Synthesis of 3-ketoacyl-CoA thiolase of rat liver peroxisomes on free polyribosomes as a larger precursor

Induction of thiolase mRNA activity by clofibrate

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The site of synthesis and induction by clofibrate of peroxisomal 3-ketoacyl-CoA thiolase (acetyl-CoA acyltransferase; EC 2.3.1.16) was investigated. Free and membrane-bound polyribosomal RNA species from the livers of normal rats and rats treated with clofibrate, a hypolipidaemic drug that causes marked proliferation of peroxisomes, were translated in a nuclease-treated rabbit reticulocyte-lysate cell-free protein-synthesizing system with [35 S]methionine as label. The cell-free translation products were immunoprecipitated with monospecific rabbit anti-thiolase serum and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and fluorography. Thiolase mRNA was found predominantly in free polyribosomes, in both normal and clofibrate-treated rats. Clofibrate treatment increased mRNA activity for thiolase approx. 20-fold. The translation product of clofibrate-induced thiolase mRNA migrated slightly faster in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis than did the translation product of normal thiolase mRNA. Both the normal and the clofibrate-induced translation products were approx. 6000 Da larger than the 41 000-Da subunit of the purified enzyme. Immunoblot analysis of liver homogenates, isolated peroxisomes and the purified enzyme indicated that the thiolase subunit was approx. 41 000 Da in all samples, ruling out proteolysis during the purification of thiolase. Thiolase biogenesis thus differs from that of rat liver peroxisomal proteins studied previously in that it is synthesized as a larger precursor, implying post-translational import of thiolase into peroxisomes with proteolytic processing. Clofibrate apparently alters the size as well as the amount of the translation product.

Previous studies on the biogenesis of rat liver peroxisomes have dealt with matrix enzymes, including catalase (Higashi & Peters, 1963; Redman *et al.*, 1972; Lazarow & de Duve, 1973*b*; Goldman & Blobel, 1978; Robbi & Lazarow, 1978, 1982; Tobe & Higashi, 1980), acyl-CoA oxidase and the bifunctional enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (Furuta *et al.*, 1982*a*; Rachubinski *et al.*, 1983, 1984), carnitine octanoyltransferase (Ozasa *et al.*, 1983), and the crystalloid core protein, urate oxidase (Goldman & Blobel, 1978). These proteins are all synthesized on free polyribosomes at their mature sizes. We have demonstrated the synthesis of a major integral membrane polypeptide of rat liver peroxisomes on

free polyribosomes: it is also made at its mature size (Fujiki *et al.*, 1983, 1984*a*).

One apparent exception to this rule is 3-ketoacyl-CoA thiolase (acetyl-CoA acyltransferase; EC 2.3.1.16), which in dimeric form (Miyazawa *et al.*, 1981) catalyses the last reaction of the peroxisomal system of fatty acid β -oxidation. Furuta *et al.* (1982*a*) reported that the thiolase cell-free translation product was about 3000 Da larger than the purified peroxisomal thiolase subunit, suggesting the possibility of a rat liver peroxisomal protein-processing machinery. This result was reminiscent of previous findings concerning catalase biogenesis: Robbi & Lazarow (1978) observed that the catalase cell-free translation product was 4000 Da larger than the purified peroxisomal catalase subunit. However, this difference was due

Abbreviation used: SDS, sodium dodecyl sulphate.

entirely to an artifactual proteolytic cleavage of catalase during the purification procedure. When proteolysis was avoided by the immunochemical isolation of peroxisomal catalase, the cell-free translation product and the subunit of the mature enzyme were the same size (Robbi & Lazarow, 1978; Goldman & Blobel, 1978) and were indistinguishable in one- and two-dimensional peptide mappings (Robbi & Lazarow, 1982).

The glyoxysome, a related plant organelle, also contains proteins that are very susceptible to proteolysis, including isocitrate lyase in *Neurospora* (Zimmermann & Neupert, 1980) and catalase in cucumber (Kindl, 1982a). For rat liver catalase, the proteolysis appears to be caused by a lysosomal enzyme, probably cathepsin B (Mainferme & Wattiaux, 1982).

We have therefore investigated further the biogenesis of thiolase under conditions that prevent artifactual proteolysis. In this paper, we report the size comparison of authentic thiolase and the thiolase translation product *in vitro*, as well as the subcellular site of thiolase mRNA. We also describe the effect on thiolase mRNA activity of clofibrate, a hypolipidaemic drug that causes a striking proliferation of peroxisomes (Hess *et al.*, 1965) and an induction of the peroxisomal β -oxidation system (Lazarow & de Duve, 1976; Lazarow, 1977; Lazarow *et al.*, 1982). Some of these results have appeared in abstract form (Fujiki *et al.*, 1984b).

Materials and methods

Preparation of antiserum

A rabbit was immunized with purified thiolase from di-(2-ethylhexyl) phthalate-treated rats (Miyazawa *et al.*, 1980) (generously given by Dr. T. Hashimoto) by injection into the popliteal lymph nodes, followed by one boost as described previously (Goudie *et al.*, 1966; Fujiki *et al.*, 1984a). Rabbit anti-(rat serum albumin) was purchased from United States Biochemical Corp. (Cleveland, OH, U.S.A.) and the preparation of rabbit anti-(rat liver catalase) was described previously (Lazarow & de Duve, 1973a). Immunoblot analyses were performed by the modification (Fujiki *et al.*, 1984a) of the procedure of Burnette (1981).

Isolation of RNA and cell-free translation

The preparation of RNA from free and membrane-bound polyribosomes of normal and clofibrate-treated rat livers was described previously (Fujiki *et al.*, 1984a; Rachubinski *et al.*, 1984). For the study of the effect of clofibrate on thiolase mRNA activity, total RNA was isolated by guanidinium thiocyanate/guanidinium chloride

extraction (Chirgwin *et al.*, 1979) from the livers of four male rats (Fisher F-344; Charles River Breeding Laboratories, Wilmington, MA, U.S.A.), two of which were given clofibrate (5 g/kg of chow) for 2 weeks (Mortensen & Lazarow, 1984). Total RNA prepared from a post-mitochondrial fraction of the liver of a clofibrate-treated male F-344 rat by phenol/chloroform extraction (Shore & Tata, 1977) was included in some experiments; this RNA is enriched in free polyribosomal RNA relative to bound. RNA (final concn. 200–600 μ g/ml) was translated with [35 S]methionine (1000–1300 Ci/mmol; Amersham Corp., Arlington Heights, IL, U.S.A.) as labelled amino acid in a nuclease-treated mRNA-dependent rabbit reticulocyte-lysate cell-free protein-synthesizing system (Pelham & Jackson, 1976; Fujiki *et al.*, 1984a).

Analysis of cell-free products

Thiolase synthesized *in vitro* was immunoprecipitated from post-ribosomal supernatants of translation mixtures with anti-thiolase and *Staphylococcus aureus* cells, and was analysed by SDS/polyacrylamide-gel electrophoresis followed by fluorography on pre-exposed film as described by Fujiki *et al.* (1984a). Control immunoprecipitations of catalase and preproalbumin were carried out similarly. Quantification was carried out by densitometric scanning of the fluorograms with a Bio-Rad model 1650 densitometer.

Preparation of cell fractions

Normal rat liver was homogenized in 0.25M-sucrose containing 5mM-Hepes/KOH, pH 7.3, 0.1% ethanol and 15 kallikrein-inhibitor units of aprotinin/ml. The liver homogenate of a clofibrate-treated rat was prepared in the same solution, with the addition of 10 μ g of cycloheximide/ml, 20 μ M-dithiothreitol, 2mM-methionine, 250 ng of leupeptin and antipain/ml and 125 ng of chymostatin and pepstatin/ml. Peroxisomes were isolated from normal liver as described by Leighton *et al.* (1968) and from livers of clofibrate-treated rats as described previously (Lazarow *et al.*, 1982).

Materials

Proteinase inhibitors and molecular-mass marker proteins [rabbit muscle phosphorylase *b* (97400 Da), bovine serum albumin (68000), ovalbumin (45000), bovine erythrocyte carbonic anhydrase (29000), bovine pancreas trypsinogen (24000), soya-bean trypsin inhibitor (20000) and egg-white lysozyme (14300)] were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

Results

Immunoblots

Rabbit antiserum raised against thiolase was used to probe rat liver fractions by immunoblotting (Fig. 1). Antibodies reacted with purified thiolase (Fig. 1*b*, lane 2), with a band in clofibrate-induced peroxisomes that had previously been tentatively identified as thiolase (Lazarow *et al.*, 1982) (lane 1) and with one band in the liver homogenate of a clofibrate-treated rat (lane 3). At this level of sensitivity, no thiolase was detected in the liver homogenate of a normal rat (lane 4). Antibodies did not bind to any other peroxisomal polypeptide, nor to any other homogenate protein. These results demonstrate that the antiserum is specific for thiolase, and they confirm the previous identification of thiolase among peroxisomal proteins.

The purified thiolase co-migrated with the thiolase present in purified peroxisomes as well as the thiolase of the unfractionated homogenate (Fig. 1*b*). The apparent molecular mass of the thiolase subunit was estimated to be $41\,000 \pm 800$ Da ($n = 3$), in agreement with the published value of 40 000 Da (Miyazawa *et al.*, 1981).

A small amount of thiolase could be detected by immunoblotting in normal peroxisomes (Fig. 1*c*).

Cell-free synthesis of thiolase

Total RNA from the liver of a clofibrate-treated rat was translated *in vitro*. Immunoprecipitation with anti-thiolase serum yielded a single 35 S-labelled band of 47 000 Da on SDS/polyacrylamide-gel electrophoresis (Fig. 2, lane 2). This 35 S-

Fig. 1. Immunoblot analysis with anti-thiolase serum

(a) SDS/polyacrylamide gel stained with Coomassie Brilliant Blue. Lanes: 1, isolated peroxisomes (10 µg of protein) from livers of rats treated with clofibrate (CP); 2, purified peroxisomal 3-ketoacyl-CoA thiolase (TH) (1 µg); 3, liver homogenate (50 µg) of clofibrate-treated rat (CL); 4, homogenate (50 µg) of normal rat liver (NL). (b) Immunoblot with antiserum and 125 I-protein A of duplicate samples as in panel (a). (c) Immunoblot of lanes: 5, normal peroxisomes (NP) (50 µg); 6, clofibrate-induced peroxisomes (CP) (30 µg); 7, purified thiolase (TH) (1 µg). Numbers to the left of each panel indicate the positions of molecular-mass markers (kDa, STD).

Fig. 2. Cell-free synthesis of thiolase

Lanes 1–3, fluorogram: 1, immunoprecipitate with preimmune serum; 2, immunoprecipitate with immune serum; 3, immunoprecipitation as lane 2, except that 10 µg of purified thiolase was present during the immunoprecipitation. Lane 4, purified thiolase (2 µg), Coomassie Blue-stained (CB). SDS/polyacrylamide-gel electrophoresis molecular-mass markers (kDa) are at left (STD). Translation products (24 µl, 4×10^5 c.p.m.) encoded by 'free-enriched' total RNA of clofibrate-treated rat liver were subjected to immunoprecipitation.

labelled polypeptide was approx. 6000 Da larger than the authentic 41 000 Da subunit of the purified enzyme (lane 4). The labelled cell-free product was not immunoprecipitated by preimmune serum (lane 1), and was displaced by unlabelled authentic thiolase during immunoprecipitation (lane 3).

Intracellular site of synthesis

The products of translation *in vitro* of free polyribosomal RNA and membrane-bound polyribosomal RNA fractions from livers of normal and clofibrate-treated rats were subjected to immunoprecipitation with anti-thiolase serum (Fig. 3). Thiolase was encoded predominantly by free polyribosomal RNA (lanes 5) rather than by membrane-bound polyribosomal RNA (lanes 6) in both normal and clofibrate-treated rats. Preproalbumin, which is visible among the total translation products, was synthesized by membrane-bound polyribosomal RNA (Fig. 3, arrows).

Quantification by densitometric scanning of the fluorograms showed that 90–94% of the thiolase mRNA activity was found in free polyribosomal RNA. [35 S]Preproalbumin and [35 S]catalase were similarly immunoprecipitated (Fig. 3, insets) and quantified: approx. 99% of the preproalbumin was found in membrane-bound polyribosomal RNA translation products, whereas 94–96% of the catalase was detected in free polyribosomal RNA translation products. This confirms the adequate separation of free and membrane-bound polyribosomes.

Induction of thiolase mRNA by clofibrate

Thiolase mRNA activity was assayed by cell-free translation of total liver RNA (Fig. 4). Clofibrate greatly increased the amount of cell-free product made (cf. lanes 3 and 4 with 5 and 6). In addition, the drug treatment led to the synthesis of a translation product that migrated slightly faster than the normal translation product (cf. lanes 3

Table 1. Induction of thiolase mRNA activity by clofibrate

Immunoprecipitated translation products were quantified by SDS/polyacrylamide-gel electrophoresis, fluorography and scanning densitometry.

RNA	Thiolase <i>in vitro</i> product	Fluorographic intensity (arbitrary units)		
		Normal	Clofibrate-induced	Ratio
Total RNA*		88 ± 3	1900 ± 97	22
Free polyribosomal RNA†		150	2850	19

* RNA extracted from rat liver homogenate by guanidinium thiocyanate/guanidinium chloride extraction (Chirgwin *et al.*, 1979). Results are means ± range for two rats.

† Scans of Fig. 3, lanes 5.

Fig. 3. Site of synthesis of thiolase

(a) RNA from normal rat liver; (b) RNA from clofibrate-treated rat liver. Lanes 1–3, total translation products (4×10^5 c.p.m.). Arrows indicate preproalbumin. Lanes 4–6, immunoprecipitates (IMPT; each from 4×10^5 c.p.m. of translation products). T, total RNA; F, free polyribosomal RNA; B, membrane-bound polyribosomal RNA. Arrowheads indicate the position of purified thiolase. Insets at the bottom show the immunoprecipitated preproalbumin and catalase translation products from total, free and membrane-bound polyribosomal RNA. Radioactive ink dots mark the positions of albumin and catalase. Total RNA preparations used were guanidinium thiocyanate-extracted total RNA from normal rat liver homogenate and 'free-enriched' total RNA from the post-mitochondrial fraction of clofibrate-treated rat liver. The fluorogram of (b) was over-exposed in order to detect the [35 S]thiolase from membrane-bound polyribosomal-RNA translation products. STD, molecular-mass markers (kDa).

and 4 with 1, 2, 5 and 6). The normal 35 S-labelled translation product was displaced during immunoprecipitation by unlabelled purified thiolase (results not shown) in the same fashion as for the clofibrate-induced product (Fig. 2, lane 3).

The results of Fig. 4 were quantified by scanning densitometry; total liver thiolase mRNA activity was 22 times greater in the clofibrate-treated rats than in the controls (Table 1). An mRNA activity ratio of 19 was determined for the free polyribosomal RNA of Fig. 3, lanes 5.

Discussion

The results reported herein demonstrate that the biogenesis of thiolase in part resembles, and in part

differs from, that of other rat liver peroxisomal proteins. The resemblance is in the site of synthesis: thiolase mRNA was found predominantly in free polyribosomes, in both normal and clofibrate-treated rats. We interpret the small amount of mRNA coding for thiolase in the membrane-bound polysomal RNA fractions to be due to the residual cross-contamination of free and bound polyribosomes, as observed previously (Goldman & Blobel, 1978; Shore *et al.*, 1979; Rachubinski *et al.*, 1980; Borgese & Gaetani, 1980; Okada *et al.*, 1982; Colman *et al.*, 1982; Rachubinski *et al.*, 1983; Fujiki *et al.*, 1984a).

The difference between thiolase and other peroxisomal proteins investigated thus far is that thiolase is synthesized *in vitro* as a precursor

Fig. 4. *Induction of thiolase mRNA activity by clofibrate*. Lanes 3 and 4, mRNA from two normal (NL) rats; lanes 5 and 6, mRNA from two clofibrate (CL)-treated rats; immunoprecipitation from 40 μ l of translation mixture (approx. 4×10^5 c.p.m.). Lanes 1 and 2, same as 5 and 6, except that immunoprecipitation was from 4 μ l. Arrows indicate cell-free thiolase translation products. Radioactive ink dot indicates position of purified thiolase in the SDS/polyacrylamide gel. STD, molecular-mass markers (kDa).

approx. 6000 Da larger than the mature enzyme subunit. As pointed out in the introduction, proteolysis during enzyme purification has in the past caused apparent, but artifactual, differences of this sort for other proteins, such as peroxisomal catalase (Robbi & Lazarow, 1978) and isocitrate lyase of glyoxysomes (Zimmermann & Neupert, 1980). Therefore it is essential in reporting a larger precursor for a peroxisomal protein that proteolysis should be excluded. We approached this problem by determining the size of mature thiolase in isolated peroxisomes and homogenates by SDS/polyacrylamide-gel electrophoresis and immunoblotting. Unlike in the procedures of enzyme purification or immunoprecipitation, thiolase was not maintained for long times at 0°C in the presence of other proteins, including lysosomal

proteinases, nor was it incubated at 37°C, 26°C or 0°C in homogenates with detergents and anti-serum. Rather, homogenates and peroxisomes were frozen promptly after preparation and boiled in SDS/polyacrylamide-gel electrophoresis sample buffer immediately after thawing. The liver homogenate of a clofibrate-treated rat in Fig. 1, lane 3, was prepared in the presence of several proteinase inhibitors, including leupeptin, which has been shown to protect catalase against degradation (Mainferme & Wattiaux, 1982). The immunoblotting results, that (1) a thiolase of approx. 41 000 Da was observed in all samples, and (2) there were no traces of a larger thiolase protein, make it unlikely that proteolysis has occurred.

The size difference that we observe for thiolase synthesized *in vitro* is twice as large as the 3000 Da reported by Furuta *et al.* (1982a) for thiolase induced by the plasticizer, di-(2-ethylhexyl) phthalate. This discrepancy is due mainly to a difference in the cell-free product (47 000 versus 43 000 Da); the measured values for mature thiolase nearly agree (41 000 and 40 000 Da). Chatterjee *et al.* (1983) reported a molecular mass of 43 000 Da for the thiolase translation product induced by another hypolipidaemic drug, Wy-14,643; no comparison was made with the mature protein.

Thiolase is the only known exception to the rule that peroxisomal proteins are synthesized at their final sizes. The same rule applies to the biogenesis of the proteins of the related plant organelle, the glyoxysome, including isocitrate lyase and malate synthase (Zimmermann & Neupert, 1980; Kruse *et al.*, 1981; Kindl, 1982a,b; Lord & Roberts, 1982; Desel *et al.*, 1982). These proteins are likewise synthesized on free polyribosomes. Here too there appears to be an exception, namely malate dehydrogenase (in watermelon and cucumber), which is reported to be made *in vitro* as a precursor 5000–8000 Da larger than the purified enzyme subunit or the protein labelled *in vivo* (Walk & Hock, 1978; Riezman *et al.*, 1980; Hock & Gietl, 1982). The presence of phenylmethanesulphonyl fluoride during homogenization did not affect the size of the immunoprecipitated protein (Hock & Gietl, 1982).

Cucumber catalase synthesized *in vitro* has been reported to be 1000 Da larger than the mature protein subunit (Riezman *et al.*, 1980). However, cucumber catalase has a sensitivity to proteinase similar to that of rat liver catalase and may be observed in a variety of sizes (Kindl, 1982a).

It was very surprising to find that the immunoprecipitated thiolase cell-free translation product encoded by mRNA of normal rat liver migrates slightly slower in SDS/polyacrylamide-gel electrophoresis than does the product encoded by mRNA

of clofibrate-treated rat liver. Because all steps of this experiment were carried out in parallel, it is difficult to imagine what artifact could be responsible. This raises the possibility that clofibrate switches on a different (but closely related) thiolase gene instead of increasing transcription from the gene that is used in the control state. Another possibility is that clofibrate interferes in some unknown fashion with the processing that is presumed to occur in the formation of thiolase mRNA from nuclear transcripts. Close scrutiny of Fig. 1(c) leaves open the possibility that the mature thiolase subunit might not have exactly the same size in normal and clofibrate-induced peroxisomes; further investigation is required.

The thiolase mRNA activity ratio of clofibrate-treated/normal rats is approx. 20. This may be compared with mRNA activity ratios of approx. 60 for the bifunctional enoyl-CoA hydratase/ β -hydroxyacyl-CoA dehydrogenase and approx. 3 for catalase (Mortensen & Lazarow, 1984). Chatterjee *et al.* (1983) reported that another hypolipidaemic peroxisome-proliferating drug, Wy-14,643, increased thiolase mRNA activity 20-fold. In their paper, a slight difference in size between the thiolase translation products encoded by normal and induced mRNA may be visible. Furuta *et al.* (1982a,b) found that the industrial plasticizer di-(2-ethylhexyl) phthalate, which is also a peroxisome proliferator, induced thiolase mRNA activity 13-fold. No thiolase translation product from normal mRNA can be seen in the published fluorogram, so the possibility of a size difference cannot be evaluated.

The finding that thiolase is made as a larger precursor *in vitro* raises the possibility that the polypeptide extension might play a role in the translocation of thiolase into peroxisomes, as well as the possibility of a peroxisomal proteolytic processing machinery. Very recently, Miura *et al.* (1984) reported that the thiolase precursor was processed in isolated hepatocytes, but this proteolytic processing was not coupled to import into peroxisomes. Further work is required to elucidate the significance of the larger thiolase precursor and its role, if any, in intracellular translocation.

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Yamaguchi *et al.* (1984) observed that catalase is synthesized as a 4000 Da larger precursor in glyoxysomes and leaf peroxisomes of pumpkin cotyledons.

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