w/w) with a solvent system of benzene and acetone (4:1, v/v). Detection and elution of extracted steroids were carried out by methods described previously [1]. The recovery of the initial radioactivity was approx. 92% at this step.

Quantitation of radioactivity and protein

Radioactivity of each steroid fraction was measured with a liquid scintillation spectrometer (Packard TRI-CARB 4530 with 460 CD dpm option) in 10 ml Econofluor (New England Nuclear Corp., Boston, MA). The counting efficiency was 51% for ^{3}H and 56% for ^{14}C.

The protein content of the enzyme preparations was measured according to the method of Bradford using BSA as a standard [6].

Expression of C17-20 lyase activity

The C_{19} metabolites from substrate 17α-hydroxyprogesterone detected by autoradiography of the thin layer chromatograms were androstenedione and testosterone. Therefore, the sum of the latter two steroid metabolites was used as the index of C17-20 lyase activity after correction for losses during the analytical procedures.

Identification of metabolites

The identification of metabolites was carried out by demonstrating constant specific radioactivities through repeated recrystallization with corresponding authentic preparations and derivative formation by oxidation and acetylation [1]. Once the identification of the metabolite was established, steroids obtained in the subsequent experiments using the same enzyme preparation and nucleotides were identified by the chemical derivative method.

Chemicals

[4-^{14}C] 17α-hydroxyprogesterone (50 mCi/mmol), [1,2-^{3}H] androstenedione (46 Ci/mmol) and [1,2-^{3}H] testosterone (49 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA) and purified by a Sephadex LH-20 microcolumn before use. Non-radioactive steroids and pyridine nucleotides were obtained from Sigma Chemical Co. (St Louis, MO). Steroids were recrystallized at least three times before use. All organic solvents were redistilled and other chemicals were of analytical grade.

RESULTS

Effect of NADH on C17-20 lyase in the human testis

A time course study (Fig. 1) revealed a linear increase of product formation catalyzed by the lyase in the presence of NADH alone and a linear response for the synergistic enhancement by NADH of the NADPH-mediated lyase activity. To minimize the possibility of the presence of contaminating NADPH and/or NADP in the commercial NADH preparation, further purification of NADH was carried out using a DEAE cellulose column (10 × 180 mm) which was equilibrated and eluted with a 20 mM potassium phosphate buffer (pH 7.4) to separate NADH from NADP and NADPH [7]. The NADH thus purified activated the lyase activity (Table 1, Expt 1). NADP by itself showed no effect on the lyase activity but enhanced the lyase activity in the presence of NADH (Table 1, Expt 2).

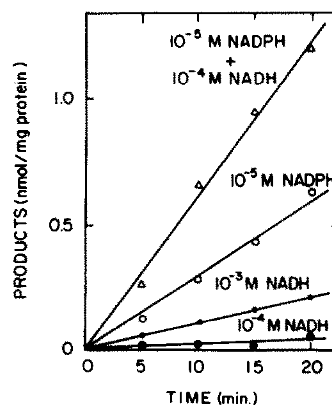

Fig. 1. Time course of C17-20 lyase activity. The enzyme preparation, containing 0.4 mg protein, was incubated with [4-^{14}C]17α-hydroxyprogesterone (6×10^5 dpm, 12 μM) in the presence of pyridine nucleotide(s) as indicated. An aliquot (0.7 ml) was taken from the incubation medium and added to a flask containing 10 ml of dichloromethane at the indicated times. The experiment was repeated with similar results.

Table 1. Effect of pyridine nucleotides on C17-20 lyase activity

Pyridine nucleotide	Concentration (10^{-6} M)	Lyase activity (pmol/mg protein/min) Expt 1	Expt 2
none	0	0.0	0.0
NADPH	6	—	18.5
NADH			
commercial	100	4.2	4.5
purified	100	4.3	—
NADP	6	—	0.0
NADPH NADH	6 100	—	53.5
NADP NADH	6 100	—	17.0

The enzyme preparation containing 1.0 mg of protein was incubated in the presence of pyridine nucleotides, as indicated in the table, under the conditions described in the text. Values represent the mean of duplicate incubations.

Kinetic study of C17-20 lyase for pyridine nucleotides

NADH increased C17-20 lyase activity dose-dependently (Fig. 2A). The synergistic effect of NADH on NADPH-mediated lyase activity also depended on its concentration; the activity increased with increasing concentrations of NADH, reached a plateau at 3×10^{-5} M NADH and then decreased at concentrations of NADH over 2×10^{-4} M (Fig. 2A).

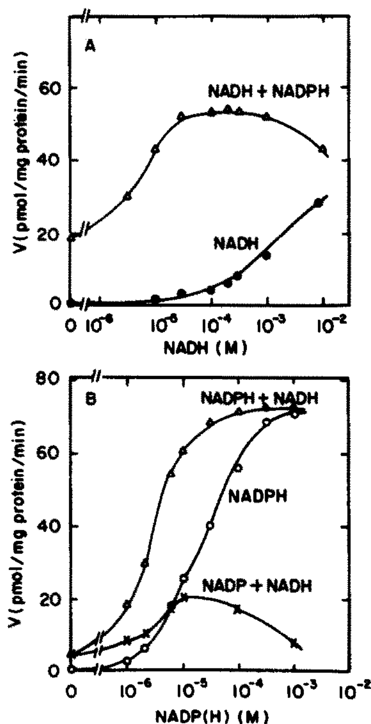

Fig. 2. Kinetic study of C17-20 lyase for pyridine nucleotides. The enzyme preparation, containing 1.0 mg protein, was incubated in the presence of pyridine nucleotide(s) at the concentrations indicated. Panel A indicates the results of a kinetic study for NADH in the presence or absence of 6×10^{-6} M NADPH. Panel B indicates the kinetics of the lyase for NADPH in the presence or absence of 10^{-4} M NADH and for NADP in the presence of 10^{-4} NADH.

Table 2. Effect of cations on C17-20 lyase activity

Additive	Concentration (10^{-3} M)	Relative activity (%) NADPH	NADH
none	—	100	100
EDTA	1	94	98
KCl	10	102	107
NaCl	10	102	105
$MgCl_2$	5	57	118
$MnCl_2$	5	49	120
$CaCl_2$	5	69	137

The enzyme preparation containing 0.8 mg of protein was incubated in a buffer containing 50 mM Tris (pH 7.4), 0.25 M sucrose, 5×10^{-4} M NADPH or 10^{-3} M NADH and an additive as noted under conditions described in the text.

The apparent K_m of NADH for C17-20 lyase was estimated as approx. 10^{-3} M.

As shown in Fig. 2B, NADH enhanced NADPH-mediated lyase activity and decreased the apparent K_m of NADPH for the lyase, but the maximum velocity of NADPH-mediated lyase activity was not influenced by NADH. The apparent K_m of NADPH for the lyase was determined as 1.6×10^{-5} M. NADP also enhanced the enzyme activity in the presence of NADH but showed inhibitory effects at higher concentrations.

Effect of cations on C17-20 lyase

A preliminary study (Table 2) revealed that NADPH-mediated activity of C17-20 lyase was inhibited by various divalent cations whereas the lyase activity in the presence of NADH was enhanced by their addition. To confirm this finding, the effect of Ca^{2+} and Mg^{2+} on C17-20 lyase activity was examined using different concentrations of cations (Table 3). The results showed a dose dependent inhibition of NADPH-mediated lyase activity by both cations. Lyase activity in the presence of either NADPH alone, NADPH and NADH, or NADP and NADH was inhibited by either cation. In contrast, the lyase activity in the presence of NADH was enhanced by either cation at the concentrations examined.

Effect of carbon monoxide on C17-20 lyase activity in the presence of NADH

As shown in Table 4, NADPH-mediated lyase activity was inhibited by carbon monoxide under reduced pressure of oxygen. Similarly, the presence of carbon monoxide had an inhibitory effect on the lyase activity mediated by NADH.

Enhancement of NADPH-mediated lyase activity by NADH supplied through enzymic dehydrogenation of a 3β-hydroxysteroid

Because of the synergistic effect of NADH on NADPH-mediated lyase activity (Fig. 1), coupling of NADPH-mediated C17-20 lyase with microsomal Δ^5-3β-hydroxysteroid dehydrogenase (and Δ^5–Δ^4 isomerase) as a possible source of NADH production from NAD was examined using dehydroepiandrosterone as a substrate for the dehydrogenase. As

Table 3. Effects of Ca^{2+} and Mg^{2+} on C17-20 lyase activity

Pyridine nucleotide	Concentration (10^{-6} M)	$CaCl_2$ (10^{-3} M) 0	1	2	5	$MgCl_2$ (10^{-3} M) 0	1	2	5
NADH	1000	100	122	137	136	100	111	115	112
	100	100	115	126	136	100	118	122	134
NADPH	1000	100	85	77	63	100	70	60	42
	6	100	87	76	55	100	80	73	54
NADH	100								
NADPH	6	100	87	80	63	100	77	65	48
NADH	100								
NADP	6	100	102	100	77	100	93	85	63

The enzyme preparation containing 0.9 mg of protein was incubated in a buffer containing 50 mM Tris (pH 7.4), 0.25 M sucrose, and additives as indicated in the Table. Activity, expressed as per cent of control value, represents the mean of duplicate incubations.

shown in Table 5, the lyase showed no distinct activity in the presence of NAD alone under conditions where NADH would be supplied by dehydrogenation of dehydroepiandrosterone. This suggests the production of insufficient amounts of NADH to activate the C17-20 lyase by itself. In contrast, when NAD was added to incubation medium containing both NADPH and dehydroepiandrosterone, a marked increase of C17-20 lyase activity was observed, indicating the synergistic effect to the NADPH-mediated lyase activity of NADH supplied by Δ^5-3β-hydroxysteroid dehydrogenase.

DISCUSSION

The present study demonstrates the activation of C17-20 lyase in the presence of NADH alone using human testis microsomal fractions. Since the lyase activity is markedly enhanced by the addition of NADPH to the incubation medium containing NADH, the activation of the lyase by NADH might be explained by a small amount of NADPH supplied by the incubation system used in the present study. This could result from the presence of endogenous NADPH in the enzyme preparation, the contamination by NADPH of the commercial NADH preparation, or NADPH formed by transhydrogenation of NADH to endogenous NADP. To minimize these possibilities the microsomal preparations were washed repeatedly with a KCl containing buffer to remove endogenous pyridine nucleotides [4] and the NADH preparations further purified of NADPH and NADP were also tested. The lyase activity was still observed in the presence of purified NADH.

Table 4. Effect of carbon monoxide on C17-20 lyase activity in the presence of NADH or NADPH

Gas phase	% Lyase activity NADH (10^{-3} M)	NADPH (10^{-3} M)
O_2–Ar = 95:5	100	100
O_2–CO = 95:5	96	101
O_2–Ar = 5:95	100	100
O_2–CO = 5:95	70	45

The enzyme preparation containing 1.0 mg of protein was incubated in duplicate in the indicated atmosphere under the conditions described in the text. Mean values are given in the Table.

The effect of cations was used further to rule out the alternative explanations for the NADH mediated effect. The NADPH-mediated lyase activity as well as transhydrogenase [8] action are inhibited by divalent cations. However, the cations enhanced the lyase activity in the presence of NADH alone. Therefore, it is concluded that NADH-mediated C17-20 lyase activity is present in the human testis although NADH-mediated C17-20 lyase activity is not present in the rat testis [4].

Inhibition of NADPH-mediated C17-20 lyase activity by NADH at high concentrations and of NADH-mediated lyase activity by carbon monoxide suggests further that common sites for the C17-20 lyase are present in the electron transport system involving cytochrome P-450 from both pyridine nucleotides. The finding is in agreement with the involvement of NADPH-cytochrome P-450 reductase in electron transport to monooxygenases from NADH at high concentration [9].

The synergistic effect of NADH on the NADPH-mediated lyase activity is shown in the present study. This might be explained by the effect of an increased supply of NADPH through an NADPH-generating system created by adding NADH to the enzyme system. In the present study, however, the attempt to demonstrate such an NADPH-generating system by adding NADP and NADH failed; the lyase activity was not enhanced to the levels observed by addition of NADPH with NADH (Fig. 2B). Furthermore, NADPH-linked lyase activity decreased at higher

Table 5. Effect on C17-20 lyase activity of coupling with dehydrogenation catalyzed by Δ5-3β-hydroxysteroid dehydrogenase

Pyridine nucleotide	Concentration (10^{-6} M)	Lyase activity (pmol/mg protein/min) Average	(Range)
NAD	20	0.5	0.5–0.5
NADPH	6	8.7	8.4–9.5
NAD	20		
NADP	6	0.3	0.2–0.4
NAD	20		
NADPH	6	13.4	12.7–13.9

The enzyme preparation containing 0.9 mg of protein was incubated in duplicate or triplicate in the presence of dehydroepiandrosterone (1.2×10^{-5} M) and the indicated pyridine nucleotide(s) under the conditions described in the text.

concentrations of added NADH (Fig. 2A). This would be difficult to explain on the basis of an NADPH-generating system activated by the addition of NADH. Therefore, it appears unlikely that the synergistic enhancement of NADPH-mediated lyase activity by NADH is attributable to transhydrogenation alone.

Although transhydrogenase can be ruled out as the major mechanism for the NADH-mediated lyase activity and NADH synergistic effect, the finding that C17-20 lyase activity was increased by the addition of NADP to the incubation medium in the presence of NADH, even though NADP alone had no effect, suggests the presence of transhydrogenation in the enzyme preparation to produce NADPH. The data from the present experiments, however, do not resolve the question of whether the transhydrogenase involved is supplied by the microsomes of the human testis or results from contamination by other subcellular components. Transhydrogenase has been reported in mitochondrial subparticles [10, 11].

Two electrons are required for the reaction catalyzed by monooxygenases [12]. The synergism of NADH with NADPH-mediated C17-20 lyase activity in the rat or pig testis [4, 13] and with NADPH-mediated monooxygenases in the liver [14] has been shown to involve cytochrome b_5 and cytochrome b_5 reductase in the transport system for these monooxygenases for the second electron from NADH [4, 13, 14]. In addition, cytochrome b_5 itself has been reported to have a stimulating effect on NADPH mediated C17-20 lyase activity [15]. The present finding of the action of NADH on reducing the K_m value of NADPH for the lyase while synergizing the NADPH effect is consistent with the proposal that NADH is preferred to NADPH as a donor of the second electron for the lyase [4]. Further the unchanged V_{max} of the lyase in the presence of both nucleotides suggests that NADPH is preferred to NADH as a first electron donor for the lyase and that the V_{max} of the lyase is defined by the efficiency of the first electron transport. Ohba *et al.* [4] further proposed and demonstrated the synergistic effect of coupling the reactions catalyzed by 3β-hydroxysteroid dehydrogenase and C17-20 lyase. Evidence for this coupling effect is also presented in the present study of the human testis.

In conclusion, in additon to NADPH-mediated C17-20 lyase activity, the microsomal fraction of the human testis has NADH-mediated C17-20 lyase activity and demonstrates synergism of NADH to the NADPH-mediated C17-20 lyase activity.

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