Biosynthesis and maturation of peroxisomal \(\beta\)-oxidation enzymes in fibroblasts in relation to the Zellweger syndrome and infantile Refsum disease

(acyl-CoA oxidase/peroxisomal 3-oxoacyl-CoA thiolase/peroxisomal biogenesis/catalase)

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**ABSTRACT** The biosynthesis of the peroxisomal enzymes acyl-CoA oxidase, 3-oxoacyl-CoA thiolase (acyetyl-CoA acyltransferase, EC 2.3.1.16), and catalase (EC 1.11.1.6) was studied in cultured skin fibroblasts from a control subject and from patients with Zellweger syndrome and the infantile form of Refsum disease, inherited disorders in which peroxisomes are deficient and certain peroxisomal functions are impaired. The results of continuous labeling and pulse–chase experiments indicate that in control fibroblasts, as in rat liver, acyl-CoA oxidase is synthesized as a 72-kDa precursor that is converted to two polypeptides of 52 and 20 kDa and 3-oxoacyl-CoA thiolase is synthesized as a 44-kDa precursor that is converted to the 41-kDa mature protein. In fibroblasts from the patients the precursors of the two enzymes are formed but their maturation is impaired, and they are rapidly degraded. In contrast, the biosynthesis of catalase is not impaired. We conclude that functional peroxisomes are required for the maturation and stability of acyl-CoA oxidase and 3-oxoacyl-CoA thiolase but not for catalase.

In 1973, Goldfischer et al. (1) discovered that morphologically distinct peroxisomes are absent in liver and kidney of patients with the cerebrohepato renal (Zellweger) syndrome, an autosomal-recessive disease usually leading to death within the first year of life. Peroxisomes are also greatly reduced in number in cultured skin fibroblasts from patients with the classic form of the Zellweger syndrome (2). The deficiency of peroxisomes is accompanied by a number of biochemical abnormalities, including an accumulation in tissues and/or body fluids of very long-chain fatty acids (3), of lecithin (4), and of intermediates in the biosynthesis of bile acids (5) and a deficiency in tissue plasminogen (6). These biochemical abnormalities are in accordance with findings showing that peroxisomes play an essential role in the oxidation of very long-chain fatty acids (7), the catabolism of lecithin (J. M. F. Trijbels, personal communication), and the biosynthesis of bile acids (8) and ether phospholipids (9).

We have found (10, 11) that dihydroxyacetone phosphate acyltransferase and alkylidihydroxyacetone phosphate synthase, two peroxisomal enzymes (9) required for the introduction of the ether bond in ether phospholipids, are deficient in the Zellweger syndrome (see also ref. 12). Both enzymes are membrane bound (13). In contrast, the soluble peroxisomal matrix enzymes catalase (EC 1.11.1.6), L-α-hydroxy acid oxidase ([L]-2-hydroxy-acid oxidase, EC 1.1.3.15), and D-amino acid oxidase (EC 1.4.3.3) are not deficient in liver from the patients (14) but are found in the cytosol (15). In Zellweger fibroblasts, too, all (14, 16) or almost all (2) of the catalase is in the cytosol.

We have shown (17) by immunoblotting that the peroxisomal \(\beta\)-oxidation enzyme proteins acyl-CoA oxidase (18), the bifunctional protein with enoyl-CoA hydratase (EC 4.2.1.17) and with 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) activities (19), and 3-oxoacyl-CoA thiolase (acyetyl-CoA acyltransferase; EC 2.3.1.16) (20) are deficient in liver from Zellweger patients (see also ref. 21). Small amounts of high molecular weight, precursor forms of 3-oxoacyl-CoA thiolase (22, 23) were detected in the patients. These results suggest that functional peroxisomes are required for the maturation and stability of peroxisomal \(\beta\)-oxidation enzymes. To investigate this possibility, we have, therefore, carried out pulse-labeling experiments with cultured skin fibroblasts from a control subject and patients with the Zellweger syndrome and the infantile form of Refsum disease (24), a related disorder (25-27) in which peroxisomal functions are impaired (25-30) and peroxisomes are also deficient (31, 32).

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions.** The following fibroblast cell lines were used: Fc, cells from a control subject; KrZw 84-118, MA 85 AD, and W78/515, cells from Zellweger patients (obtained from L. A. Mohnnens, H. Przyrembel, and W. H. H. Tegelaers, respectively); FRM84AD, cells from a patient with the infantile form of Refsum disease (obtained from J. M. Saudubray and B. T. Poll-Thé). The cells were grown in Ham's F10 medium (Flow Laboratories), 10% (vol/vol) fetal calf serum and antibiotics.

**Continuous Labeling of Cells with \[^{14}C\]Leucine.** Three days after the cells had reached confluency, the medium was replaced with medium containing 0.19 mM leucine and \[^{14}C\]leucine (15 μCi per flask; specific activity, 3000 mCi/mmol; 1 Ci = 37 GBq). The cells were harvested after 4 days and treated further as described below.

**Pulse–Chase Experiments with \[^{35}S\]Methionine.** Three days after confluence the cells were preincubated for 1 hr with methionine-free medium and pulsed for 1 hr with the same medium containing \[^{35}S\]methionine (100 μCi per flask; specific activity, 1100 Ci/mmol) and 5% (vol/vol) dialyzed fetal calf serum. The cells were either extracted immediately or chased for 24 hr in medium containing unlabeled methionine (0.2 mM) and 10% (vol/vol) fetal calf serum before extraction with a solution containing 50 mM Tris-HCl, 5 mM MgCl₂, 0.5% Triton X-100, and 50 mM NaCl (pH 7.5). The extracts were treated further as described below.

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Immunoprecipitation, Electrophoresis, and Fluorography.

The extracts were centrifuged for 1 hr in a Spinco centrifuge at 50,000 \( \times g \), and the supernatants were used for immunoprecipitation of labeled proteins as described (33). The antibodies used were an anti-bovine liver catalase antiserum (17), an anti-rat liver acyl-CoA oxidase IgG fraction (18, 34), and an anti-rat liver 3-oxoacyl-CoA thiolase IgG fraction (34). The procedures for NaDodSO4/PAGE and fluorography were as described (33).

RESULTS

Fig. 1 shows an experiment in which fibroblasts were labeled continuously for 4 days with \(^{[3]C}\)leucine. After immunoprecipitation with anti-catalase serum, the main band seen in all cell lines was \( \approx \) 62 kDa (Fig. 1A; cf. refs. 20, 35, and 36). The smaller sized bands represent degradation products. In the control cell line (Fig. 1B, lane C), the immunoprecipitate obtained from anti-acyl-CoA oxidase IgG contained a prominent 52-kDa band corresponding to component B of rat liver acyl-CoA oxidase (18) and minor amounts of 72- and 20-kDa bands, corresponding to components A and C, respectively (18). In contrast, the immunoprecipitates from a patient with infantile Refsum disease (Fig. 1B, lane R) and two patients with the Zellweger syndrome (Fig. 1B, lanes Z) contained only the 72-kDa band, and its intensity was higher than that of the same band in the other cell lines.

Immunoprecipitation of a homogenate of control cells with anti-3-oxoacyl-CoA thiolase IgG yielded three bands of 44, 42, and 41 kDa (Fig. 1C, lane C). The 44- and 41-kDa bands correspond to precursor and mature 3-oxoacyl-CoA thiolase in rat liver (22, 23, 34) and the 42-kDa band presumably represents an intermediate form. Only the 44-kDa band was detected in immunoprecipitates of fibroblasts from the patients (Fig. 1C, lanes R and Z).

The biosynthesis of acyl-CoA oxidase was studied further in pulse-chase experiments. In control cells pulsed for 1 hr with \(^{[35]S}\)methionine a 72-kDa labeled protein was detected in the immunoprecipitate obtained with anti-acyl-CoA oxidase IgG; this component was converted to a 52-kDa form after a 24-hr chase (Fig. 2, lanes C). The 20-kDa component could not be detected (cf. ref. 34). In cells from patients with the Zellweger syndrome (Fig. 2, lanes Z) and infantile Refsum disease (Fig. 2, lanes R), a normal amount of the 72-kDa precursor and a very small amount of the 52-kDa form were found after the 1-hr pulse but very little conversion to the 52-kDa form occurred during the 24-hr chase period. Instead, the total amount of labeled protein decreased after a 24-hr chase. It should be noted that a considerable amount of the 72-kDa form was still present in fibroblasts from the patients after the 24-hr chase period.

Analogous results were obtained with 3-oxoacyl-CoA thiolase. In control fibroblasts the 44-kDa precursor formed after a 1-hr pulse was converted in part during a 24-hr chase to the 41-kDa mature protein, whereas this conversion was diminished in fibroblasts from Zellweger and infantile Refsum patients (not shown).

The relative amounts of the 72- and 52-kDa components of acyl-CoA oxidase were quantified by cutting out the bands in an experiment similar to that shown in Fig. 2 and estimating the radioactivity in a liquid scintillation counter. The ratio of radioactivity in the 72-kDa band to that in the 52-kDa band decreased in control cells from a value of 10.3 after the pulse to 0.17 after the chase. In contrast, the ratio decreased only slightly in fibroblasts from the patients (from 6.1 to 4.3 in Zellweger fibroblasts and from 2.3 to 1.6 and to 1.5 in fibroblasts from two patients with infantile Refsum disease).

DISCUSSION

Osumi et al. (18) have shown that acyl-CoA oxidase isolated from rat liver contains three components, A, B, and C, of Mr 72,500, 50,100, and 19,000, respectively. Figure 3 of ref. 18 suggests that the 50.1-kDa polypeptide is the main component. Evidence was presented (18, 34) showing that components B and C are derived from component A by proteolytic cleavage. Treatment of the isolated enzyme with trypsin led to disappearance of component A without a change in specific activity. The results presented in this paper suggest that in human skin fibroblasts acyl-CoA oxidase is synthesized as a 72-kDa precursor that is converted to the mature 52-kDa form of the enzyme (+20-kDa peptide). Similarly, 3-oxoacyl-CoA thiolase is synthesized as a 44-kDa precursor that is converted to the 41-kDa mature enzyme.

Thus in mammalian cells the maturation of at least two peroxisomal enzymes involves proteolytic processing. Fur-
thermore, our results show that functional peroxisomes are required for efficient processing of the two enzymes; in fibroblasts from patients with the Zellweger syndrome (2, 14) and infantile Refsum disease (30–32), in which peroxisomes are deficient, processing of these enzymes is impaired.

Miura et al. (34) have found in pulse-labeling and fractionation experiments with isolated rat hepatocytes that the precursor and mature forms of 3-oxoacyl-CoA thiolase are equally distributed between the cytosolic and particulate fractions. This result suggests that processing of 3-oxoacyl-CoA thiolase is not linked to transport into the peroxisome. Possibly the putative processing protease is present on the outer face of the peroxisome and transport into the peroxisome takes place after proteolytic processing.

Finally, the absence of peroxisomes and concomitant impairment of processing of acyl-CoA oxidase and 3-oxoacyl-CoA thiolase in fibroblasts from patients with the Zellweger syndrome (and infantile Refsum disease) is accompanied by breakdown of the newly synthesized precursors of these enzymes. This is in accordance with the deficiency of cross-reactive material observed in immunoblots of liver from the Zellweger patients (17).

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