cDNA clones of rat peroxisomal 3-ketoacyl-CoA thiolase were isolated. By blotting analysis using the cDNAs as probes, the mRNA for this enzyme was estimated to be about 1.9-kilobase pairs. Elevation of mRNA levels in the liver with administration of di(2-ethylhexyl)phthalate was also evident. Sequencing analysis revealed 1,272 bases of the open reading frame which encoded 424 amino acid residues. Amino acid sequence data on six tryptic peptides and the amino terminus of the purified enzyme confirmed the cDNA sequence. The precursor of peroxisomal thiolase contains at its amino terminus a peptide extension of 26 residues. The mature enzyme is composed of 398 amino acids and the molecular weight is 41,074. The sequence has a net positive charge, lacks a long stretch of hydrophobic residues, and contains a cluster of serine residues. When the primary structure of the precursor was compared to structure of known peroxisomal proteins, there was no common homologous sequence. Peroxisomal thiolase exhibits a significant sequence homology with the mitochondrial thiolase. Possible location of the transport signal of the peroxisomal thiolase is discussed. Acyl-CoA binding sites were also located on primary structures of the two thiolases. The occurrence of interrupting sequences in several clones likely originates from intron sequences.

3-Ketoacyl-CoA thiolase (EC 2.3.1.16; referred to as thiolase herein) catalyzes the final reaction of fatty acid β-oxidation. Peroxisomal thiolase of rat liver is distinct from the mitochondrial counterpart both molecularly and catalytically (1–3). Peroxisomal thiolase has a molecular weight of 89,000 and consists of two identical subunits, whereas mitochondrial thiolase has a molecular weight of 154,000 and is composed of four identical subunits (2). Peroxisomal thiolase exhibits different preferences for various chain length substrates, as compared with the mitochondrial counterpart (e.g. much lower $V_{\text{max}}$ value to acetoacetyl-CoA (2)). Peroxisomal thiolase is markedly induced by various hypolipidemic compounds (e.g. clofibrate (4) and DEHP\(^1\) (3)) in parallel with the other two enzymes of the peroxisomal β-oxidation system, acyl-CoA oxidase (EC 1.3.3.6) and enoyl-CoA hydratase (EC 4.2.1.17); 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) bifunctional enzyme. This induction occurs at the level of transcription (5). Moreover, most of these hypolipidemic compounds also cause a marked proliferation of peroxisomes (6), thereby suggesting the presence of a coordination between the induction of peroxisomal β-oxidation enzymes and the proliferation of the organelle. Therefore, the peroxisomal β-oxidation system seems to be pertinent for studying the mechanism of peroxisome biogenesis and gene regulation in higher eukaryotes.

All the peroxisomal proteins heretofore reported are synthesized on free polysomes (7) and most of their primary translation products have the same molecular weights as the mature forms. These products seem to be transported into peroxisomes without post-translational proteolytic processing. This is in sharp contrast to mitochondrial proteins which have cleavable leader peptides as signals for translocation into mitochondria (7, 8). Among rat peroxisomal proteins, only thiolase is synthesized as a larger precursor form and is converted to its mature form by proteolytic processing (9–11). It is not clear, however, whether this proteolytic processing is functionally coupled with the transport of the enzyme to peroxisomes.

To study the structure, localization, and induction of peroxisomal thiolase at the molecular level, we cloned and sequenced the cDNA for the enzyme. Based on the deduced primary structure of the thiolase and the directly determined amino-terminal sequence of the mature enzyme, the precursor form is concluded to be composed of 424 amino acid residues, including 26 residues of amino-terminal peptide extension. We discuss the possible structures of the transport signals of peroxisomal proteins by comparing these data with those of the peroxisomal enzymes already reported from our laboratory (12–14) and others (15–21). We also describe the isolation of clones containing interrupting sequences probably derived from introns.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Isolation and Characterization of Peroxisomal Thiolase
cDNA—To obtain a cDNA clone of rat peroxisomal thiolase,

\(^1\) Portions of this paper (including "Experimental Procedures," parts of "Results" and "Discussion," Figs. 1–4 and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-4170, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
we first screened a cDNA library from the liver of DEHP-treated rats. This was carried out by a differential colony hybridization procedure using a pair of \( ^{32} \text{P} \)-labeled cDNA probes synthesized from liver mRNAs of induced and uninduced animals, yielding 38 clones (12). These clones were further characterized by hybridization-selected translation (12). We isolated three cDNA clones, pMJ201, pMJ202, and pMJ203, for peroxisomal thiolase. These contained 1.4-, 1.5-, and 1.1-kb cDNA inserts, respectively, at the PstI site of a plasmid, pBR322. Restriction maps of these clones are shown in Fig. 1 (A, for pMJ201 and pMJ202, and B, for pMJ203). The cleavage pattern of the cDNA insert of pMJ202 was the same as that of pMJ201, except for approximately 80 bases of additional sequence about 600-bp upstream of the 3' end. The cDNA insert of pMJ203 gave a cleavage pattern different from those of pMJ201 and pMJ202 in the 3' terminal region of about 200 nucleotides. As described below, RNA blot analysis showed that mRNA for this enzyme was about 1.9-kb long (Fig. 2). To obtain cDNA clones covering the complete sequence of mRNA for peroxisomal thiolase, we screened the same liver cDNA library as above by colony hybridization using as a probe the \( ^{32} \text{P} \)-labeled cDNA insert of pMJ201. Out of about 2500 colonies, we obtained 13 positive clones (designated as pMJ204 through pMJ216). Among them, pMJ204 and pMJ206, which contained longer cDNA inserts than the others, were further characterized. The cDNA inserts of pMJ204 and pMJ206 were about 1.5- and 1.3-kb long, respectively. Based on the restriction maps, they were found to overlap with pMJ201 and pMJ202, as shown in Fig. 1A. However, as mentioned later, the cDNA sequence constructed by overlapping the sequences of these clones contained a region (about 800 bases long) interrupted by stop codons in all three reading frames. Nevertheless, the cDNA sequences upstream and downstream of this region contained long open reading frames. The sequences determined for the tryptic peptides of the purified enzyme were found in the amino acid sequences specified by these open reading frames (see Fig. 5). The amino-terminal sequence of the mature form of the enzyme was found to be encoded by the cDNA sequence near the 5' terminus of pMJ204. We concluded, therefore, that the composite cDNA sequence constructed by the above clones had a single open reading frame for peroxisomal thiolase, but was interrupted by an untranslatable sequence in its central region.

On the other hand, an open reading frame having the same nucleotide sequence as above but lacking the untranslatable region was present in pMJ203. Accordingly, the cDNA sequence of this clone was the correct copy of the mRNA sequence for peroxisomal thiolase. Out of the above 16 clones, six had uninterrupted structures. These uninterrupted cDNA clones lacked sequences specifying the amino-terminal portion of the mature enzyme and the presequence. To obtain clones which covered the 5' terminal region of the mRNA for this enzyme, we did two further cDNA cloning experiments. cDNA was synthesized by specific priming of reverse transcription, using as primers restriction fragments of isolated cDNAs. In the first experiment, a HpaII/PstI fragment of pMJ206 was used as a primer (Fig. 1A, primer I), and a HhaI/HhaI fragment of pMJ204 was used as a hybridization probe for screening the recombinant clones (Fig. 1A, probe I). A clone, pMJ227, which contained an uninterrupted cDNA insert was isolated. The second experiment was carried out using a PstI/HhaI fragment of pMJ204 as a primer (Fig. 1A, primer II), and a PstI/PstI fragment of pMJ204 as a probe (Fig. 1A, probe II). A clone which represented the complete 5' coding sequence, pMJ234, was obtained. Fig. 1 shows the restriction maps of the clones isolated. The cDNAs containing the interrupting sequence are shown in A, whereas those not containing this sequence are in B. The two maps completely overlap except for the region of the interrupting sequence.

**Analysis of mRNA of Peroxisomal Thiolase**—We determined the size of the mRNA of peroxisomal thiolase by blotting analysis, using the method of Thomas (32). Here, nick-translation cDNA inserts of pMJ203 were used as probes. The mRNA was estimated to be about 1.9-kb long (Fig. 2A). A faint 2.6-kb band was detected in the RNA sample used in the first cDNA cloning which yielded pMJ201, pMJ202, etc. (Fig. 2A, lane 1). This band was not found in any other RNA sample, i.e. either total cellular RNA or polysomal RNA from control or DEHP-fed rats (Fig. 2A, lanes 2–13). The entity of the 2.6-kb RNA species is discussed in detail below. As shown in Fig. 2A, peroxisomal thiolase mRNA was much more abundant in poly(A) RNA from DEHP-fed rats than from control rats. This finding indicates the induction of mRNA for peroxisomal thiolase by DEHP and is consistent with previously reported data (33).

The composite length of the cDNA constructed by overlapping the sequences of pMJ203, pMJ227, and pMJ234 was about 1.6 kb (Fig. 1B). The difference between this value and the size of the mRNA (1.9 kb) could be due to a long 5'-untranslated region or a long poly(A) tail at the 3' terminus. Accordingly, we determined the average length of the poly(A) tail by the procedure of Domdey et al. (34). Liver polysomal poly(A) RNA was annealed with a Sau3AI/Sau3AI fragment of pMJ203 (Fig. 3, top panel) to form a DNA-RNA hybrid. The region of the RNA hybridized with the cDNA fragment was then digested with RNase H. The size of the RNase H-resistant RNA region was determined by blotting analysis, using as a probe the fragment from the EcoRI site to the 3' PstI cloning site of pMJ203 (Fig. 3, top panel). As shown in this figure, an RNase H-resistant fragment, which should correspond to the region from the 3' proximal Sau3AI site to the 3' end of the mRNA, was detected as a diffuse band with a maximum intensity at the position corresponding to the size of about 800 nucleotides. The length of the cDNA sequence from the Sau3AI site to the poly(A) addition site is 526 bases (see below). These data suggest that mRNA for peroxisomal thiolase contains a poly(A) tail with an average length of nearly 300 nucleotides. Therefore, the lengths of the cloned cDNA and the poly(A) tail total about 1.9 kb, a value close to the estimated size of mRNA for peroxisomal thiolase.

We did a primer extension experiment using as a primer a SauI/FokI fragment (188 bp) and which was labeled with \([\gamma-^32\text{P}]\)ATP at its FokI site (Fig. 4, top panel) (25). As shown in this figure, several radioactive bands were apparent. The most prominent band appeared at the position corresponding to the size of about 260 bases (Fig. 4, indicated by arrowhead). Therefore, the 5' end of mRNA for this enzyme was considered to be located about 260 bases upstream from the labeled FokI site. This position is assigned approximately to the 5' end of the cDNA insert of pMJ234 (264 bp upstream of the FokI site).

The above results suggest that the cDNA inserts of pMJ203, pMJ227, and pMJ234, when overlapping, cover the full length of the mRNA sequence for the enzyme. Several minor products with sizes varying from about 290 to 360 bases (Fig. 4, indicated by arrows) were found in the primer extension experiment, possibly representing the minor species of a longer mRNA of the enzyme. We have not obtained cDNA clones corresponding to such mRNAs.
Fig. 5. Composite nucleotide sequence of the cDNA for rat peroxisomal thiolase and the deduced primary structure of the enzyme. The sequence typed in large letters was constructed by overlapping the sequences of pMJ203, pMJ227, and pMJ234. Two interrupting regions, a 979-bp sequence of pMJ204 and a 85-bp sequence of pMJ202, are indicated by smaller letters. Nucleotides are numbered designating the first nucleotide of the initiator methionine codon (ATG) as +1. The interrupting sequences are excluded from the numbering. Amino acid residues are numbered beginning at the amino-terminal residue of the mature enzyme. Nucleotides in the 5'-untranslated region and the amino acids in the presequence are denoted by negative numbers. The translation termination codon is indicated by "TER." The putative polyadenylation signal (AGTTAA) is marked by closed circles. The amino-terminal sequence of the mature enzyme, determined by Edman degradation, is indicated by a double underline. The determined peptide sequences are underlined. The sequence is enclosed with a box. Base variations found among different clones are as follows: G was found at position 1092 in pMJ202 and A was found at position 672 in pMJ203, instead of A and G at the respective positions in the sequence shown. These substitutions caused no amino acid change. Positions 452 and 457 were occupied by T in pMJ203 instead of G. The translation start site was assigned to the first ATG triplet (positions 1–3), for the following reasons. (i) An in-frame stop codon (TAA) (positions −24 to −22) was found 22 bases upstream of the ATG codon. (ii) The molecular weight of the polypeptide calculated from the deduced amino acid sequence starting at this position agreed well with the molecular weight of the in vitro translation product of the peroxisomal thiolase precursor already reported (9, 10). (iii) The nucleotide sequence around the ATG codon conforms to the consensus sequence of the eukaryotic translation initiation signal (35). Hence, the cDNA sequence is composed of 25 bp of 5' noncoding, 1272 bp of coding, and 283 bp of 3' noncoding regions (Fig. 5). A putative poly(A) addition signal, AGTTAA (nucleotides 1537 to 1542) (36–39), is present 13 bases upstream of the poly(A) tail (Fig. 5, indicated by closed circles).

The amino-terminal sequence of the purified peroxisomal thiolase was determined by Edman degradation. The obtained sequence matched the deduced sequence from residues 1 to 13 (shown by double underline in Fig. 5). Amino acid sequencing was also carried out on tryptic peptides of the enzyme. The six peptides analyzed gave a total of 42 residues. All of these could be assigned on the predicted amino acid sequence derived from the cDNA sequence (underlines in Fig. 5). These data confirm that the cDNAs obtained are indeed copies of the mRNA of peroxisomal thiolase.

By comparing the amino-terminal sequence of the mature enzyme with that of the deduced sequence, it was concluded that the peroxisomal thiolase precursor contains 424 amino acids, and the amino-terminal 26 residues constitute a cleavable peptide extension (referred to as a presequence) (boxed in Fig. 5). The molecular masses of the precursor and the mature enzyme are calculated to be 43,775 and 41,074 Da, respectively.
FIG. 7. Comparison of the amino acid sequence of rat peroxisomal thiolase with the sequence of rat mitochondrial thiolase. Standard one letter amino acid abbreviations are used. PT and T1 represent the sequences of peroxisomal and mitochondrial thiolases, respectively. Gaps (−) were inserted to achieve maximum homology. Amino acid residues (marked with dots) are numbered beginning at the amino-terminal serine residue of the mature enzyme in the case of peroxisomal enzyme, and at the initiator methionine in the case of the mitochondrial enzyme. Boxes indicate the amino acid residues identical between two thiolases. Sequence data of mitochondrial thiolase were taken from Ref. 50. PT and T1 represent the sequences of peroxisomal and mitochondrial thiolases, respectively. Gaps (−) were inserted to achieve maximum homology. Amino acid residues (marked with dots) are numbered beginning at the amino-terminal serine residue of the mature enzyme in the case of peroxisomal enzyme, and at the initiator methionine in the case of the mitochondrial enzyme. Boxes indicate the amino acid residues identical between two thiolases. Sequence data of mitochondrial thiolase were taken from Ref. 50.

Fig. 8. Hydrophilicity profiles of peroxisomal and mitochondrial thiolases. The averaged hydrophilicity values of hexapeptides are plotted versus the positions of the amino acid residues. Each point is placed at the center of the respective hexapeptide. Hydrophilicity values of individual amino acids were taken from Ref. 50. PT and T1 represent the profiles of peroxisomal and mitochondrial thiolases, respectively. Amino acid residues are numbered as described in the legend to Fig. 7.

The presequence of peroxisomal thiolase contains 4 basic residues (2 arginines and 2 histidines) and 1 acidic residue (glutamic acid), and therefore, has a net positive charge. It lacks a long stretch of hydrophobic residues commonly found in the signal peptides of secretory proteins. There is a cluster of 4 serine residues in the central part of this region. Some of these characteristics are shared by the amino-terminal transit peptides of mitochondrial matrix proteins. The mature protein contains 41 basic residues (22 residues of arginine, 17 residues of lysine, and 2 residues of histidine) and 36 acidic residues (19 residues of glutamic acid and 17 residues of aspartic acid). This is consistent with the high pi value (9.2) of the enzyme already reported (1).

FIG. 9. Comparison of amino acid sequences around the putative substrate-binding sites. Sequences of rat peroxisomal (PT) and rat mitochondrial (T1), and pig heart (HT) thiolases are aligned. Amino acid residues are abbreviated and numbered as described in the legend to Fig. 7. Residues, the numbers of which are multiples of 10 are marked with dots. The sequence of the pig enzyme was taken from Ref. 52. The portino where only the amino acid composition has been determined is enclosed by parentheses. Cysteine residue indicated by an asterisk is apparently involved in the substrate binding in the pig enzyme (52).
indicating that these enzymes originated from a common ancestor. The sequences of carboxyl-terminal halves are particularly well conserved. The amino acid sequence from residue 215 to 372 of peroxisomal thiolase and from residue 216 to 373 of mitochondrial thiolase are 45% homologous, without a gap. By inserting several gaps into adequate positions of the two sequences in the amino-terminal halves, a moderate level of homology was obtained, albeit less significant than in the carboxyl-terminal halves. Regions corresponding to the inserted carboxyl-terminal halves of both thiolases demonstrate similar high homology was obtained, albeit less significant than in the mitochondrial enzyme. These regions seem particularly well conserved. The amino acid sequence from residues 216 to 373 of peroxisomal thiolase (there is no homology between the two enzymes in this region) and the inserted region (residues 126–136) of the mitochondrial isozyme are relatively rich in basic residues. The other portions of the two enzymes exhibit essentially similar distributions of basic residues.

Hydropilicity analysis was also performed for the two thiolases, using the method of Hopp and Woods (50) (Fig. 8). Reflecting highly homologous amino acid sequences, the carboxyl-terminal halves of both thiolases demonstrate similar profiles. This would suggest that these regions of the two enzymes have similar three-dimensional structures. The profiles in amino-terminal halves of these enzymes, however, do not resemble each other so much as do those in the carboxyl-terminal halves, even when the sequence gaps were taken into account. Based on these data, we suggest that the sites bearing functions (or functional properties) common to the two enzymes are located in the carboxyl-terminal halves, whereas sites with functions differing from each other are located in the amino-terminal halves. Thus, intracellular transport signals of peroxisomal thiolase seem to be located in the amino-terminal half, including the presquence.

Thiolase reactions follow a ping-pong mechanism for both the peroxisomal and mitochondrial enzymes (2). A single cysteine residue per subunit may be involved in formation of the acyl enzyme intermediate, through a thioester linkage in the first step of the reaction (51). The substrate-binding site of pig heart thiolase has been identified, and the amino acid sequence around the site has been established (51). Fig. 9 shows that this sequence corresponds to amino acids 77–102 of the rat peroxisomal enzyme and 72–97 of the rat mitochondrial enzyme. We propose that Cys29 of the peroxisomal enzyme and Cys40 of the mitochondrial enzyme are involved in substrate binding.

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REFERENCES
cDNA Sequence of Rat Peroxisomal 3-Ketoacyl-CoA Thiolase


EXPERIMENTAL PROCEDURES

Materials — Restriction endonucleases were purchased from Takara Shokai, Toyko, Classhagen, and Bethesda Research Laboratories. Several transcrisptase was from Life Sciences. Other enzymes were obtained from Takara Shokai. 12p-labeling descripton products were products of Amer Handbook of Molecular Cloning Protocols. The labeled translation was purchased from Amerham.


SYNTHETIC cDNA Synthesis — Preparation of cDNA from rat-liver muscle and gonadal mRNA was performed as described (12, 22). To obtain clones which covered the 5' terminal region of the mRNA, those which contained no interrupting sequences were separated from those which contained insertions (12, 23). By the use of this method, clones which contained no insertions were obtained. After selecting the correct clones, the insertions were purified and used in the present experiments.

Isolation of cDNA Synthesis — The cloned cDNA was synthesized by the methods of Franks, J., and Catchmen, W. (1982) FEBS Lett. 141, 219-224. The cDNA synthesis was performed as described (12, 23).

Isolation of cDNA Synthesis Determination — The cloned cDNA was synthesized by the methods of Franks, J., and Catchmen, W. (1982) FEBS Lett. 141, 219-224. After the cDNA purification, RNA was digested with Rsa I, and the insertion was purified and used in the present experiments.

RESULTS

Interpreting the Results — Several cDNA clones were found to contain multiple inserts of the same size and, therefore, were not isolated as intact clones. These cDNA clones were sequenced by the method of Popescu, L., and Opperdoes, F. R. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5903-5907.

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cDNA Sequence of Rat Peroxisomal 3-Ketoacyl-CoA Thiolase

Fig. 1. Restriction maps and sequencing strategy of peroxisomal thiolase cDNAs. (A) Maps of the cDNAs containing the 787-bp interrupting sequence, and (B) maps of the cDNAs not containing the interrupting sequence. In (A) and (B), the maps of the composite cDNA were constructed by overlapping the sequences of several clones. Coding regions are indicated by solid bars, and non-coding linked flanks are shown by dashed lines. The 787-bp interrupting sequence is indicated by a broken line, and in (B), the 641 bp corresponding to the interrupting sequence is marked by a filled triangle. Regions represented by the respective cDNA clones are shown by bold lines. The additional sequence found in pMJ202 is shown by a separate short line. Only the restriction sites used for end labeling and subcloning in sequencing analysis are indicated. Names of the enzymes are abbreviated as follows: A, BamHI; B, BglII; C, EcoRI; F, PstI; H, HindIII; H, HpaII; H, HaeIII; M, MluI; M, MspI; N, NotI; P, PstI; R, Rsal; S, SalI; S, SauI; S, SstI; St, StuI; S, SstI; T, Tsp450I; Y, YaeII. Total DNA was isolated from two-month-old male rats and digested with restriction enzymes. The DNA was ligated into pBR322 vector DNA, transformed into E. coli, and finally sequenced. The DNA which was not treated with Hind III was also analyzed as a control (lane 1).

Fig. 2. blot analysis of rat liver poly(A)-RNA. The cDNA insert of pMJ203 (in [B]) and a part of 787-bp interrupting sequence of pMJ204 (in [B]) were used as probes. These are shown in the top figure, where the PstI sites at both ends of the insert of pMJ203 is bracketed to show that these are not the boundaries between the cDNA and the vector DNA. Liver poly(A)RNA from three animals were analyzed for each of control and DHP-treated groups. Lane 1, the poly(A)RNA which was used for the first DNA insert of the pMJ203 (data not shown). Lane 2, total DNA of control rats. Lane 3 and 4, total DNA of DHP-fed rats. The programs of RNA were applied to each lane. The size of the DNA was determined by comparing its electrophoretic mobility with those of the size markers (FDP3, HindIII restriction fragments of phage λ DNA). Electrophoresis, transfer, and hybridization were carried out as described by Thomas (32). After autoradiography, all filters were rehybridized to a labeled fragment of rat poly(A). Southern blot analysis of poly(A)-RNA confirmed that equal amounts of RNA were applied to each lane for each of the total RNA and the poly(A)RNA samples.
**cDNA Sequence of Rat Peroxisomal 3-Ketoacyl-CoA Thiolase**

Fig. 1. Primer extension analysis of peroxisomal thiolase mRNA. The reaction was performed as described in Materials and Methods. The 3' primer (188 bp) was labeled at the 5' termini (marked with an arrow in the top figure). Following cleavage with BstNI, the Bst/BstI fragment was separated by polyacrylamide gel electrophoresis and used as a specific primer. After desalting and annealing with 20 ng of total liver RNA of SDHF-fed rat (lane B), the primer was elongated by reverse transcription. The reaction products were separated on a 6% sequencing gel. Lane B contains the sequence of the 5' primer. The arrow indicates the position of the 5' primer product. The sequence was determined by an electrophoresis autoradiograph. A same reaction was carried out with 20 ng of RNA (lane B) instead of cDNA as a control. Lane B, labeled small fragments of pC18 DNA as size markers. The major and the minor bands in lane B are marked by an arrowhead and an arrow, respectively.