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On the Intracellular Localization of the 3β-Hydroxysteroid Dehydrogenase/Isomerase in the Rat Adrenal Cortex[1] (39418)

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Controversy exists with regard to the exact intracellular localization of the 3β-hydroxysteroid dehydrogenase/isomerase (HSD)[4] in steroid hormone-producing cells. In an early report, Beyer and Samuels (1) found that the enzyme was distributed among all subcellular fractions derived from homogenates of beef adrenal cortex but that the microsomal fraction had the highest specific activity. Since then, HSD has been highly purified from sheep adrenal cortex microsomes (2) and is now generally accepted to be a constitutive microsomal membrane-bound enzyme. On the other hand, several reports have suggested that HSD is also a mitochondrial enzyme in the adrenal cortex (3), testis (4), ovary (5), and placenta (6). Investigators seeking to resolve this descrepancy have variously concluded that mitochondrial HSD results from a direct microsomal contamination (7); or from an enzyme redistribution artifact that occurs during homogenization (8); or from an actual mitochondrial localization (4). If HSD is located in the inner mitochondrial membrane, as suggested by Sulimovici *et al.* (4), steroid oxidation might proceed utilizing NAD contained in the matrix space. In this report we show that this reaction does occur and conclude that HSD is present in the inner mitochondrial membrane in rat adrenal cortex mitochondria.

Materials and methods. Male Sprague–Dawley rats weighing 150–250 g were used in these experiments. The adrenals from 10 to 15 animals, sacrificed by decapitation, were removed and chilled in cold isotonic sucrose. The adherent fat was removed and the glands were homogenized in 0.25 *M* sucrose containing 1 m*M* EDTA and 30 m*M* Tris-HCl, pH 7.2. A mitochondrial fraction was isolated as previously described (9). The crude mitochondrial pellet was washed three times by resuspension and recentrifugation. Microsomes were isolated from the postmitochondrial supernatant fluid by centrifugation for 1 h at 105,000 *g*. The microsomal and washed mitochondrial pellets were resuspended in homogenizing medium to total volumes of 2 ml.

All incubations were performed at 37° in duplicate 1-ml portions of a medium that contained 50 m*M* sucrose, 20 m*M* KCl, 1 m*M* EDTA, 30 m*M* Tris-HCl, pH 7.4, and 0.2 to 0.5 mg of mitochondrial or microsomal protein. HSD activity was measured as described by Neville and Engel (10). For this assay the medium also contained 0.1 m*M* [^{3}H]pregnenolone (30,000 dpm). NAD, when present, was 0.5 m*M* and other additions were as indicated in the tables. Blank incubations contained no enzyme. The reaction was started by the addition of [^{3}H]pregnenolone and was stopped, after 5 min, by the addition of either 2 ml of heptane or 10 ml methylene chloride. [^{3}H]Progesterone formation was constant with time for 5 min and with tissue protein to 0.7 mg. The 21-hydroxylase activity was measured by the method of Koritz and Kumar (11). The medium also contained 0.1 m*M* [^{3}H]progesterone (30,000 dpm), 50 m*M* NADPH, 3.3 m*M* glucose-6-phosphate, and 2 IU glucose-6-phosphate dehydrogenase. The reaction was started by the addition of progesterone and was stopped,

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[4] The abbreviation used is: HSD, 3β-hydroxysteroid dehydrogenase/isomerase.

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after 10 min, by the addition of 2 ml of heptane. The 11β-hydroxylase activity was measured by the conversion of deoxycorticosterone to corticosterone as previously described (9). For this assay the medium also contained 30 *μM* [^{3}H]deoxycorticosterone. Reducing equivalents were supplied by either 10 m*M* malate or isocitrate or an NADPH-regenerating system. The reaction was started by the addition of deoxycorticosterone and was stopped, after 5 min, by the addition of 10 ml of methylene chloride.

In the three enzyme assays, aliquots of ^{14}C-labeled steroid substrates and products were added prior to extraction to correct for losses of tritiated steroid during the extraction and the chromatographic separations. The incubates were extracted twice and the organic phases were separated and evaporated to dryness. Carrier, unlabeled steroids were added to enhance visualization by UV absorption. Thin-layer chromatography was performed on Silica gel G Plates using the following solvent systems: pregnenolone and progesterone, either chloroform–methanol (99:1) or chloroform–ether (9:1); progesterone and deoxycorticosterone, either chloroform–methanol (99:1) or ether–benzene (9:1); and deoxycorticosterone and corticosterone, ether–benzene (9:1). The substrates and products of the 21-hydroxylase reaction were also separated on a 1 × 39-cm column of Sephadex LH-20 (11 g) as described by Tan and Mulrow (12). After elution from either the thin-layer plates or the Sephadex LH-20 columns, the radioactive steroids were counted in a Packard Tri-Carb scintillation counter. Quenching was estimated by internal standardization.

Results and discussion. Mitochondrial and microsomal HSD activity. HSD was assayed in mitochondria and microsomes prepared from several different rat adrenal homogenates. The mean specific activities (± SEM) are summarized in Table IA. While HSD activity was present in both the mitochondrial and microsomal fractions the specific activity of HSD was higher in the microsomes only in the presence of added NAD. This resulted from the fact that the mitochondrial HSD was active in the absence of added NAD. Exogenous NAD about doubled the mean mitochondrial HSD activity. In contrast, the microsomal HSD activity was low in the absence of NAD and was increased at least 20-fold by exogenous NAD. As shown in Table IB, these mitochondrial preparations contained measurable amounts of 21-hydroxylase, a microsomal-marker enzyme in this tissue (7). In some preparations (not shown) 21-hydroxylase activity reached values as high as 3 nmol deoxycorticosterone formed per minute per milligram of mitochondrial fraction protein. However, the ratio of HSD activity (measured in the presence of NAD) to the 21-hydroxylase activity was five to seven times greater in the mitochondrial fraction

TABLE I. THE SPECIFIC ACTIVITIES OF 3β-HYDROXYSTEROID DEHYDROGENASE (HSD) AND 21-HYDROXYLASE IN RAT ADRENAL MITOCHONDRIAL AND MICROSOMAL FRACTIONS.

Fraction	HSD activity[a]		21-Hydroxylase activity[a]	HSD (with NAD) / 21-Hydroxylase
	No NAD	0.5 m*M* NAD		
A				
Mitochondria (12)	7.5 ± 1.0	14.4 ± 1.2	–	–
Microsomes (5)	1.1 ± 0.4	26.5 ± 4.9	–	–
B				
Mitochondria (2)	13.5	20.5	1.0[b]	21
Microsomes (2)	2.1	33	10.1	3.4

[a] The enzyme assays were performed as described under Materials and Methods. The activities are in nanomoles progesterone (HSD) and deoxycorticosterone (21-hydroxylase) formed per minute per milligram of protein. The numbers in parentheses represent the number of separate experiments. Some variation in enzyme specific activity was noted among different preparations but the relationships shown under A and B always held.

[b] 11β-Hydroxyprogesterone may be formed from progesterone by mitochondria. This steroid, which migrated with deoxycorticosterone in the thin-layer chromatographic system (ether:benzene, 9:1) used for the 21-hydroxylase assay in microsomes, was separated from deoxycorticosterone by thin-layer chromatography using chloroform:methanol (99:1) or by Sephadex LH-20 chromatography (as described in Methods).

compared to the microsomal fraction. We interpret this finding to mean that the HSD activity of the mitochondrial fraction cannot be due entirely to microsomal contamination. Similar HSD/21-hydroxylase activity ratios were found by Moustafa and Koritz (7) in rat adrenal mitochondrial and microsomal fractions, but were interpreted to mean a selective contamination of the mitochondria by microsomes high in HSD and low in 21-hydroxylase activity. To our knowledge such a heterogeneity of enzyme activity has not been demonstrated in the adrenal cortex endoplasmic reticulum.

Since about half of the mitochondrial HSD activity appeared to result from an inner membrane enzyme with an active site facing the matrix space, an attempt was made to influence the activity of this enzyme by reduction of the intramitochondrial pyridine nucleotides. We have previously shown (13) that a combination of inorganic phosphate, citric acid cycle substrates, and inhibitors of mitochondrial electron transport caused a reduction of the pyridine nucleotides in these mitochondrial preparations. Table II shows that this reagent combination inhibited endogenous NAD-dependent mitochondrial HSD activity about 90%. Substrates plus inorganic phosphate alone inhibited about 75% and rotenone and cyanide alone inhibited only about 10% (not shown). In the presence of exogenous NAD, mitochondrial HSD activity was decreased about 40% on addition of the inhibitor-substrate combination. Microsomal HSD was essentially inactive in the absence of exogenous NAD and the inhibitor-substrate combination did not inhibit microsomal HSD activity significantly in either the presence or absence of NAD.

The [^{3}H]progesterone formed during the mitochondrial and microsomal HSD reactions was characterized in two different thin-layer and a Sephadex LH-20 chromatographic system (see Materials and Methods). Recovery of added tracer [^{14}C]progesterone ranged from 88 to 113%. In other experiments (not shown), we found good correlation between pregnenolone disappearance and progesterone formation. Therefore, no new or different products were formed during the mitochondrial HSD reaction.

On the basis of these results it seems reasonable to conclude that the mitochondrial progesterone formation that was inhibited by rotenone, KCN, and the citric acid cycle substrates was dependent on intramitochondrial NAD. We assume that the exogenous NAD-dependent HSD activity of these mitochondrial fractions is due either to microsomal contamination and/or to damaged mitochondria whose permeability barriers to exogenous NAD have been impaired. Moustafa and Koritz (7) reported that about one-half of the mitochondrial HSD could be accounted for by microsomal contamination. In our experiments (Table I), microsomal contamination accounted for less than 20% of the mitochondrial HSD activity. A redistribution of HSD from microsomes to mitochondria, as suggested by Cowan *et al.* (8), seems unlikely because this would require the selective transfer of HSD and not the 21-hydroxylase. A transfer of HSD from

TABLE II. The Effect of a Combination of Krebs Cycle Substrates and Respiratory Inhibitors on HSD Activity in Mitochondria and Microsomes.

	HSD activity[a]			
	No NAD		0.5 m*M* NAD	
		+Inhibitors[b]		+Inhibitors[b]
Mitochondria (6)	5.8 ± 1.1	0.60 ± 0.20	12.0 ± 1.3	7.7 ± 1.2
Microsomes (2)	0.40 ± 0.04	0.67 ± 0.11	16.1 ± 4.9	21.5 ± 7.1

[a] Mitochondrial and microsomal HSD activity was measured as described in Materials and Methods in either the presence or absence of 0.5 m*M* NAD. HSD activity is given as nanomoles progesterone produced per minute per milligram of protein ± SEM. The numbers in parentheses represent the number of different experiments.

[b] The inhibitor combination used was 10 m*M* isocitrate, 10 m*M* α-ketoglutarate, 5 μM rotenone, 0.5 m*M* cyanide, and 1 m*M* phosphate. Analysis of the entire thin-layer chromatographic plate showed that no steroid other than progesterone was formed during these incubations, i.e., the inhibitor-dependent decrease in progesterone formation was not due to the appearance of 11β-hydroxyprogesterone.

microsomes to the inner mitochondrial membrane so that the active site faced the matrix space without disruption of the mitochondria is especially difficult to imagine.

The role of mitochondrial HSD in adrenal cortex steroidogenesis is purely speculative at this point. If the mitochondrial enzyme is regulated *in vivo* by the NADH/NAD ratio of the matrix space as suggested here, the product of cholesterol side chain cleavage that leaves the mitochondrion could be either pregnenolone or progesterone. Hochberg and Lieberman (14) recently reported that progesterone is a major product of cholesterol side chain cleavage in rat adrenal mitochondria oxidizing succinate. Since NAD was not added in their experiments they suggested that HSD was present in the mitochondria. Our results support that interpretation. Further studies designed to clarify the effect of the intramitochondrial pyridine nucleotide redox state on the product of cholesterol side chain cleavage will be of interest.

Summary. In this study we have compared some properties of the microsomal and mitochondrial 3β-hydroxysteroid dehydrogenase/isomerase (HSD) from the rat adrenal cortex. The following major differences were noted: (i) the microsomal enzyme required exogenous NAD while the mitochondrial enzyme was active in the absence of added NAD. (ii) The mitochondrial enzyme was inhibited by a combination of rotenone, KCN, and citric acid cycle substrates. These agents did not influence the activity of the microsomal enzyme. (iii) The ratio of the specific activities of HSD to the 21-hydroxylase (a microsomal marker enzyme) was greater in the mitochondria than in the microsomes. We conclude that HSD is present in the inner mitochondrial membrane and, therefore, that the enzyme has a dual mitochondrial and microsomal localization in the rat adrenal cortex.

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