A second isoform of 3-ketoacyl-CoA thiolase found in *Caenorhabditis elegans* that is similar to sterol carrier protein x but lacks the sequence of sterol carrier protein 2

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Abbreviations. 17β-HSD, 17β-hydroxysteroid dehydrogenase; PTS, peroxisomal targeting signal; PXP: peroxisomal polypeptide: RACE, rapid amplification of cDNA ends; SCP, sterol carrier protein; SL, *trans*-spliced leader sequence; *unc*, uncoordinated; YAC, yeast artificial chromosome.

Enzymes. 3-ketoacyl-CoA thiolase (EC 2. 3. 1. 16), acetoacetyl-CoA thiolase (EC 2. 3. 1. 9), catalase (EC 1. 11. 1. 6), acyl-CoA oxidase (EC 1. 3. 3. 6).

Note. The nucleotide sequence reported in this work has been submitted to the DDBJ database and is available under the accession number D86473.

SUMMARY

We cloned a full-length cDNA of the nematode *Caenorhabditis elegans* that encodes a 44-kDa protein (P-44, 412 residues) similar to sterol carrier protein x (SCPx). Mammalian SCPx is a bipartite protein: its 404-residue N-terminal and 143-residue C-terminal are similar to 3-ketoacyl-CoA thiolase and identical to the precursor of sterol carrier protein 2 (SCP2; also termed nonspecific lipid-transfer protein), respectively. The 44-kDa protein, P-44, has 56% sequence identity to the thiolase domain of SCPx but lacks the SCP2 sequence. Northern blot analysis revealed only a single mRNA species of 1.4 kb, which agrees well with the length of the cDNA (1,371 bp), making it improbable that an alternative splicing produces an SCPx-like fusion protein. The sequence similarities of P-44 to conventional thiolases are lesser than that to SCPx. The purified recombinant P-44 cleaved long-chain 3-ketoacyl-CoAs (C_{8-16}) in a thiolytic manner by the ping-pong Bi Bi reaction mechanism. The inhibition of P-44 by acetyl-CoA was competitive with CoA and noncompetitive with 3-ketooctanoyl-CoA. This pattern of inhibition is shared with SCPx but not with conventional 3-ketoacyl-CoA thiolase, which is inhibited uncompetitively with respect to 3-ketoacyl-CoA. From these results, we concluded that the nematode P-44 and mammalian SCPx constitute a second isoform of thiolase, which we propose to term type II 3-ketoacyl-CoA thiolase.

Keywords : cDNA cloning; 3-ketoacyl-CoA thiolase; sterol carrier protein x; sterol carrier protein 2; *Caenorhabditis elegans*

INTRODUCTION

Sterol carrier protein 2 (SCP2), which is identical to nonspecific lipid-transfer protein [1], has been thought to participate in the intracellular transport of cholesterol and various phospholipids (for reviews, see [2, 3]). It was first described as a cytosolic protein [1], but was also reported to be located in the peroxisomal matrix of liver cells [4, 5]. Hepatic peroxisomes contain another protein that cross-reacts with anti-SCP2 antibody [4, 6]. This 58/60-kDa protein, termed SCPx, carries at the C-terminus the complete sequence of preSCP2, which includes a 20-residue presequence in addition to the 123-residue mature SCP2 [7, 8]. Two mRNA species of 2.2 kb and 0.85 kb from rat liver were detected by Northern blot analysis and shown by cDNA cloning to have sequences for SCPx and preSCP2, respectively [7]. The two mRNA species appear to be transcribed from a single gene through alternative start sites. In the human genome, the corresponding gene was mapped to 1p32 [9], and the transcription of the shorter mRNA was suggested to start within exon XII of the gene [10]. The synthesis of the longer mRNA was inducible by a peroxisome proliferator but that of the shorter mRNA was constitutive [7]. The 404-residue N-terminal of SCPx has sequence similarity to 3-ketoacyl-CoA thiolases [7, 11]. Rat SCPx as a whole showed thiolase activity with unique kinetic property and various lipid-transfer activities [12].

A peroxisomal matrix protein of the yeast *Candida tropicalis* (PXP-18) resembles SCP2 in size and sequence [13]. PXP-18 is a basic protein of 126 residues with 37% sequence identity to rat SCP2 and shows nonspecific lipid-transfer activity equivalent to that of rat SCP2 [14]. However, PXP-18 is

synthesized without any presequence except the N-terminal methionine and its synthesis is highly inducible by oleic acid or *n*-dodecane [15]. Mammalian 80-kDa 17β-hydroxysteroid dehydrogenase (17β-HSD) and the product from the *unc-24* gene of the nematode *Caenorhabditis elegans* (UNC-24) resemble SCPx in molecular construction. Porcine 80-kDa 17β-HSD is tripartite [16]: the 323 residues at the N-terminal, which is cleavable posttranslationally, catalyzes a dehydrogenase reaction not only with steroids at the C17 position but also with 3-hydroxyacyl-CoA; the central part (residues 324-596) has the 2-enoylacyl-CoA hydratase activity; and the C-terminal (residues 597-737) is similar to preSCP2. The 80-kDa 17β-HSD and the 141 residues at its C-terminal, which has 36% sequence identity to rat preSCP2 (40% to SCP2) [17], exhibited sterol- and phospholipid-transfer activities comparable to those of SCPx and SCP2, respectively [16]. UNC-24 is a bipartite protein encoded by a single gene [18]. The N-terminal (residues 1-272) resembles stomatin (band 7.2), the erythrocyte integral membrane phosphoprotein, and the 143 residues at the C-terminal has 23-24% sequence identity with respect to PXP-18, preSCP2 and the SCP2-like part of 80-kDa 17β-HSD.

SCPx has been reported only for mammals [7, 8, 19] and an avian species [20] thus far. A survey of expressed genes of *C. elegans* [21], however, provided a short cDNA sequence that encodes a polypeptide similar to the N-terminal of SCPx. We describe here the isolation and sequencing of the full-length cDNA and the structural and catalytic properties of its protein product of 44 kDa (P-44).

MATERIALS AND METHODS

Materials. Avian myeloblastosis virus reverse transcriptase, calf thymus terminal transferase and ribonuclease-free bovine pancreatic deoxyribonuclease I were obtained from Life Technology, while *Taq* DNA polymerase, Klenow fragment of *Escherichia coli* DNA polymerase I and bacteriophage T4 DNA ligase were purchased from Takara. Restriction endonucleases were also obtained from these companies. Oligonucleotides used as the primer in PCR amplification were products of Sawady Technology. The DNA-sequencing kit, nonradioactive DNA-labeling-and-detection kit and random-primer DNA-labeling kit were purchased from Applied Biosystems, Boehringer and Takara, respectively. [α-^{32}P]dCTP was supplied by ICN Biomedicals and CoA and acetyl-CoA by Sigma. Other 3-ketoacyl-CoAs were prepared as described [22]. The *C. elegans* λ ZAPII cDNA library, made with poly(A)-rich RNA from the whole animal, was the generous gift of Dr. Alan Coulson (The Sanger Center, Cambridgeshire).

Organisms. *C. elegans* Bristol strain (N2) was used throughout the work according to the methods compiled by Sulston and Hodgkin [23]. The *E. coli* strains XL1-Blue (Stratagene) and JM109 (Takara) were utilized to multiply the phage λ ZAP II (Stratagene) and plasmids (described below), respectively.

RNA preparation and Northern blot analysis. To isolate RNA from *C. elegans,* a method for preparing yeast RNA [24] was adopted. Cells of mixed-stage animals were disrupted by vortexing at 0-4°C with glass beads (0.5 mm) in 200 mM Tris/HCl pH 7.5, containing 1% SDS, 0.5 M NaCl and 10 mM EDTA. Proteins in the mixture were extracted with phenol-chloroform-isoamylalcohol

(25:24:1), and nucleic acids were precipitated with ethanol from the aqueous phase. The resulting pellet was dissolved in 10 mM Tris/HCl pH 7.4, containing 1 mM EDTA, 10 mM $MgCl_2$ and 1 mM dithiothreitol, and DNA was digested with deoxyribonuclease I. After extraction with phenol-chloroform-isoamylalcohol, the remaining nucleic acid was precipitated with ethanol and dissolved in water treated with diethylpyrocarbonate. This preparation was used as the total RNA. Poly(A)-rich RNA was prepared as described [15]. The total RNA was examined by Northern blot analysis [25] with a probe generated by random priming of a cDNA fragment using [α-^{32}P]dCTP at 3,000 Ci/mmol.

Screening of the cDNA library. The primers S1 (sense, 5'-AGGTATA CATCGTTGGAGTC) and A1 (antisense, 5'-CGATAATTTGCTTTCCGAGG) amplified a 285-bp sequence when the cDNA library was used as the template for PCR; the sequence is a portion of cm19a5 [21] that seemed to encode an SCPx-like protein. The library was divided into 20 pools (equivalent to 10^4 plaques each) and used to amplify the 285-bp sequence by PCR. One positive pool out of three was further divided into 20 subpools (about 500 plaques each) and the amplification repeated. The plaques from one positive subpool were probed with the 285-bp PCR product labeled nonradioactively. A resulting positive λ ZAP II derivative was excised *in vivo* to produce a derivative of the plasmid Bluescript (Stratagene).

Amplification of the 5'-end of cDNA. The rapid amplification of cDNA ends (RACE) [26] was applied to obtain the 5'-end of the cDNA end with the antisense primer A2 (5'-TTGTCTACAGAAAGAGCCCG) and the poly(A)-rich RNA preparation. The resulting single-strand cDNA was extended with an oligo(dA) tail by terminal transferase. It was converted to the double strand and

amplified with the primer A3 (5'-AGGATCCCGAGGCGCAGGC) and 17-mer of oligo(dT) by PCR. To select the correct 5'-end of the target cDNA, the PCR product was further amplified with the inside primer A4 (5'-GCAGATCTGAGCAG TAGTGACGGCCTCC) and oligo(dT). The product was cloned into the plasmid T7Blue T-vector (Novagen).

Gene coding for the hexahistidine-tagged P-44. The primers S2 (5'-CTC<u>CCCGGG</u>**ATG**ACGCCAACCAAGCC) and A5 (5'-CTC<u>CCCGGG</u>TGC**TCA**A ATCTTGGACTG) were used to amplify the entire region of the ORF of P-44. The PCR product was cloned into the plasmid pUC119 (Takara) using the *Sma* I sites in the primers. The resulting plasmid, as the template, and the primers S3 (5'-GACTCTAGAGGATCCCCGG<u>CC**ATG**G</u>TG*CACCACCACCACCACCAC*ACG CCAACCAAGCCAAAGGTATAC) and A6 (5'-CTC<u>CTGCAG</u>TGC**TCA**AATCTTGG ACTG) were used to introduce the valyl-hexahistidine tag and the *Nco* I site at the 5'-end of the ORF and the *Pst* I site at the 3'-end. In the above primer sequences, nucleotides corresponding to the start and stop codons are boldfaced, those to hexahistidine are italicized and those to the restriction sites are underlined. The amplified sequence was cloned between the sites of *Nco* I and *Pst* I of the plasmid pKK233-2 (Clontech); the resulting plasmid is pEMB728.

Isolation of the hexahistidine-tagged P-44. The cells of *E. coli* JM109 transformed with pEMB728 were grown aerobically at 37°C in 1-l LB medium containing 1% tryptone (Difco), 0.5% yeast extract (Difco), 1% NaCl and 100 μg/ml ampicillin. At a mid-exponential growth phase (the optical density at 600 nm was 0.7-0.9), isopropyl-1-thio-β-D-galactoside was added at a final concentration of 1.5 mM, and the incubation was continued for a further 5 h to induce the hexahistidine-tagged P-44. The cells were harvested by

centrifugation, suspended in 50-ml buffer-S (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 2 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride), and sonicated at 160 watts at 0-4°C seven times (1 min each at 3-min intervals). The resulting extract was centrifuged at 12,000 x g at 0-4°C for 20 min, and the supernatant was loaded onto a column of Ni-nitrotriacetate resin (Qiagen, open bed of 5.2 x 1.0 cm) equilibrated with buffer-S, washed with 50 mM sodium phosphate pH 6.0, containing 300 mM NaCl, 20% (mass/vol) glycerol and 2 mM 2-mercaptoethanol, and then eluted with a linear concentration gradient of 0-0.5 M imidazole. The fractions containing the essentially pure tagged P-44 were pooled and ultrafiltrated by Centriprep-10 (Amicon) to concentrate the protein and the buffer changed to 0.1 M potassium phosphate pH 7.5, 50% glycerol, 2 mM 2-mercaptoethanol and 1 mM EDTA. The final preparation was stored at –80°C.

Miscellaneous methods. Procedures for the amplification and sequencing of DNA [25], SDS/PAGE, the staining of proteins and the assay of the protein with bovine serum albumin as the standard [27] were as described. The activity of thiolase was determined by measuring initial velocities of the decrease of 3-ketoacyl-CoA at 303 nm at 25°C as described [22]. The standard reaction mixture contained the following in a final volume of 1.0 ml: 100 μmol Tris/HCl pH 8.3, 25 μmol $MgCl_2$, 50 μmol KCl, 50 nmol CoA and 15 nmol 3-ketoacyl-CoA. One unit of thiolase activity is defined as the amount that utilizes 1 μmol of 3-ketoacyl-CoA/min. The molar extinction coefficients [28] used are: acetoacetyl-CoA, 21,400 M^{-1} cm^{-1}; 3-ketohexanoyl-CoA, 16,600 M^{-1} cm^{-1}; 3-ketooctanoyl-CoA, 14,400 M^{-1} cm^{-1}; 3-ketodecanoyl-CoA, 13,900 M^{-1} cm^{-1}; 3-ketododecanoyl-CoA, 11,000 M^{-1} cm^{-1}; 3-ketotetradecanoyl-CoA, 11,600 M^{-1} cm^{-1}; 3-ketohexadecanoyl-CoA, 9,900 M^{-1} cm^{-1}.

RESULTS AND DISCUSSION

Cloning and sequencing of the full-length cDNA encoding a *C. elegans* homologue of SCPx. The cDNA clone cm19a5, obtained by a survey of expressed genes in *C. elegans* and sequenced preliminarily [21], showed a stretch of 120 amino acid residues that was highly similar to the N-terminal sequence of rat SCPx. Using primers designed by referring to the published, tentative nucleotide sequence, a 285-bp sequence from a *C. elegans* cDNA library was amplified by PCR. The amino acid sequence deduced from the PCR product had 56% identity to that of the corresponding sequence of rat SCPx. Three pools (1 x 10^4 plaques each) out of 20, of the divided library, provided the same 285-bp PCR product, when used as the template. One of them was subdivided in order to select a positive pool of about 500 plaques, from which a single positive plaque was isolated by hybridization with the 285-bp PCR product as the probe. The plasmid pEMB673, which was derived from the positive plaque by *in vivo* excision, was identified to carry the sequence consistent with that of cm19a5 (Fig. 1A). Since the cDNA lacked the translation start codon, the 5'-end of the mRNA was amplified by the 5'-RACE procedure. From the PCR product (439-bp) the specific 5'-proximal sequence of 147 bp, which contained the *trans*-spliced leader sequence 1 (SL1), was reamplified and cloned as the plasmid pEMB757 (Fig. 1A). The presence of the complete spliced leader sequence [29] at the 5'-end and the potential polyadenylation signal (AATAAA) and poly(A) sequence at the 3'-end verifies the cloning of a full-length cDNA.

The 1,371-bp cDNA revealed an ORF of 1236 bp terminated with a TGA stop codon (Fig. 1B). The 44-kDa protein (P-44) deduced from the ORF

exhibited significant similarity to mammalian SCPx (see below). Unexpectedly, the ORF terminated in the middle of the presequence of SCP2 and thus lacked the entire sequence of SCP2. It is possible that an alternative splicing generates two distinct protein products: one which contains the SCP2 sequence at its C-terminus and one which does not. This possibility was examined by Northern blot analysis with RNA prepared from a mixed-phase culture of the animal (Fig. 2). Only a 1.4-kb single RNA species was detected, when probed with a 1.2 kbp fragment encompassing almost the entire region of the ORF. The size agrees well with the length of the cDNA cloned. This result does not support the hypothesis of alternative splicing stated above, though it is possible that the splicing occurs under a particular metabolic condition. This point will be clarified when the genomic sequence of the gene becomes available. The gene, which is on the clone cm19a5, has been mapped to the right border of the gene cluster of linkage group II, the region covered by the YAC clones of both Y57A10 and Y70F3 [30].

Primary structure and catalytic property of P-44. The deduced 412 amino acid residues of P-44 showed significant similarities to various thiolases; the highest identity was to SCPx. P-44 shared 231 residues with the thiolase part of SCPx at identical positions (56% identity); although it lacked the SCP2 sequence. An unrooted neighbor-joining tree [31] for 15 related proteins showed that P-44 was positioned closest to the thiolases of SCPx (Fig. 3A), where the mature SCP2 sequence was deleted from SCPx. The four proteins constituted a group that was far apart from groups of conventional thiolases. A multiple alignment of all 15 sequences, generated by the CLUSTAL W program [32], revealed the marked

conservation of the cysteine residue believed to be involved in the substrate binding of conventional thiolase [33]. In the sequence of P-44, this is Cys87; see Fig. 3B, in which four sequences of rat enzyme are presented as the representatives of each group: SCPx (the SCP2 part was deleted), mitochondrial thiolase, peroxisomal thiolase and acetoacetyl-CoA thiolase.

In the examination of catalytic activity, the recombinant P-44 tagged with a hexahistidine at the N-terminal expressed in *E. coli* cells was purified to near homogeneity by affinity chromatography with nickel ions. P-44 displayed efficient thiolytic cleavage of 3-ketoacyl-CoA ranging from eight to 16 in carbon number of the acyl group (Fig. 4); the highest activity was observed with 3-ketotetradecanoyl-CoA (specific activity of 41 units/mg). Acetoacetyl-CoA and 3-ketohexanoyl-CoA were ineffective as the substrate. Both of the double reciprocal plots of initial velocities versus concentrations of one of the two substrates (CoA and 3-ketooctanoyl-CoA) were essentially parallel when the concentration of the other substrate was varied (Fig. 5). These results reveal that P-44 catalyzes thiolysis by the ping-pong Bi Bi reaction mechanism, as SCPx [12] and conventional 3-ketoacyl-CoA thiolase [34] do. This mechanism implies that the binding of 3-ketooctanoyl-CoA to the enzyme is followed by the release of acetyl-CoA and that the subsequent entrance of CoA causes the production of 3-ketohexanoyl-CoA. According to the reaction mechanism, the maximal velocity was calculated to be 0.1 mmol/min per mg with K_m values of 8×10^{-5} M for 3-ketooctanoyl-CoA and 7×10^{-5} M for CoA.

Thiolysis catalyzed by P-44 was inhibited by acetyl-CoA (Fig. 6), as expected from its reaction mechanism. The inhibition was competitive with CoA (Fig. 6A) and noncompetitive with 3-ketoacyl-CoA (Fig. 6B). The noncompetitive

inhibition is also observed with the thiolase of SCPx [12] but not with conventional peroxisomal 3-ketoacyl-CoA thiolase, which is inhibited uncompetitively [34]. Although the properties of P-44 may be modulated by the hexahistidine tag at the N-terminus, P-44 resembles the thiolase of SCPx not only in primary structure but also in kinetic property. The reported observation that SCPx more readily employs 3-ketooctanoyl-CoA as a substrate over 3-ketotetradecanoyl-CoA [12] may be due to the higher substrate concentration in the assay mixture (35 μM) than that used in the present study (15 μM). Long-chain 3-ketoacyl-CoA tend to precipitate at higher concentrations. Thus, we conclude that P-44 and the thiolase of SCPx are distinct from conventional 3-ketoacyl-CoA thiolases and constitute a second isoform of 3-ketoacyl-CoA thiolase, which we propose to term type II 3-ketoacyl-CoA thiolase.

Biological significance of P-44. Discussions as to the biological significance of the type II 3-ketoacyl-CoA thiolase of *C. elegans* must be based on knowledge obtained mainly from other eucaryotes, because little is known about peroxisome biogenesis and fatty acid metabolism in this organism. The C-terminal tripeptide of P-44, Ser-Lys-Ile (Fig. 3A), which resembles the peroxisomal targeting signal 1 (PTS1; typically Ser-Lys-Lue) [35, 36], suggests that this enzyme is imported into peroxisomes. Peroxisomes have not been reported, however, for any tissues of *C. elegans* thus far. Thin sections of the intestine revealed some peroxisome-like vesicles surrounded by a single unit-membrane when examined by electron microscopy (data not shown), but the vesicles were not stained clearly enough by 3,3'-diaminobenzidine, which is commonly used to detect the activity of catalase, the marker enzyme of peroxisomes. Nevertheless, it is likely that P-44 is

peroxisomal, since its mammalian homologue, SCPx, has PTS1 (Ala-Lys-Leu) [7, 8, 19] and has been located in the matrix of peroxisomes [5, 6]. Although the conventional (i. e., type I) peroxisomal thiolase lacks PTS1 at its C-terminus (see X in Fig. 3B), it has PTS2 at the N-terminus instead [37].

The relationship between SCPx and P-44 can be explained alternatively. One explanation is that SCPx is the original form of type II 3-ketoacyl-CoA thiolase and P-44 is the derivative that has lost the C-terminal domain. A new promoter sequence for SCP2 could be created in the preceding intron [10]. The other is that P-44 represents the origin of type II 3-ketoacyl-CoA thiolase and SCPx resulted from a gene fusion that connected this protein to SCP2. The properties of several proteins suggest that the latter explanation is more likely. First, the gene of the yeast protein PXP-18, which is a structural and functional homologue of SCP2, encodes no extra sequence [14]. This indicates that the lipid-transfer activity of PXP-18 is intrinsic and independent of thiolytic activity. Second, at least two other proteins, mammalian 80-kDa 17β-HSD [17] and nematode UNC-24 [18], carry the SCP2-like sequence at the C-terminus. The recombinant SCP2-like protein of the 17β-HSD showed a significant lipid-transfer activity [16]; although the corresponding activity of UNC-24 has not been tested. Though the preceding parts of SCPx and 80-kDa 17β-HSD are involved in lipid metabolism, that of UNC-24 is a stomatin-like integral membrane protein and unrelated to the metabolism, implying that the SCP2-like part functions structurally. Third, two eucaryotic ribosomal proteins, S27a and L40, are fused at their N-terminus to ubiquitin [38, 39], which is a heat shock protein and also encoded by its own genes, which are independent of ribosomal proteins and tandem aligned at another locus. The ubiquitin part of the fusion proteins

appears to function as an intramolecular chaperone during ribosome biogenesis [40]. Another ribosomal protein S30 is synthesized fused to a 74-residue ubiquitin-like protein in mammal but is unfused in yeast [41]. Finally, there is a parallelism between the phylogenetic complexity of an organism and the molecular complexity of the acyl carrier protein, which is, in enterobacteria, free from the other seven enzymes required for fatty acid synthesis. In yeast this protein together with two other enzymes comprises the α subunit of fatty acid synthase with the other five in the β subunit, while in mammals it is a domain of the single polypeptide that includes all eight components [42]. Thus, the assemblage of SCPx in mammals seems to be a result of an evolutionary trend.

In conclusion, we propose that P-44 is the prototype of type II 3-ketoacyl-CoA thiolase and that a gene-fusion event created vertebrate SCPx. It is plausible that the fusion to SCP2 makes the thiolase ready to be built into a putative enzyme complex for the β-oxidation. In this context, it is interesting that the yeast homologue of SCP2 interacts with peroxisomal acyl-CoA oxidase and protects it from thermal denaturation *in vitro* [43].

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FIGURE LEGENDS

Fig. 1. Structure and sequence of the full-length cDNA coding for P-44. (A) The clones pEMB757 and pEMB673 were obtained by 5'-RACE and plaque hybridization, respectively. The open bar represents the ORF, in which the 285-bp PCR product is shown by a thick bar, and the open rectangles at its 5'- and 3'- ends denote SL1 and poly(A) sequence, respectively. Positions of sequences that correspond to some PCR primers are marked by short horizontal arrows with their names. The nucleotides on the mRNA-like strand are numbered in the 5' to 3' direction; position +1 is A of the start codon of the ORF. (B) Nucleotide sequence of the cDNA. The SL1 sequence (at –5) and the potential polyadenylation signal (at +1314) are underlined. The start and stop codons are marked by thick bars. Sequences used to design some PCR primers are indicated by arrows with their names.

Fig. 2. Northern blot analysis. The total RNA (about 8 μg) prepared from a mixed-phase culture of the animal was electrophorized in the presence of 2.2 M formaldehyde, transferred onto a nylon membrane and probed with the ^{32}P-labeled cDNA fragment ranging from +20 to +1242 (lane 1). RNA Ladder (Life Technology) was electrophorized on the same gel as the size marker and stained with ethidium bromide (lane 2). The sizes of markers are shown on the right and that of the transcript detected is on the left.

Fig. 3. Amino acid sequence of P-44 and the comparison with other thiolases. (A) Unrooted neighbor-joining tree for P-44 (I) and 14 related

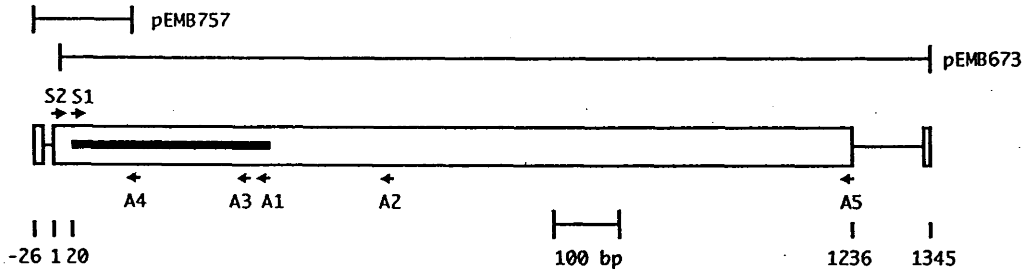
proteins: three forms of SCPx (II-IV; the SCP2 sequence was deleted) from *Gallus gallus* (L09231), *Rattus norvegicus* (P11915) and *Homo sapiens* (B40407); two mitochondrial thiolases (V, VI) from rat (A29452) and human (S43440); one bacterial thiolase (VII) from *E. coli* (P21151); four peroxisomal thiolases (VIII-XI) from *S. cerevisiae* (P27796), *C. tropicalis* (P33291), rat (P21775) and human (P09110); and four acetoacetyl-CoA thiolases (XII-XV) from *S. cerevisiae* (S28144), *C. tropicalis* (S01099), rat (P17764) and human (A37233). The EMBL/GenBank/DDBJ accession number of each sequence was indicated in parentheses. (B) Amino acid sequences of P-44 (I) and those of four rat proteins: SCPx (III), mitochondrial thiolase (V), peroxisomal thiolase (X), and acetoacetyl-CoA thiolase (XIV). The sequences were aligned by the CLUSTAL W program [32] together with the other ten sequences shown in (A) and thereafter five (I, III, V, X and XIV) were taken from the final alignment. Amino acid residues (expressed by the single-letter code) not identical to those of P-44 are in lower case. The cysteine residue believed to be involved in the substrate binding is marked with an arrowhead.

Fig. 4. Substrate specificity of P-44 with respect to the chain-length of 3-ketoacyl group. The thiolase activity was assayed with 0.15-0.76 μg of the purified recombinant P-44 in the standard reaction mixture containing 15 μM of various 3-ketoacyl-CoAs: C4, acetoacetyl-CoA; C6, 3-ketohexanoyl-CoA; C8, 3-ketooctanoyl-CoA; C10, 3-ketodecanoyl-CoA; C12, 3-ketododecanoyl-CoA; C14, 3-ketotetradecanoyl-CoA; C16, 3-ketohexadecanoyl-CoA. No activity was detected with any substrate in the absence of the P-44 preparation or with the use of a similar preparation from *E. coli* transformed with pKK233-2.

Fig. 5. Lineweaver-Burk plots for CoA (A) and 3-ketooctanoyl-CoA (B). The thiolase activity was assayed with 0.38 μg of the purified recombinant P-44 in the standard reaction mixture. The concentration of CoA was varied and that of 3-ketooctanoyl-CoA was fixed as indicated in (A), while that of 3-ketoacyl-CoA was varied and that of CoA was fixed as indicated in (B). The initial velocity (v) is expressed by specific activity (units/mg).

Fig. 6. Inhibitory effects of acetyl-CoA with respect to CoA (A) and 3-ketooctanoyl-CoA (B). The thiolase activity was assayed with 0.66 μg of the purified recombinant P-44 in a reaction mixture containing acetyl-CoA at the concentration indicated. The concentration of CoA was varied and that of 3-ketooctanoyl-CoA was fixed at 10 μM in (A), while that of 3-ketoacyl-CoA was varied and that of CoA was fixed at 20 μM in (B). The initial velocity (v) is expressed by specific activity (units/mg).

A



B

```
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S2                 S1
GATATGGTAAAGGAGGCCGTCACTACTGCTCTTGATGATTGCAAGATGAAGTATTCTGATATTCAGCAGGCTACAGTTGGATACCTTTTT  180
                     A4
GGAGGAACCTGCTGTGGTCAACGTGCTCTATATGAGGTTGGACTTACCGGAATCCCAATTTTCAACGTGAACAATGCCTGCGCCTCGGGA  270
TCCTCTGGACTATTCCTCGGAAAGCAAATTATCGAAAGTGGAAACTCCGATGTGGTTCTCTGCGCCGGATTTGAGCGCATGGCTCCGGGA  360
A3                A1
TCGCTAGAAAATTTGGCTGCTCCAATTGATGATCGGGCTCTTTCTGTAGACAAACACATTTCTGTCATGTCAGAGACTTATGGGCTCGAG  450
                                           A2
CCGGCTCCGATGACGGCTCAGATGTTTGGAAATGCAGCGAAAGAGCATATGGAGAAGTATGGTTCAAAACGCGAGCATTACGCCAAAATC  540
GCCTACAAGAACCATCTTCACTCAGTCCACAATCCAAAATCCCAGTTCACCAAGGAATTCTCTCTGGATCAGGTGATCAATGCCCGTAAG  630
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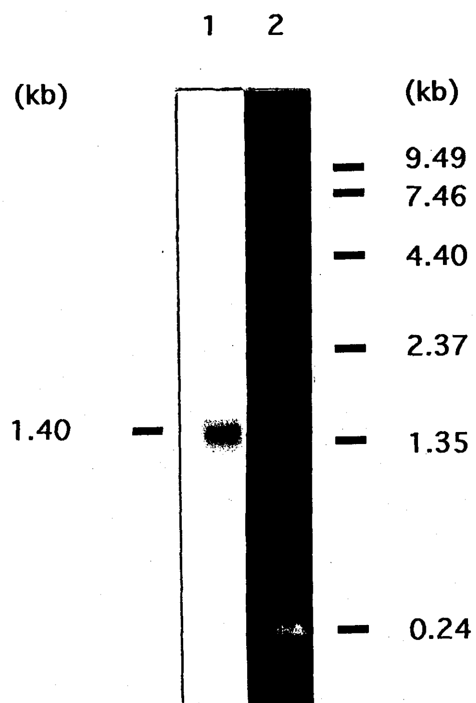

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(kb)
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4.40
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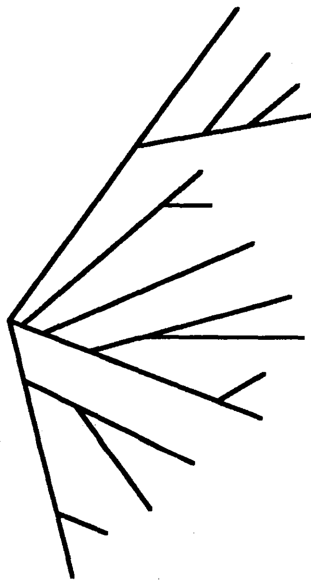
1.35
0.24

A



I P-44
II SCPx *G.gallus*
III SCPx *R.norvegicus*
IV SCPx *H.sapiens*

V 3-ketoacyl-CoA thiolase *R.n.* (mitochondrial)
VI 3-ketoacyl-CoA thiolase *H.s.* (mitochondrial)
VII 3-ketoacyl-CoA thiolase *E.coli*

VIII 3 ketoacyl-CoA thiolase *S.cerevisiae*
IX 3-ketoacyl-CoA thiolase *C.tropicalis*
X 3-ketoacyl-CoA thiolase *R.n.* (peroxisomal)
XI 3-ketoacyl-CoA thiolase *H.s.* (peroxisomal)

XII acetoacetyl-CoA thiolase *S.c.*
XIII acetoacetyl-CoA thiolase *C.t.*
XIV acetoacetyl-CoA thiolase *R.n.*
XV acetoacetyl-CoA thiolase *H.s.*

B

```
I      1   -----------------------------------------MTPTKPKVYIVGVGMTKFC--KPGSVPGWDYPDMVKEAVTTALDDCKMKYS----DIQQ   53
III    1   -----------------------------mpsva------lnsPrlPrVfvVGVGMTKFm--KPGgensrDYPDlaKEAgqkALaDrqipYS----aveQ   59
V      1   ------------------------------------------mallrgVfIVaakrTpFg-aygGllkdftatDltefAaraALsagKvppe----tIds   53
X      1   msesvgrtsamhrlqvvlghlagrpesssalqaapcs-atfpqasasdVvvVhgrrTpigragrGgfkdttpdellsavlTavLqDv----klkpeclgd   95
XIV    1   ----------maalavlhgvvrrpllrgllqevrclgrsyaskPTlndVvIVsatrTpig-sflGSlasqpatklgtiAiqgAiekagipke----evke   85

                                                 ▼
I     54   ATVGYLFGGTCCGQRALYE-VGLTGIPIFN----VNNACASGSSGLFLGKQIIESGNSDVVLCAGFERMAPGSLENLAAPIDDRALS-VDKHISVMSETY  147
III   60   AcVGYvyGestCGQRAiYhslGLTGIPIiN----VNNnCstGStaLFmaqQlvqgGlancVLalGFEkMekG---sLgtkysDRsnp-leKHIdVlinkY  151
V     54   viVGnvmqsssdaaylarhvglrvGvPtetgaltlNrlCgSGfqsivsGcQeIcSkdaeVVLCgGtEsMsqs---pysvrnvrfgtkfgldlkledtlwa  150
X     96   isVGnvlepg-aGavmariaqfLsGIPetvplsaVNrqCsSGlqavaniaggIrnGsyDigmacGvEsMsls---Nrgn---------pgnISs-rlle  180
XIV   86   vymGnviqGg-eGQaptrqatlgaGlPIatpcttVNkvCASGmkaimmasQslmcGhqDVmvagGmEsMsnv---pyvms--rgAtpyggvkledlivkd  179

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III  152   GmsacPfapQlFGsAgKEHMEtYGtKvEHfAKIgwKNHkHSVnN-PySQFqdEySLDeimksR---pvfDFltvLqCcPTSDGAAAAivsSEeFvqKhg-  246
V    151   GltdqhvklpM-GmtAenlaaKYnisREdcdryAlqsqqrwkaaneagyFneEmapieVktkk---gkqtmqvdehar--pqttleqlqnlppvfkKe--  242
X    181   sdkardclipM-GitsenvaErfGisRqkqdafAlasqqkaasaqsKgcFraEivpvtttvlddkgdrktitvsqdegvrpsttmeglkalkpafkdg--  277
XIV  180   GLtdvynkihM-GNcAentakKlsisREeqdKyAigsytrSkeawdagkFanEitpitisvk----gkpDvvvkedeey-krvdfskVpklktvfqKen-  272

I    244   LKAQAVEIVGLKLGTDEPSVFAENSNIKMIGFDMIQKLAKQLWAETKLTPNDVQVIELHDCFAPNELITYE--AIGLCPVGQG-HHIVDRNDNTYGG-KW  339
III  247   LqskAVEIVaqemvTDmPStFeEkSvIKMvGyDMskeaArkcyeksgLgPsDVdVIELHDCFstNELlTYE--AlGLCPeGQG-galVDRgDNTYGG-KW  342
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X    248   ---gsttagnssqvsDgaaavllarrsKaeelglpilgvlrsyAvvgvpPdimgigpayaipAalqkagltvndIdifeineafasqalycveklGipae  374
XIV  273   ---gtVtaanastlnDgaaavvlmtaeaaqrlkvkplariaafAdaavdPiDfplapayavpkvlkyaglkkedIamweVneafsvvVlaNikmleidpq  369

I    340   VINPSGGLISKGHPIGATGVAQAVELSNQLRGKCGKRQVPNCKVAMQHNIGIGGAGVVGLYRLGFPGAAQSKI---------                   412
III  343   VINPSGGLISKGHPlGATGlAQcaELcwQLRGeaGKRQVPgaKVAlQHNlGLGGAaVVtLYRmGFPeAAsSfrthqisaapt                   424
V    340   ktNvSGGaIalGHPlGgsGsritahLvheLRrr-G-----gkyavgsacIGgGqgisliiqnta------------------                   397
X    375   kvNPlGGaIalGHPlGcTGarQvVtLlNeLkrrgr-----raygvvsmcIGtGmgaaavfeypGn-----------------                   434
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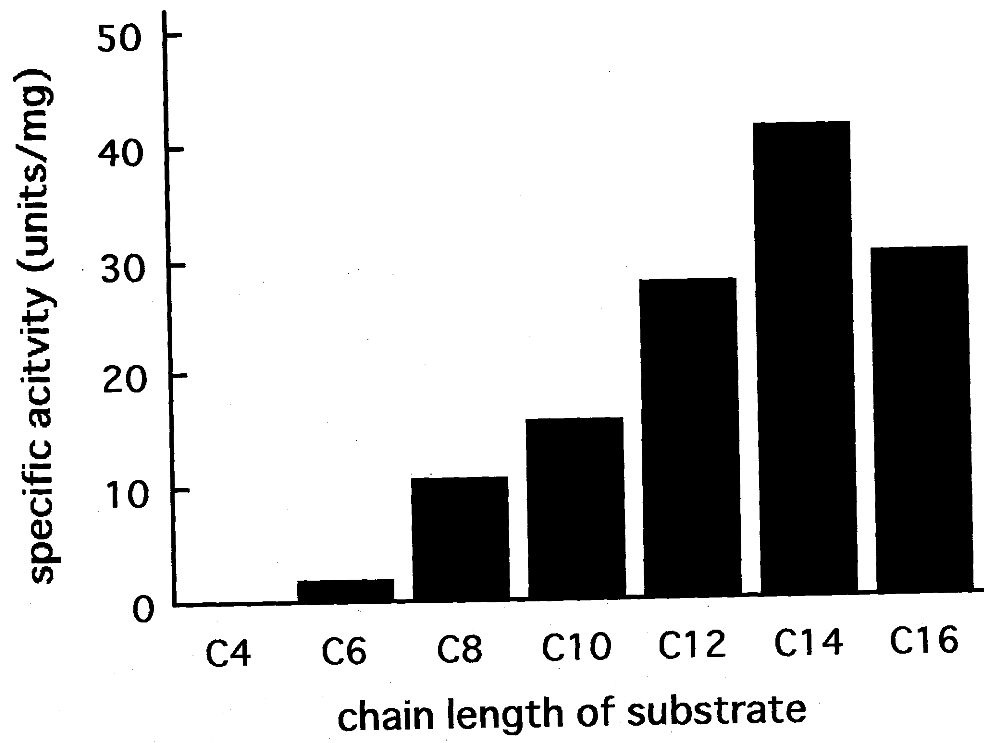

specific acitvity (units/mg)
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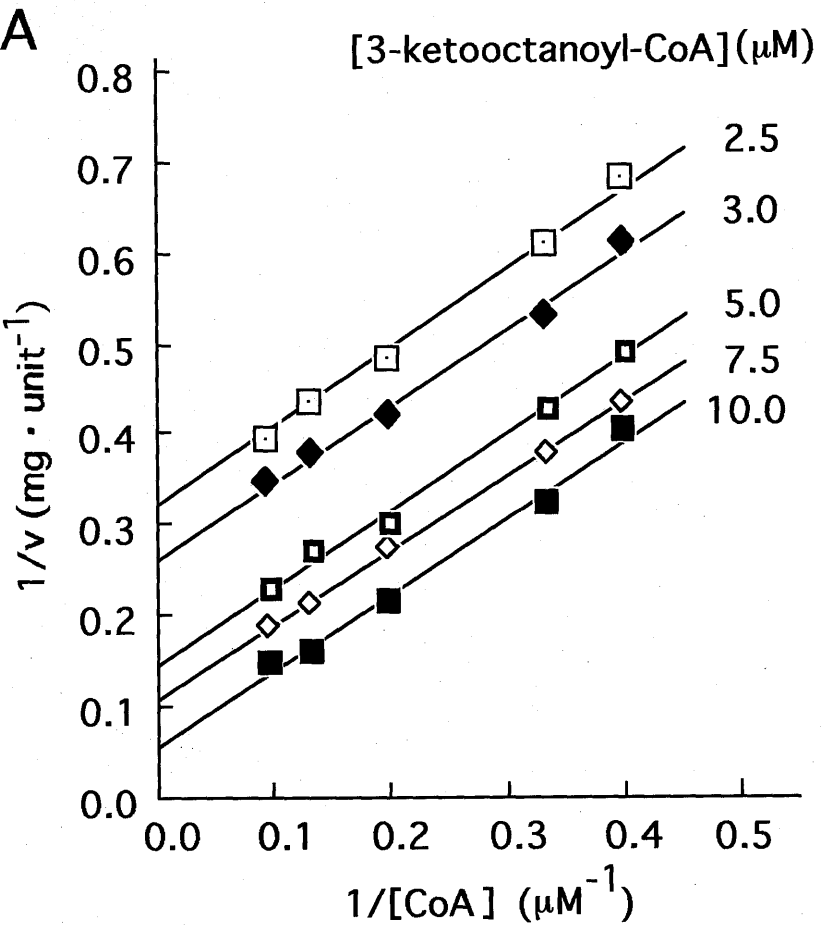
30
20
10
0
C4
C6
C8
C10
C12
C14
C16
chain length of substrate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5.0
7.5
10.0
1/v (mg · unit-1)
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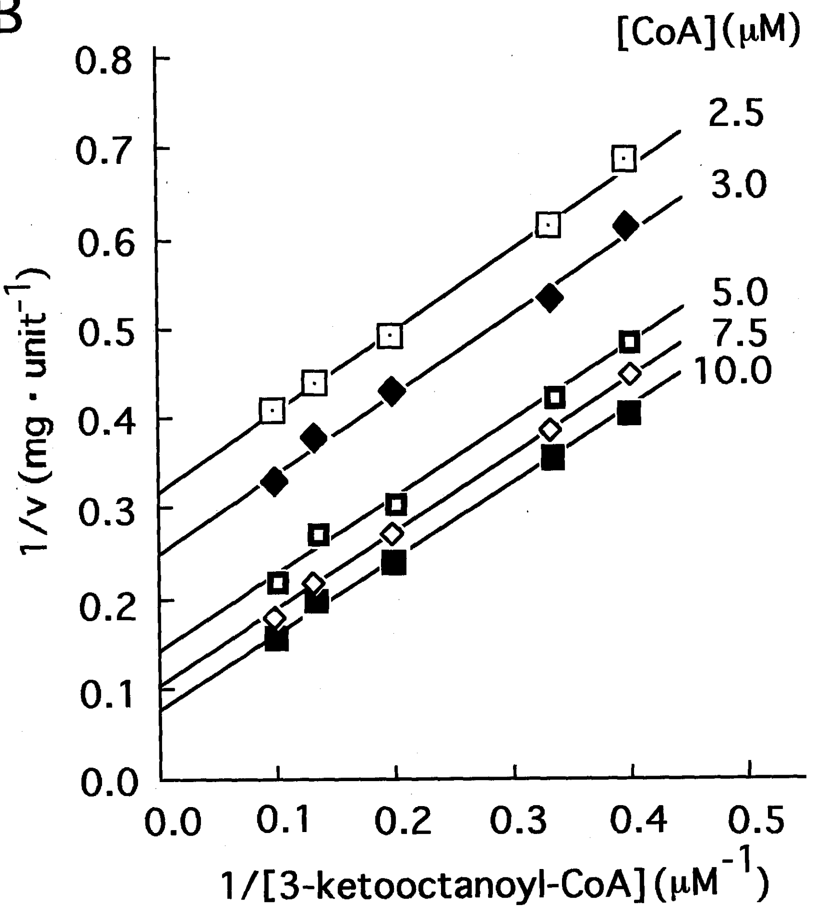
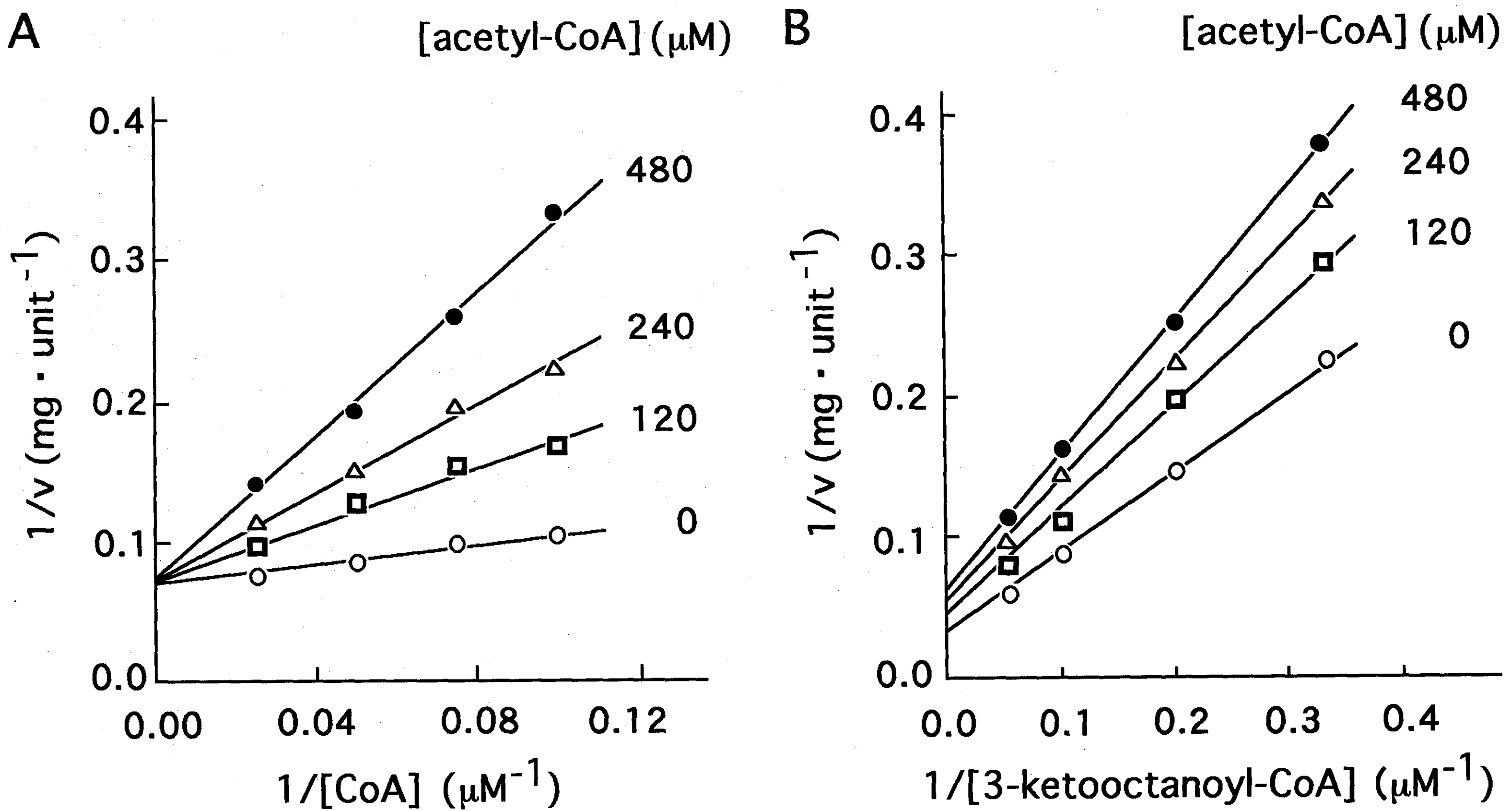
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.0
0.1
0.2
0.3
0.4
0.5
1/[CoA] (μM-1)


B
[CoA](μM)
2.5
3.0
5.0
7.5
10.0
1/v (mg · unit-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.0
0.1
0.2
0.3
0.4
0.5
1/[3-ketooctanoyl-CoA](μM-1)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120
0
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0.1
0.2
0.3
0.4
0.00
0.04
0.08
0.12
1/[CoA] (μM⁻¹)
B
[acetyl-CoA] (μM)
480
240
120
0
1/v (mg · unit⁻¹)
0.0
0.1
0.2
0.3
0.4
1/[3-ketooctanoyl-CoA] (μM⁻¹)