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COFACTOR REQUIREMENTS OF MICROSOMAL 3β-HYDROXY-STEROID OXIDOREDUCTASE, 5-ENE-ISOMERASE FROM HUMAN PLACENTA

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SUMMARY

Cofactor requirements of 3β-hydroxysteroid oxidoreductase, 5-ene-isomerase were studied in lyophilized microsomes and in supernatants obtained by washings with buffer solution. Following 1 h incubation with lyophilized microsomes the activity with NAD or NADP was almost equal. However, rate measurements using 1 or 5 min incubations showed that NAD was the most active cofactor. In 120,000 *g* supernatants of three successive washings the activity was mainly NAD dependent.

The possibility that the activity found in the presence of NADP is mediated by a microsomal transhydrogenase is discussed.

INTRODUCTION

Human placental microsomes contain an enzymic system which catalyses the conversion of 5-ene-3β-hydroxysteroids to 4-ene-3-keto-steroids (Koide and Torres, 1965; Davies and Ryan, 1972). This conversion consists of two reactions: (1) oxidation of the hydroxy group at C-3 (3β-ol-dehydrogenase) and (2) a shift of double bond from C-5 to C-4 (5-ene-isomerase) (Samuels, 1960; Cheatum and Warren, 1966). Whereas it is generally accepted that the above conversion is NAD dependent (Dorfman and Ungar, 1965; Davies and Ryan, 1972), Koide and Torres (1965) have shown that both NAD and NADP may be cofactors in the conversion of certain 5-ene-3β-hydroxysteroids to 4-ene-3-keto-steroids. In order to investigate these observations in more detail, we have attempted to determine the cofactor requirements of 3β-hydroxysteroid oxidoreductase, 5-ene-isomerase (3βOH, 5-I) of human placental microsomes and of soluble extracts derived from these microsomes.

EXPERIMENTAL

Materials

All reagents and solvents were of analytical grade. Solvents were redistilled before use; water was double distilled. Pyridine nucleotide cofactors were obtained from Sigma Chemical Co. The steroids were obtained from Ikapharm (Ramat-Gan, Israel). Radioactivity was counted in 10 ml of a scintillation fluid consisting of 4 g PPO and 0·1 g POPOP (Packard) per liter of toluene. The counting efficiency for ^3H was 53% and the S.D. was 2·5% in a Packard scintillator spectrometer, model 3380. Scanning of radioactivity on chromatograms was performed on a Berthold, LB 2723 Dunnschicht scanner.

METHODS

Preparation of placental microsomes

All procedures were performed at 0–4°C. Fresh human placentas, obtained up to 1 h after delivery, were washed with ice-cold H_2O and cut into small pieces; blood vessels and connective tissue were excluded. The tissue (300 g) was suspended in 150 ml phosphate buffer (0·2 M pH 7·2) containing 0·25 M sucrose and 0·04 M nicotinamide and was homogenized for 1 min in a Waring Blender. The cell debris was removed by centrifuging for 30 min at 1000 *g*; the resulting supernatant was then centrifuged for 60 min at 10,000 *g* to remove the mitochondria. Microsomes were obtained by centrifuging the second supernatant for 60 min at 105,000 *g*. The precipitate, containing the microsomes, was washed twice with buffer solution and once with H_2O by resuspension in 10 ml of the appropriate fluid, and centrifuged for 60 min at 105,000 *g*. The final precipitate was suspended in 20 ml of H_2O and then lyophilized. The yield of lyophilized microsomes was about 500 mg per 300 g wet weight placenta. The dry preparation consisted of approximately 50% protein. (Protein was determined according to the procedure of Lowry *et al.*, 1951).

Extraction of 3βOH,5-I from microsomes

The microsomes (125 mg) were suspended in 16 ml 0·2 M phosphate buffer containing 0·154 M KCl. The suspension was then homogenized by 2–3 passages of a Teflon piston homogenizer and then centrifuged for 60 min at 120,000 *g*. The resulting supernatant (referred to as supernatant I) was decanted. The precipitate was resuspended twice in 16 ml of buffer and centrifuged each time at 120,000 *g* for 60 min. The supernatants (referred to as supernatants II and III, respectively) were decanted and kept separately.

Table 1. Activity of 3β-hydroxysteroid oxidoreductose, 5-ene-isomerase in lyophilized microsomes and 120.000*g* supernatants, following 3 successive washings

Fraction	Total protein (mg)	Activity* nmoles product/mg prot./60 min. from pregnenolone: no cofactor	NADP	NAD	from DHA: no cofactor	NADP	NAD
Microsomes 120.000 × *g* supernats	63·0	0·5	3·8	4·2	0·4	3·5	3·8
I.	12·0	<0·1	0·8	6·0	<0·1	0·5	4·0
II.	6·0	<0·1	0·7	16·0	<0·1	0·6	10·0
III.	3·0	<0·1	0·8	30·6	<0·1	0·6	24·0
microsomes following 3 washings	40·0	0·4	4·0	4·2	0·38	3·6	3·7

Enzymic activity was measured as the conversion of [7-^{3}H]-pregnenolone to [7-^{3}H] progesterone and as the conversion of [7-^{3}H]-DHA to [7-^{3}H]-androstenedione. Each beaker contained 0·2 μCi/2 μg of the appropriate substrate, 1·5 μmoles of either NAD or NADP and 0·15 mg protein. Incubations were carried out in 1·2 ml of 0·2 M phosphate buffer pH 7·2 containing 0·154 M KCl at 37°C for 60 min.

* The values shown are the means of two duplicates which in no case differed from one another by more than 10% of the mean value.

Enzymic activity was determined in the first microsomal suspension, in the 3 supernatants and in the microsomes remaining after the third centrifugation. In the supernatants the protein concentration gradually decreased with each successive washing of the microsomes (Table 1). Thus the first extract contained approximately 20% of the protein estimated in the original microsomes, the second extract contained 10%, and the third contained 5%, respectively.

Incubation procedure

The activity of 3βOH,5-I was determined by measuring the conversion of pregnenolone to progesterone and also by the conversion of dehydroisoandrosterone (DHA) to androstenedione. An ethanolic solution of 0·2 μCi [7-^{3}H] (S.A. 19·8 Ci/mmol) and 2 μg of crystalline pregnenolone or 0·2 μCi of [7-^{3}H] DHA (S.A. 20·5 Ci/mmol) and 2 μg of crystalline DHA, was transferred to 10 ml beakers. The ethanol was evaporated under a stream of N_2 and the steroid residue dissolved in 0·2 ml of 0·2 M phosphate buffer (pH 7·2) containing 0·154 M KCl and 1·5 μmol of either NAD or NADP. Samples of 0·15 mg protein from the various enzymic preparations in phosphate buffer were added to a final volume of 1·2 ml. The incubations were carried out in air at 37°C for 1 h in a Dubnoff metabolic shaker. Rate measurements for the conversion of pregnenolone to progesterone (Fig. 1) in presence of NAD or NADP, were carried out by incubating 0·1 μCi of [7-^{3}H]-pregnenolone at 5 or 6 different concentrations of crystalline pregnenolone for 1 min (NAD) or 5 min (NADP) at 37°C in air. The protein content was 0·025 mg (with NAD) or 0·25 mg (with NADP) in a total incubation volume of 0·7 ml. The incubations were terminated by placing the samples in an ice bath and adding 0·2 ml of ethanol containing the appropriate standard carriers *i.e.* 50 μg pregnenolone and 50 μg progesterone or 50 μg DHA and 50 μg androstenedione.

Extraction and separation of pregnenolone and progesterone

The incubation mixture was extracted in 10 ml ether. The ether was washed with 1 ml of H_2O, transferred to a small tube and evaporated to dryness under a stream of N_2. The extracted steroids were chromatographed on thin layer (Ladany and Finkelstein, 1963). Pregnenolone was separated from progesterone by developing the chromatograms in chloroform–ethyl acetate (3:1, v/v) while DHA was separated from androstenedione in the solvent system of benzene–methanol (9:1, v/v). The radioactive zones were located by scanning; standards of pregnenolone and DHA were detected by their reaction with concentrated sulphuric acid (Finkelstein, 1968). The progesterone and androstenedione standards were located under U.V. light (240 nm). The zones of radioactivity corresponding to the crystalline standards were scraped, eluted with ethanol and their radioactivity counted. The sum of the radioactivity in the zones accounted for about 95% of the total radioactivity taken for incubation. In the control incubation (in absence of enzyme) about 95% of the radioactivity was found in the pregnenolone or DHA zones. No radioactivity could be detected in either the zone corresponding to progesterone or androstenedione.

Determination of enzymic activity

The enzymic activity was determined by measuring the percent of conversion of [7-^{3}H]-pregnenolone or [7-^{3}H]-DHA of a known S.A. to [7-^{3}H]-progesterone or [7-^{3}H]-androstenedione respectively. Based on the percent conversion, the amount of product was calculated. The enzymic activity was expressed as nmol

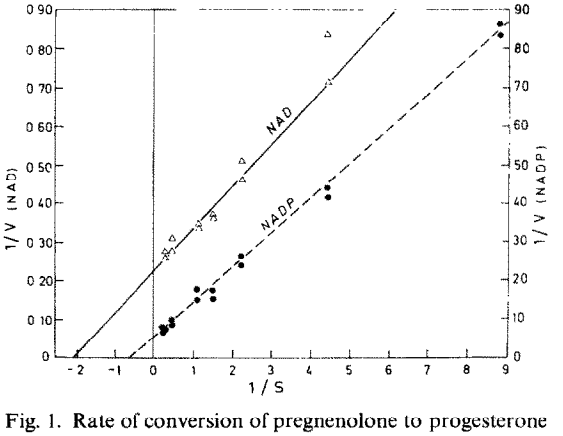

Fig. 1. Rate of conversion of pregnenolone to progesterone by lyophilized placental microsomes with NAD(△) or NADP(●) as cofactors. The left ordinate refers to NAD and the right ordinate refers to NADP. V is expressed as nmoles of product formed/mg protein/1 min. S is expressed as μM. The incubations were carried out with 0·1 μCi of [7-^{3}H]-pregnenolone at different concentrations of crystalline pregnenolone at 37°C pH 7·5 for 1 min (NAD) or 5 min (NADP). Each beaker contained 1·5 μmol of either NAD or NADP and 0·025 mg protein (with NAD) or 0·25 mg protein (with NADP).

of progesterone or androstenedione produced per 60 min per mg of protein. The kinetic data were plotted according to the method of Lineweaver–Burk (1934). The Michaelis constant (K_m) and the maximal velocity (V_{max}) were calculated by an unweighed linear regression and computed.

RESULTS

The activity of 3βOH,5-I in lyophilized microsomes and in 120,000 *g* supernatants following 1 h incubation is summarized in Table 1. In the native microsomes the activity of 3βOH,5-I was approximately equal with either NAD or NADP as cofactors; the activities measured by the conversion of pregnenolone to progesterone were 4·2 and 3·8 nmol/mg/h, respectively. In the absence of cofactors only a relatively low activity was found (0·5 nmol/mg/h). Similar results (Table 1) were obtained when the conversion of DHA to androstenedione was measured.

In the presence of NAD the amount of progesterone formed in the successive supernatants were 6, 16 and 30 nmol/mg/h, respectively. When these supernatants were examined for 3βOH,5-I with NADP the activities were significantly lower (0·8 nmol/mg/h) and were constant. In the absence of exogenous cofactors no activity could be detected (less than 0·1 nmol/mg/h). When the activity was measured by the conversion of DHA to androstenedione in the presence of NAD or NADP, a similar pattern was found although the values were 20–30% lower than those found for the production of progesterone (Table 1).

The activity of 3βOH,5-I remaining in the microsomal pellet after the third extraction was comparable to that found in original microsomes (Table 1): for pregnenolone in the presence of either NAD or NADP the activities were 4·2 and 4·0 nmol/mg/h, respectively; in the absence of NAD or NADP, the activity of 3βOH,5-I was 0·4 nmol/mg/h. Similar but slightly lower results were found for the conversion of DHA (Table 1).

Initial rate measurements of conversion of pregnenolone to progesterone in presence of either NAD or NADP are shown in Fig. 1. The K_m values were 0·44 μM with NAD and 1·73 μM with NADP. The V_{max} values were 4·2 and 0·2 nmol/mg protein/min with NAD and NADP, respectively.

DISCUSSION

The results of the present study show that in lyophilized human placental microsomes either NAD or NADP may act as a cofactor in the conversion of pregnenolone to progesterone or of DHA to androstenedione. One hour incubation with either of these cofactors increased the activity of 3βOH,5-I approximately 7 fold (Table 1). However, initial rate measurements of the conversion of pregnenolone to progesterone (Fig. 1) showed that NAD was more efficient than NADP as a cofactor. This is indicated by the marked difference in both K_m and V_{max} for the reaction in presence of NAD or NADP. While the activity of 3βOH,5-I in the microsomes in 1 h incubation was almost equal with either NAD or NADP, the activity in the 120,000*g* supernatant was mainly NAD dependent. Thus, the activity in the 120,000*g* supernatant, following the third wash, was 40-fold higher with NAD than with NADP. In addition, the activity with NAD as the cofactor increased with each successive washing whereas the activity with NADP remained consistently low. This increase in activity with NAD (as the cofactor) may be because the more readily soluble extraneous proteins are extracted in the earlier washings and contribute to the total protein measured, whereas in the last supernatant only a small amount of protein is extracted containing a relatively high proportion of 3βOH,5-I.

A review of the literature indicates that the cofactor requirements for the conversion of 3β-hydroxysteroids to 4-ene-3-ketosteroids appears to depend on the preparation used. Evidence for activity in the presence of either NAD or NADP has been found by Koide and Torres (1965) for human placental microsomes, by Tamaoki *et al.* (1969) for rat testicular microsomes and by Sulimovici and Boyd (1969) for rat ovarian microsomes. On the other hand others have found solely a NAD dependence: Cheatum and Warren (1966) in a purified microsomal preparation of bovine corpus luteum, Davenport and Mallette (1966) in rabbit ovarian microsomes and Weidenfeld and Ben-Uzilio (1973) in the microsomal fraction of the human ovary.

The results of the present study suggest that 3βOH,5-I of human placental microsomes are primarily NAD dependent. This follows from the marked difference in K_m and V_{max} values in the presence of the respective cofactors; the increase in specific activity of 3βOH,5-I with NAD in the sequential 120,000*g* supernatants supports this idea.

The activity of 3βOH,5-I with NADP as cofactor may be the result of a dual cofactor specificity of the enzyme, however, the great difference in activities in the presence of the respective cofactors observed with the 120,000*g* supernatants indicate that this is probably not the case. Another possibility is that the microsomes contain different forms of 3βOH,5-I having different cofactor specificities and which also differ in the ease with which they can be solubilized. This would account for the difference in cofactor dependent activity found in the microsomes, supernatants, and final pellet. A third possibility is that the activity of 3β-OH,5-I observed in the presence of NADP is due to the activity of a microsomal transhydrogenase catalyzing the reaction NADP + NADH → NADPH + NAD. As we have shown, there is a small amount of conversion of pregnenolone to progesterone in the absence of added cofactors which suggests the presence of a small amount of endogenous cofactor bound to the microsomes. If this bound cofactor were NAD, then, its regeneration from NADH by a transhydrogenase would be sufficient to account for the activity seen in the presence of NADP. Similarly,

the low activity of the 3βOH,5-I in the 120,000 g supernatant with NADP as a cofactor may be explained on the basis that only residual transhydrogenase activity was released to the 120,000 g supernatant.

The presence of 3βOH,5-I in the 120,000 g supernatants raises the question whether these enzymes are truly solubilized or whether they are still bound to small microsomal particles which do not sediment at 120,000 g. The latter possibility has been suggested by Neville and Engel (1968), who worked with deoxycholate treated bovine adrenal microsomes, and by others who used other solubilization techniques (Ewald *et al.*, 1964a, 1964b). It would be expected, however, that if the 120,000 g supernatant contained small microsomal particles, other membrane bound enzymes such as the enzyme complex catalyzing the aromatization of testosterone to estradiol-17β would be detectable. We have attempted to estimate such activity in both the microsomes and in the 120,000 g supernatant (Weidenfeld and Ben-Uzilio, 1973) but aromatizing activity was not found in the supernatants. Moreover, the difference in cofactor specificity between the 3βOH,5-I found in the native microsomes and that found in the 120,000 g supernatants suggests that the supernatant enzyme is no longer microsomally bound; were it bound, it would be expected that the cofactor requirements would remain the same as in the native microsomes. These arguments, however, do not exclude the possibility that the supernatant 3βOH,5-I activity is still bound to small membrane or particulate components, not necessarily identical with the native microsomes, and which can utilize NADP as a cofactor.

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