ULTRASTRUCTURAL EVIDENCE FOR THE BIOGENESIS OF D-AMINO ACID OXIDASE-CONTAINING PEROXISOMES IN 'NORMAL' FETAL MOUSE LIVER IN MUSCULAR DYSGENESIS

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D-amino acid oxidase (DAAO) cytochemistry was undertaken in the 'normal' liver of fetal mice obtained from mothers carrier of muscular dysgenesis. Developing and differentiating hepatocytes revealed that DAAO is released from mitochondria and immediately enveloped by smooth membrane in the cytoplasm to form DAAO-containing peroxisomes. DAAO synthesized in the cytosol may be relocated in mitochondria before being released back into cytosol.

Peroxisomes (microbodies) are respiratory organelles present in the cytoplasm of eukaryotes. They normally range from 0.1 to 0.5 μm in diameter and are delimited by a single membrane. The organelle is made up of homogenous granular matrix containing dense nucleoid or crystalline core within. The matrix may sometimes condense to form matrical plates usually in pathological conditions (4, 5, 7). It is interesting that enzymes (catalase and oxidases) present in peroxisomes are not synthesized in peroxisomes but in the cytosol and then channeled to peroxisomes. Peroxisomal enzymes have not been cytochemically localized at the sites of their synthesis or in their pathways to peroxisomes. There has been a considerable debate over the biogenesis of peroxisomes. Morphologically shown continuities between microbodies and smooth endoplasmic reticulum (ER) in the hepatocytes (10, 11) suggest the possibility that microbodies arise from the ER. Different chemical compositions of peroxisomal membrane and ER (8) and absence of cytochemical connections between them (12) may rule out that possibility. Thus, morphological and cytochemical evidence for the biogenesis of peroxisomes is lacking. Since peroxisomes are formed during tissue development and differentiation, developmental stage may unravel the phenomenon of peroxisome biogenesis. We have previously reported DAAO-positive peroxisomes in the cytoplasm of developing and differentiating hepatocytes and also noticed a mysterious occurrence of some of them in close proximity to mitochondria (6). DAAO-reactive peroxisomes have also been spotted in close apposition to mitochondria in the fat body of Drosophila larva (13). Our further observations as reported in this publication unfold this mystery.

MATERIALS AND METHODS

Only 'normal' fetuses of 19 days gestation from mother mice carrier of muscular dysgenesis were used. They were delivered by hysterectomy under anesthesia and their livers were dissected out and cut into small pieces to fix for 1/2 hr in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4. With the rinse in buffer the tissue was processed as follow for DAAO cytochemistry using modified cerium chloride technique (2, 14). Preincubation was done for 1/2 hr at 25°C in the presoak containing 0.084 g 3-amino-1,2,4-triazole (AT), 0.012 g cerium chloride, 10 ml 0.1 M Tris-maleate buffer at pH 7.4. Final incubation was done for 1/2-2 hr at 25°C in the medium containing 0.084 g AT, 0.012 g cerium chloride, 10 ml 0.1 M Tris-maleate buffer at pH 7.4 and 0.057 g D-proline as a substrate (All chemicals were from Sigma, St. Louis, Mo.). Controls were made with medium lacking the substrate. The tissue was then washed in a slightly acidic 0.1 M Na-cacodylate buffer, pH 6.0, to remove cerium hydroxide precipitate. For electron microscopy, the tissue was then postfixed for 1/2 hr in
Fig. 1. Liver of 'normal' fetus. Hepatocyte showing DAAO reaction product within and on the mitochondria (M). Note that DAAO being expelled out of the mitochondrion and enwrapped with smooth membrane (large arrow head). Fully formed DAAO-containing peroxisome (P) anchored with stretched membrane (small arrow head) seems to be moving away from the mitochondrion. ×67,000

Fig. 2. Liver of 'normal' fetus. Hepatocyte with large accumulations of DAAO reaction product attached to the mitochondrial membrane. One being expelled out and enclosed with tubular smooth membrane (arrow head). DAAO-negative peroxisome (P) larger in size with crystalline core is also seen. M: mitochondria. ×67,000
1% aqueous osmium tetroxide, dehydrated in graded ethanol followed by propylene oxide and embedded in araldite (Durcupan, ACM Fluka). Thin sections cut on Porter-Blum MT-2 were stained with uranyl acetate and lead citrate; and examined with electron microscopes (Hitachi, Philips).

RESULTS

Liver was fixed for only 1/2 hr (by immersion) as the enzyme is known to be extremely sensitive to the glutaraldehyde fixative. DAAO-reactive peroxisomes were seen distributed in the cytoplasm of hepatocytes; a number of them in close vicinity of mitochondria. DAAO reaction product was seen within and attached to the membranes of mitochondria (Figs. 1, 2). Some appeared as being expelled out of mitochondria and enwrapped with smooth membrane (Figs. 1, 2). Staining of this membrane with lead citrate was crucial. Fully formed peroxisomes (P, Fig. 1) seemed to pull away from mitochondria. Controls did not show any DAAO reaction either in peroxisomes or mitochondria.

DISCUSSION

DAAO is a flavoprotein which catalyzes the oxidation of D-amino acid in the presence of oxygen liberating H$_2$O$_2$, NH$_3$ and the corresponding keto acid (1). Like catalase DAAO is also a marker enzyme of peroxisomes. In a cytochemical reaction with cerium chloride technique, DAAO in the cell oxidizes substrate D-proline to generate H$_2$O$_2$. Enzyme-generated H$_2$O$_2$ then get trapped by cerium ions to form insoluble cerium perhydroxide which is electron dense and can be visualized as a site of DAAO. As a result peroxisomes were turned positive for DAAO; some in close apposition to mitochondria. However, few peroxisomes remained inactive for DAAO which may contain other peroxisomal enzymes. Enzymatic heterogeneity among peroxisomes exists (2, 6). Results also indicate that DAAO reaction product within and on mitochondria is truly enzymatic. It seems that DAAO moves towards the periphery of mitochondria and crosses the membrane into the cytoplasm. The released enzyme then gets enveloped by a smooth membrane which suddenly appears in the vicinity and may have a specific receptor for DAAO. Fully formed peroxisomes (about 0.13 μm) then move away from mitochondria. Peroxisome formed by this mode may exclusively contain DAAO. Peroxisomal and mitochondrial β-oxidation systems are remarkably similar and shuttling of metabolites between these respiratory organelles do occur. Like most mitochondrial proteins, peroxisomal proteins are translated on free cytosolic polyribosomes and then imported into peroxisomes (9). It may be possible that DAAO synthesized in the cytosol may be relocated in mitochondria before being released back into the cytosol though there is no biochemical evidence yet shown. ‘Normal’ fetal mouse liver was from muscular dysgenesis. Muscular dysgenesis (mdg) is recessive lethal in mouse. Mutation causes developmental arrest (3). Our ‘normal’ fetuses were carrier (+/mdg) or actually normal (+/+). Mutants (mdg/mdg) were not used in this study. Qualitative and quantitative data on peroxisomes in ‘normal’ and mutants need to be obtained to assess whether or not peroxisomes are affected in muscular dysgenesis. Finally, the events of biogenesis of DAAO-containing peroxisomes thus recorded during development may prove to be a significant observation in peroxisome biology.

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