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THE INTRACELLULAR LOCALIZATION OF ADRENAL 3β-HYDROXYSTEROID DEHYDROGENASE/Δ^5-ISOMERASE BY DENSITY GRADIENT PERTURBATION

Denis R. Headon#, Joyce Hsiao, and Frank Ungar

Department of Biochemistry, University of Minnesota Medical School,

Minneapolis, MN 55455

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The intracellular localization of 3β-hydroxysteroid dehydrogenase/Δ^5-isomerase (Δ^5-3β-OHD) in bovine adrenal cortex was determined by density perturbation with density gradient centrifugation and the use of marker enzymes. Mitochondrial rich fractions after incubation with succinate and iodonitrotetrazolium (INT) were loaded with insoluble reduced formazan and separated on a linear sucrose gradient. A shift in median density was observed in the INT-treated mitochondria for protein, succinic dehydrogenase activity, and for Δ^5-3β-OHD activity, but not for 21-hydroxylase, a microsomal enzyme marker. Density perturbation and gradient fractionation reveals a dual location of Δ^5-3β-OHD in both mitochondrial and microsomal fractions.

INTRODUCTION

The localization of hydroxysteroid dehydrogenase/D^5-isomerase (Δ^5-3β-OHD) at a subcellular level in steroid producing cells has been the subject of some controversy. The highest specific activity is present in the microsomal fraction (1) which has been the source of the enzyme for purification studies (2) although several subcellular fractions prepared from homogenates of adrenal contained activity. The detection of Δ^5-3β-OHD activity in the mitochondrial fraction was generally attributed to contamination by endoplasmic reticulum fragments (3,4,5) or to a redistribution of the activity during the homogenisation process (6). Nonetheless, a number of investigators have suggested that the Δ^5-3β-OHD activity is also located within mitochondria in addition to its presence in endoplasmic reticulum (7-10). These suggestions have been derived primarily from circumstantial evidence, since it has not been possible to prepare a pure mitochondrial fraction free from microsomal fragments.

#Present address: Department of Biochemistry, University College Galway, Ireland

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In recent years density perturbation techniques coupled with density gradient centrifugation procedures have been employed in the identification of cholesterol containing membranes in liver (11,12) and in skeletal muscle (13). Other density perturbation procedures include precipitation of calcium oxalate within vesicles of muscle microsomal preparations capable of transporting calcium (14), the formation of lead phosphate precipitate at the sites of phosphatase activity (15) and the development of affinity density perturbation procedures (16). An increase in mitochondrial density has been accomplished by the formation of an insoluble mitochondrial contamination of synaptosomal plasma membrane preparations (17,20). We have utilized the formation of insoluble reduced iodonitrotetrazolium in the mitochondrial fraction by succinate oxidation coupled with analytical fractionation in sucrose density gradients, and marker enzyme analysis to determine the subcellular localization of Δ^5-3β-OHD. By the use of these procedures, we present evidence for the localization of Δ^5-3β-OHD activity in mitochondria as well as in endoplasmic reticulum of the bovine adrenal cortex.

MATERIALS AND METHODS

(4-^{14}C)-Pregnenolone (NEC-375, 52.8mM/mM) and (4-^{14}C)-progesterone (NEC-081, 52.8μCi/mM) were obtained from New England Nuclear Corp. (Boston). Pyridine nucleotides (nicotinamide adenine dinucleotide, NAD, and nicotinamide adenine dinucleotide phosphate, reduced, NADPH) and iodonitrotetrazolium (INT, 2-(p-iodophenyl)-3-(p-nitrophenyl-5-phenyl tetrazolium) chloride were obtained from Sigma Chemical Co. (St. Louis, MO).

A 10% w/v homogenate of fresh scraped bovine adrenal cortex tissue (10gm) was prepared in cold 0.25 M sucrose, buffered at pH 7.4 with 3 mM imidazole HCl (buffered sucrose) with a hand-operated Potter homogenizer. Centrifugations were carried out in a refrigerated Beckman JA21 centrifuge using the JA20 rotor. A mitochondrial rich fraction was prepared from a post 1,000 xg 10 minute supernatant by centrifugation at 10,000 xg for 10 min. The pellet was washed twice by suspension in buffered sucrose and sedimentation at 10,000 xg for 10 minutes. The final washed pellet was resuspended in 10 ml buffered sucrose. A microsomal fraction was sedimented from the supernatant at 105,000 xg for 60 min.

Density gradient centrifugations were carried out at 5°C in a Beckman SW 27 rotor operated with the large buckets. Linear sucrose density gradients of 30 ml volume were prepared from 0.5 to 2.0 M sucrose buffered with 3 mM imidazole-HCl, pH 7.4. Samples were applied to the gradient in 5 ml of buffered 0.25 M sucrose.

Density Perturbation in Analytical Centrifugation: The washed mitochondrial rich fraction was divided into two equal portions which served as the control and analytical. The control sample in 5 ml volume was incubated in an equal volume of medium containing 50 mM succinic acid and 100 mM tris buffer, pH 7.4,while analytical samples contained, in addition, 0.1% INT. Incubation was for 20 minutes

at 37°C. The reduced formazan and tissues were pelleted by centrifugation at 10,000 xg for 10 min. The pellets resuspended in 5 ml of buffered sucrose were centrifuged on a linear sucrose gradient. Gradients were collected in 2 ml fraction by puncturing the bottom of the tube and displacing the contents with 2.0 M sucrose. Analysis: Protein was determined by the Lowry procedure (21). Succinate dehydrogenase activity was determined by the Method of Pennington (22) as modified by Porteous and Clarke (23). Sucrose densities were obtained from the refractive index values using a Bausch and Lomb Refractometer and by consulting standard tables.

The results of analytical fractionation are expressed in the form of histograms where the abscissa represents a density scale (p) and the ordinate gives the frequency within the corresponding span of density (12). Recoveries were determined for the various contituents analyzed and ranged between 93 and 105%.

Assay for Δ^5-3β-OHD and 21-Hydroxylase Activity. The use of INT-loading as a means of preparative fractionation was also employed. The washed mitochondrial fraction was loaded with reduced formazan by the incubation procedure described above. Linear sucrose gradients of 30 ml volume were prepared from 0.85 to 1.4 M sucrose buffered with 3 mM imidazole-HCl, pH 7.4. A 7.5 ml aliquot of resuspended treated mitochondrial fraction equivalent to 10 g fresh tissue was layered over the gradient and centrifuged at 25,000 rpm for 1 hour at 5°C to give a pellet at the bottom of the centrifuge tube. This pellet was resuspended in 8.5 ml of buffered sucrose for use in steroid enzyme assays.

Aliquots of the appropriate fractionated tissues were incubated in Krebs-Ringer-bicarbonate buffer, pH 7.4, in 25 ml Erlenmeyer flasks in a Dubnoff incubator with a 95% O_2 - 5% CO_2 gas phase at 37°C for 0,15 and 30 min. (4-^{14}C)-Pregnenolone (0.5μCi/2.1μg) or (4-^{14}C)-progesterone (0.5μCi/5.0μg) were incubated with 1.0 mg of NAD, or NADPH, respectively, in 2.0 ml buffer containing 0.5 mg of enzyme protein. After incubation, carrier steroids (10 μg each) were added and the flask contents were extracted with 6 ml of methylene dichloride. The steroid substrates and products were separated on thin layer chromatography plates using a solvent system of methylene dichloride; acetone (9:1). The radioactivity contained in the steroid carrier zones was detected with a Packard Model 385 Radiochromatogram Scanner. The carrier steroid zones located with ultra violet light at 240 nm were removed and the radioactivity determined in a toluene solution of 0.05% POPOP and 0.5% PPO on a Beckman Model LS-230 Scintillation counter. Counting efficiency for ^{14}C was 70%.

RESULTS AND DISCUSSION

The conversion of pregnenolone to progesterone was determined by radiochromatogram scans of incubates with mitochondria, INT-treated mitochondria and with a microsomal fraction. Similarly, 21-hydroxylation of progesterone was monitored in the mitochondrial fraction and was shown to be considerably less than that obtained with microsomal tissue. In Figure 1 the rates of conversion of precursor to products in 15 min. are shown for Δ^5-3β-OHD and 21-hydroxylase enzymes.

The distribution of protein in the control and iodonitrotetrazolium treated samples after density gradient centrifugation is shown in Figure 2-A. There is

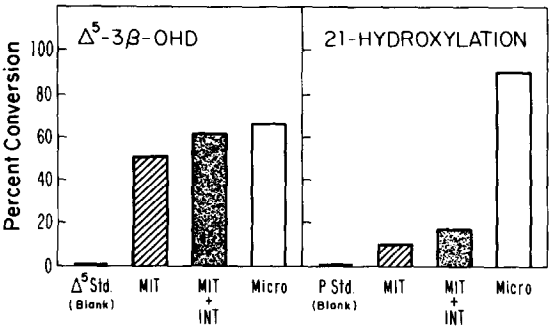

Figure 1. Conversion of [4-^{14}C]-pregnenolone to progesterone (Δ^5-3β-OHD) and [4-^{14}C]-progesterone to 21-hydroxylated products, deoxycorticosterone and corticosterone, (21-hydroxylase) in 15 min incubations with mitochondria, INT-treated mitochondria, and microsomes. Values are the average of duplicate incubations expressed in percent total radioactivity recovered after thin layer chromatography

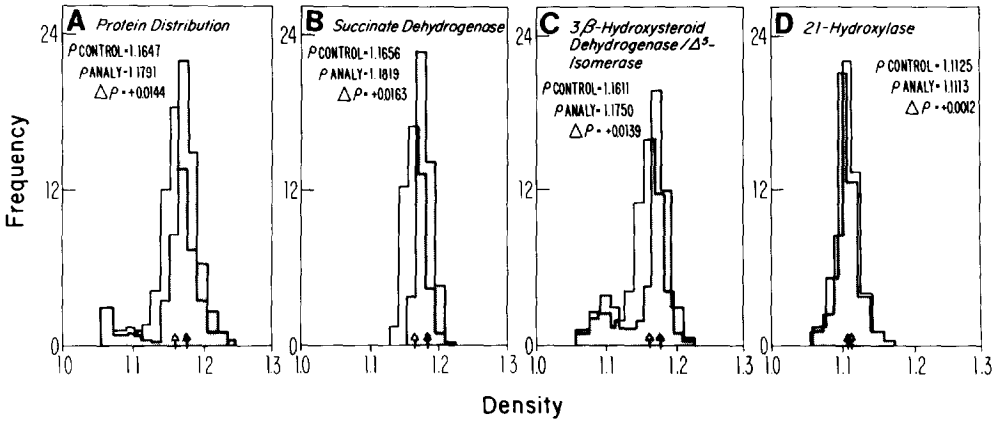

Figure 2. Influence of treatment with iodonitrotetrazolium and succinate on the density distribution patterns present in a mitochondrial fraction prepared from bovine adrenal cortex. The shaded area represents the distribution of the treated material with the median density by a black arrow. The median density value for the control sample is indicated by the white arrow: (A) Protein, (B) Succinic Dehydrogenase activity, (C) Δ^5-3β-OHD activity, (D) 21-Hydroxylase activity.

an increase in median density of 0.0144 for the protein distribution in the INT-treated fraction (1.1791) compared to the control (1.1647). The distribution of succinate dehydrogenase activity (Figure 2-B) gave a median density of 1.1656 for the control and 1.1819 for the INT - treated sample. The density increase of

0.0163 obtained for this mitochondrial marker enzyme would have been expected for protein also, if all of the protein in the mitochondrial fraction had been associated with succinic dehydrogenase activity. On this basis it appears that 87% of the protein in the mitochondrial fraction is associated with components having succinate dehydrogenase activity.

21-Hydroxylase activity was used as an endoplasmic reticulum marker enzyme (4). The histograms for this enzyme activity shown in Figure 2-D, for the control and the INT-treated tissue do not differ significantly, indicating that the distribution of this enzyme was not affected by density perturbation.

The distribution of hydroxysteroid dehydrogenase/Δ^5-isomerase after sucrose density gradient centrifugation of the control and iodonitrotetrazolium treated fraction is shown in Figure 3-C. The median density of this enzyme activity in the control lies between that of 21-hydroxylase activity (endoplasmic reticulum) and succinate dehydrogenase activity (mitochondria). Following treatment with iodonitrotetrazolium the median density distribution of Δ^5-3β-OHD in mitochondria is increased from 1.1611 to 1.1750 in contrast to no shift in distribution for 21-hydroxylase. The extent of the increase (+0.0139) is not as great as that of succinate dehydrogenase. Therefore, while part of the Δ^5-3β-OHD activity is associated with mitochondria, some of the activity in this preparation is due to contamination with fragments of the endoplasmic reticulum. This is supported by the 21-hydroxylase activity observed after incubation with mitochondria (Fig.1). Thus, analytical fractionation with marker enzymes and density perturbation procedures indicate a dual subcellular location of Δ^5-3β-OHD activity in bovine adrenal cortex. The density data obtained is summarized in Table 1.

The relative activities of Δ^5-3β-OHD in the mitochondrial and microsomal fractions of rat adrenal cortex has been evaluated recently by Kream and Sauer (1976) based on a differential effect by the addition of exogenous NAD to the two fractions. The Δ^5-3β-OHD activity in mitochondria, in contrast to the activity in microsomes, responded less to exogenous NAD, and was specifically inhibited by inhibitors such as rotenone. The amount of Δ^5-3β-OHD activity in mitochondria

Table 1. Median densities of constituents in control and iodonitrotetrazolium treated mitochondrial fractions after centrifugation in sucrose density gradients.

CONSTITUENT	MEDIAN DENSITY		DENSITY DIFFERENCE ($\times 10^4$)
	CONTROL	INT-TREATED	
Protein	1.1647	1.1791	+ 144
Succinate Dehydrogenase	1.1656	1.1819	+ 163
21-Hydroxylase	1.1125	1.1113	- 12
Δ^5-3β-OHD	1.1611	1.1750	+ 139

was much greater than could be accounted for by microsomal contamination which, based on the microsomal marker 21-hydroxylase activity, was estimated to be less than 20%. In another study of mitochondrial Δ^5-3β-OHD activity, Moustafa and Koritz (4) estimated a microsomal contamination of their fraction to be on the order of 50%. Based on 21-hydroxylase activity and on density gradient perturbation in our study the microsomal contamination in the mitochondrial fraction was less than 20 percent.

Since the original observations on the isolation and distribution of Δ^5-3β-OHD in mammalian endocrine tissues (1,25) the importance of the enzyme in steroid hormone formation has been amply substantiated. The demonstration of Δ^5-3β-OHD activity first in rat liver by Ungar et al. (26) and subsequently, in peripheral target tissues (27) has extended the significance of this reaction to other tissues for the expression of steroid biological activity. The isolation of a solubilized Δ^5-3β-OHD system from the mitochondrial fraction of human placenta (28) and the report of Δ^5-3β-OHD activity accompanying cholesterol side chain cleavage in rat adrenal mitochondria by Hochberg et al. (29) are two instances where the subcellular localization of the enzyme may have special implications. The distribution of Δ^5-3β-OHD activities in both mitochondrial and microsomal fractions of the adrenal cortex should provide further insight into the effect of cellular compartmentation on the regulation of steroid biogenetic pathways.

REFERENCES

1. Beyer, K.F. and Samuels, L.T., J. Biol. Chem. 219, 69, (1956).

2. Ford, H.C. and Engel, L.L., J. Biol. Chem. 249, 1363, (1974).
3. deDuve, C., J. Cell Biol. 50, 20D, (1971).
4. Moustafa, A. and Koritz, S., Proc. Soc. Exp. Biol. Med. 149, 823, (1975).
5. Hayano, M., Saba, N., Dorfman, R.I. and Hechter, O., Recent Progress in Hormone Research, 12, 79, (1956).
6. Cowan, R., Giles, C., and Grant, J., J. Steroid Biochem. 5, 607, (1974).
7. Kodie, S. and Torres, M., Biochem. Biophys. Acta, 105, 115, (1965).
8. Sulimovici, S., Bartoov, B. and Lunenfeld, B., Biochem. Biophys. Acta, 321, 27, (1973).
9. Armstrong, D.T., Jackanicz, T.M., and Keyes, P. L., in 'The Gonads' (K.W. McKerns, ed.), Appleton-Century-Crofts, New York (1967).
10. Basch, R.S. and Finegold, M.J., Biochem, J. 125, 983, (1971).
11. Thines-Sempoux, D., Amar - Costesec, A., Beaufay, H. and Berthet, J., J. Cell Biol. 43, 189, (1969).
12. Amar-Costesec, A., Wibo, M., Thines-Sempoux, D., Beaufay, H. and Berthet, J. Cell Biol. 62, 717, (1974).
13. O'Flaherty, J., Barrett, E.J., Bradley, D.P. and Headon, D.R., Biochem. Biophys. Acta. 401, 177, (1975).
14. Greaser, M.L., Cassens, R.G., Hoekstra, W.G. and Briskey, E.J., J. Cell. Physiol. 74, 37, (1969).
15. Leskes, A., Siekevitz, P. and Palade, G.E., J. Cell Biol. 49, 288, (1971).
16. Wallach, D.F.H., Kranz, B., Ferber, E. and Fischer, H., FEBS Letters, 21, 29, (1972).
17. Davis, G.A. and Bloom, F.E., J. Cell Biol. 47, 46a, (1970).
18. Davis, G.A. and Bloom, F.E., Anal. Biochem. 51, 429-434, (1973).
19. Cotman, C.W. and Taylor, D., J. Cell Biol. 55, 696, (1972).
20. Cotman, C.W. in Methods in Enzymology, Vol. XXXI, Part A. (S. Fleischer and L. Packer, editors) Academic Press, New York, 1974.
21. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem. 193, 265, (1951).
22. Pennington, R.J., Biochem J. 80, 649, (1961).
23. Porteous, J.W. and Clark, B.J., Biochem. J. 96, 159, (1965).
24. Kream, B.E. and Sauer, L.A., Proc. Soc. Exptl. Biol. & Med. 152, 459, (1976).
25. Samuels, L.T., Hemrich, M.L., Lasater, M.B. and Reich, H. Science 113, 490, (1951).
26. Ungar, F., Miller, A.M. and Dorfman, R.I., J. Biol. Chem. 206, 597, (1954).
27. Dao, T.L., Varela, R. and Morreal, C. in Estrogen Target Tissues and Neoplasia (T.L. Dao, ed) University of Chicago Press, (1972).
28. Edwards, P.P., O'Conner, J.L., Bransome, E.D., and Braselton, Jr. W.E., J. Biol. Chem. 251, 1632, (1976).
29. Hochberg, R. Ladany, S., Welch, M., and Lieberman, S., Biochemistry 13, 1938, (1974).