Molecular Characterization of a Glyoxysomal Long Chain Acyl-CoA Oxidase That Is Synthesized as a Precursor of Higher Molecular Mass in Pumpkin*

A cDNA clone for pumpkin acyl-CoA oxidase (EC 1.3.3.6; ACOX) was isolated from a lgt11 cDNA library constructed from poly(A)+ RNA extracted from etiolated cotyledons. The inserted cDNA clone contains 2313 nucleotides and encodes a polypeptide of 690 amino acids. Analysis of the amino-terminal sequence of the protein indicates that the pumpkin acyl-CoA oxidase protein is synthesized as a larger precursor containing a cleavable amino-terminal presequence of 45 amino acids. This presequence shows high similarity to the typical peroxisomal targeting signal (PTS2). Western blot analysis following cell fractionation in a sucrose gradient revealed that ACOX is localized in glyoxysomes. A partial purification of ACOX from etiolated pumpkin cotyledons indicated that the ACOX cDNA codes for a long chain acyl-CoA oxidase. The amount of ACOX increased and reached to the maximum activity by day 5 of germination but decreased about 4-fold on the following days during the subsequent microbody transition from glyoxysomes to leaf peroxisomes. By contrast, the amount of mRNA was already high at day 1 of germination, increased by about 30% at day 3, and faded completely by day 7. These data indicated that the expression pattern of ACOX was very similar to that of the glyoxysomal enzyme 3-ketoacyl-CoA thiolase, another marker enzyme of the β-oxidation spiral, during germination and suggested that the expression of each enzyme of β-oxidation is coordinately regulated.

There are at least three types of microbodies in higher plants (glyoxysomes, leaf peroxisomes, and unspecialized microbodies) that are distinguishable by their enzyme complements (1, 2). During the postgerminative growth of pumpkin seedlings and upon exposure to light, etiolated cotyledons turn green; at the same time, a functional transition from glyoxysomes to leaf peroxisomes occurs (3, 4). In fat-storing plants such as pumpkin, lipid bodies are present in seed cells that store triacylglycerols, which are subsequently converted to fatty acids by lipase. Fatty acids represent the main energy and carbon sources for germinating seedlings. In glyoxysomes, fatty acids are degraded to acetyl-CoA via the β-oxidation pathway, and acetyl-CoA is metabolized by the glyoxylate cycle bypassing the decarboxylating steps of the Krebs cycle. We have shown previously that the expression of glyoxysomal enzymes and leaf peroxisomal enzymes are regulated not only at the transcriptional level but also at the posttranscriptional level during the microbody transition (5, 6). The gene expressions of the enzymes of the β-oxidation and glyoxylate cycles seem to be coordinately regulated. In a recent paper, we reported the nucleotide and deduced amino acid sequences of the cDNA for 3-ketoacyl-CoA thiolase (7). The time course for thiolase mRNA and thiolase levels during germination and postgerminative growth implied that the regulation of expression of this enzyme is similar to that of glyoxylate cycle enzymes, e.g. malate synthase (8) and citrate synthase (9). The glyoxysomal β-oxidation spiral consists of three different proteins: acyl-CoA oxidase (ACOX), enoyl-CoA hydratase/3-hydroxy acyl-CoA dehydrogenase (bifunctional protein), and 3-ketoacyl-CoA thiolase (thiolase). ACOX converts acyl-CoA into trans-2-enoyl-CoA in the first step of the β-oxidation spiral and corresponds to the acyl-CoA dehydrogenase present in mitochondria of mammalian cells. Both enzymes are flavoproteins. Some plant ACOXs have been purified and characterized (10, 11) and have been shown to have different substrate specificities (for long, medium, and short chain acyl-CoAs, respectively). To further investigate the β-oxidation enzymes at the molecular level, we cloned a cDNA coding for a long chain ACOX, which is localized in glyoxysomes. Here, we report the nucleotide and deduced amino acid sequences of the cDNA. Developmental changes in the level of mRNA and protein were also determined in pumpkin cotyledons during seed germination and subsequent postgerminative growth.

EXPERIMENTAL PROCEDURES

Plant Materials—Pumpkin (Cucurbita sp. Kurokawa Amakuri) seeds were purchased from Aisan Seed Co. (Aichi, Japan). Seeds were soaked in running tap water overnight and germinated in Rock-Fiber soil (66R; Nitto Boseki, Chiba, Japan) at 25 °C in darkness. Some seedlings were transferred to light after 5 days.

Construction of the lgt11 cDNA Library—Total RNA was extracted from etiolated cotyledons of 4-day-old dark-grown seedlings by the SDS-phenol method. Poly(A)+ RNA was prepared by column chromatography on oligo(dT) cellulose (Becton Dickinson). cDNA transcribed

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF002016.

To whom correspondence should be addressed. Tel.: 81-564-55-7500; Fax: 81-564-55-7505; E-mail: mikosome@nibb.ac.jp.

The abbreviations used are: ACOX, acyl-CoA oxidase; PTS, peroxisomal targeting signal; thiolase, 3-ketoacyl-CoA thiolase; PRISCOX, pristanoyl-CoA oxidase; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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from the poly(A)⁺ RNA was constructed using the Agt11 system (Amersham, Tokyo, Japan).

**Amino Acid Sequence Analysis**—Determination of the amino-terminal sequence of glyoxysomal acyl-CoA oxidase was performed essentially as described by Matsuda et al. (12). Isolated glyoxysomes were homogenized in extraction buffer (0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% SDS), the homogenate was centrifuged at 15,000 × g for 20 min, and the supernatant was subjected to SDS-PAGE. Then, an immunoblot analysis was performed by the method of Towbin et al. (18). Immunoreactions were detected by monitoring the activity of horseradish peroxidase (ECL system; Amersham) or of alkaline phosphatase (Organon Teknika, West Chester, PA). The intensity of the signal was quantitated with a densitometer. The antisera against thiolase was prepared as described previously (5). Protein was quantitated with a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo).

**RESULTS**

**Cloning and Characterization of a cDNA for acyl-CoA Oxidase**—Initially, we isolated glyoxysomal membranes from pumpkin cotyledons from seedlings grown in the dark for 5 days. The membranes were treated with 0.1 M NaCl. The soluble proteins were separated by SDS-PAGE and blotted electrophoretically onto a polyvinylidene difluoride membrane. Polypeptides were stained with Coomassie Brilliant Blue, and a protein band of approximately 73 kDa was cut out and subjected to protein sequencing by automated Edman degradation. The amino-terminal amino acid sequence (AAGKAKAKIEVD-) was used to design degenerate primers; a 1.6-kilobase pair DNA fragment was obtained by polymerase chain reaction using a cDNA library from 4-day-old etiolated pumpkin cotyledons as a template. The DNA fragment showed a high similarity with ACOX from animals. Then, the fragment was used as a probe to screen a Agt11 cDNA library from 4-day-old etiolated pumpkin cotyledons. Several positive recombinant phages were recovered, including one containing the longest insert of approximately 2.4 kilobase pairs.

The nucleotide sequence for the putative pumpkin ACOX cDNA is presented in Fig. 1. The total length is 2313 base pairs, with an open reading frame of 2073 base pairs encoding a polypeptide of 690 amino acids and with a deduced molecular mass of 77,319 Da. Amino-terminal sequencing indicated that the molecular mass of the mature protein is 72,414 Da (Fig. 1). In the figure, the presequence is double underlined. Moreover, a putative flavin mononucleotide binding motif seems to be present from amino acid 447 to 462 (dotted line in Fig. 1) (19) and a putative cAMP/cGMP-dependent protein kinase phosphorylation site at amino acids 512–515 (20). The calculated amino acid composition of the mature protein is similar to that reported for the purified cucumber acyl-CoA oxidase (10) except for a few amino acids (data not shown).

**Comparison of Pumpkin ACOX with Related Proteins**—The polypeptide encoded by the pumpkin ACOX cDNA shows the highest sequence identity (76%), with a putative Phalaenopsis ACOX. The cDNA clone of Phalaenopsis was isolated as one of senescence-related genes in Phalaenopsis petals (21) and was not characterized in detail. The identity with ACOXs from animal sources is about 30%, e.g. 30% with rat pristanoyl-CoA oxidase, 29% with rat trihydroxycoprostanoyl-CoA oxidase, and 28% with rat palmitoyl-CoA oxidase. This led Do and Huang (21) to postulate that the Phalaenopsis cDNA codes for an ACOX.

The sequences of pumpkin ACOX, Phalaenopsis ACOX, and rat pristanoyl-CoA oxidase (PRISCOX) are aligned for comparison in Fig. 2. PRISCOX is a peroxisomal protein that oxidizes the CoA-esters of 2-methyl-branched fatty acids, e.g. pristanic acid, and straight long chain acyl-CoAs (22). Pumpkin ACOX buffer 0.1% SDS for 15 min, in 0.1× SSC/0.1% SDS for 15 min, and in SSC buffer at 60 °C twice for 15 min each. X-ray film was exposed to the washed membrane, and radioactivity was measured on the imaging plate of a BioImaging analyzer (FUJIX BAS 2000, Fuji Photo Film, Tokyo). After 18 h exposure, immunoblot hybridization—Pumpkin cotyledons were homogenized in extraction buffer (0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% SDS), the homogenate was centrifuged at 15,000 × g for 20 min, and the supernatant was subjected to SDS-PAGE. Then, an immunoblot analysis was performed by the method of Towbin et al. (18). Immunoreactions were detected by monitoring the activity of horseradish peroxidase (ECL system; Amersham) or of alkaline phosphatase (Organon Teknika, West Chester, PA). The intensity of the signal was quantitated with a densitometer. The antisera against thiolase was prepared as described previously (5). Protein was quantitated with a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo).
and _Phalaenopsis ACOX_ are represented in their mature forms (putative for _Phalaenopsis ACOX_). The overall identity of the amino acid sequences looks low, but it is possible to recognize a stretch of identical amino acids from amino acids 444 to 488. This portion also shows high identity with the amino acid sequences of rat trihydroxycoprostanoyl-CoA oxidase, rat palmitoyl-CoA oxidase, and _Caenorhabditis elegans_ acyl-CoA oxidase (data not shown). In addition, this region includes the putative flavin mononucleotide binding site (19) and six of the eight amino acids that represent the acyl-CoA dehydrogenase protein signature 2 (PS2: [QE]-x(2)-G-[GS]-x-G-[LIVMFY]-x(2)-[DEN]-x(4)-[KR]-x(3)-[DE]) (20). In respect to the acyl-CoA dehydrogenase protein signature 1 (PS1: [GAC]-[LIVM]-[ST]-E-x(2)-[GSAN]-G-S-D-x(2)-[GSA]) (20), it is possible to find seven of the nine amino acids in all three sequences of Fig. 2. This confirms the low homology between acyl-CoA oxidases and acyl-CoA dehydrogenases and suggests that the two protein stretches are involved in the FAD binding and/or in the binding with substrate.

**Pumpkin ACOX Is Synthesized as a Larger Precursor**—Some peroxisomal proteins are synthesized as larger precursors such as malate dehydrogenase (23, 24), thiolase (7, 25), and citrate synthase (9). These precursors are cleaved at a site near the amino-terminal end of the protein. An amino-terminal sequence comparison of the precursor protein with the mature protein, determined by protein sequencing following SDS-PAGE, indicates that pumpkin ACOX precursor protein is cleaved at the carboxyl side of amino acid 45 to give into the mature protein size. In recent studies, it was shown that the amino-terminal presequences of peroxisomal proteins also act as peroxisomal targeting signals (PTS2) (26, 27). Fig. 3 shows the alignment of the amino-terminal regions of pumpkin glyoxysomal ACOX, _Phalaenopsis ACOX_, and pumpkin glyoxysomal proteins that are synthesized as precursors of higher molecular mass (malate dehydrogenase (23, 24), citrate synthase (9), and thiolase (7, 25)). However, the cleavage site for the _Phalaenopsis ACOX_ has not yet been confirmed. The targeting consensus sequence, R-[I/L/Q]-x5-H-L, is highly conserved. Thus, it is suggested that pumpkin ACOX and, by analogy, _Phalaenopsis ACOX_ contain a PTS2-type targeting signal.

**Activity of ACOX in Etiolated Pumpkin Cotyledons**—To confirm that the ACOX cDNA actually codes for an ACOX, a crude extract from 5-day-old etiolated pumpkin cotyledons was subjected to hydrophobic interaction chromatography on a phenyl-Sepharose column (Fig. 4). Three peaks of ACOX activity were detected.
detected. The first peak (circles) was obtained using hexanoyl-CoA (C6) as a substrate and thus indicates the presence of a short chain ACOX. Two overlapping peaks were obtained with palmitoyl-CoA (C16) (squares) and decanoyl-CoA (C10) (triangles) as substrates and thus indicate a long/medium chain ACOX. Western blotting with polyclonal antiserum raised against the pumpkin ACOX expressed in *E. coli* (Fig. 4B) shows clearly that the antiserum recognizes a protein of approximately 73 kDa only in fractions showing long/medium chain ACOX activity. Because it was reported that plant long chain ACOX has a subunit molecular mass of approximately 72 kDa (10), and the medium chain ACOX has a subunit molecular mass of 62 kDa (11), we conclude that the isolated pumpkin cDNA encodes for a long chain ACOX.

Subcellular Localization of ACOX in Etiolated Pumpkin Cotyledons—To investigate the subcellular localization of the ACOX protein, enzyme activity and immunoblotting analyses were performed after fractionation by sucrose density gradient centrifugation of a pumpkin organelle homogenate (Fig. 5). Catalase and thiolase were used as glyoxysomal markers, and cytochrome c oxidase was used as a mitochondrial marker. ACOX activities were present in the supernatant and the glyoxysomal fractions. A small peak of activity was also detected in the mitochondrial fractions (namely fraction 11) but did not overlap with the activity of cytochrome c oxidase. As a small catalase activity was present in the fraction, these activities might be due to the contamination of glyoxysomes. The immunoblotting analysis confirmed that a protein of 73 kDa, corresponding to ACOX, is mainly present in the supernatant and in the glyoxysomal fractions (Fig. 5B). Interestingly, a similar pattern was also obtained for thiolase, another β-oxidation enzyme.

**FIG. 2.** Alignment of amino acid sequences of pumpkin ACOX (pumpkin), *Phalaenopsis* ACOX (*Phalaenopsis*), and rat pristanoyl-CoA oxidase (rat PRISCOX). The region of high homology is underlined, and asterisks mark amino acids common to the two protein signatures of acyl-CoA dehydrogenase (protein signature 1 and protein signature 2, respectively).

**FIG. 3.** Alignment of the amino-terminal presequence of pumpkin ACOX with other presequences of microbody proteins that are synthesized as larger precursors. pumACOX, pumpkin ACOX; phaACOX, *Phalaenopsis* ACOX (21); pumMDH, pumpkin glyoxysomal malate dehydrogenase (24); pumCS, pumpkin glyoxysomal citrate synthase (9); pumTHI, pumpkin glyoxysomal 3-ketoacyl-CoA thiolase (7). Conserved amino acids are shown in **bold**. Processing sites of presequences, determined by sequencing of the amino-terminal amino acids of mature proteins, are shown by arrowheads.

ACOX has a subunit molecular mass of approximately 72 kDa (10), and the medium chain ACOX has a subunit molecular mass of 62 kDa (11), we conclude that the isolated pumpkin cDNA encodes for a long chain ACOX.

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Time Course of mRNA and Protein Levels during Germination in Pumpkin Cotyledons—During greening of pumpkin cotyledons, the composition of matrix enzymes in microbodies changes dramatically, glyoxysomal enzymes decrease, and leaf peroxisomal enzymes are synthesized. Like thiolase, ACOX is a
part of the fatty acid β-oxidation spiral. Therefore, we followed the changes in the level of ACOX and thiolase mRNA and protein during the postgerminative growth of seedlings (Figs. 6 and 7). The relative levels of ACOX mRNA in dark-grown seedlings during the 9-day period after germination are indicated by Northern blot in Fig. 6A (top panel) and are quantified by densitometry (bottom panel). ACOX mRNA levels reached a maximum after 3 days and thereafter gradually disappeared. When some of the seedlings were transferred to light after 5 days (middle panel), the pattern did not change. Similar results were obtained for thiolase mRNA (Fig. 6B). Notably, the total amount of ACOX mRNA was much lower than that of thiolase mRNA. The ACOX protein levels are shown in Fig. 7A, in which the three panels correspond to the three mRNA panels in Fig. 6A. The curve for the dark-grown seedlings (closed circles, bottom panel) shows that the peak in the ACOX protein was delayed with respect to the mRNA peak, reaching a maximum level at day 5 after germination and subsequently decreasing. Similar results were obtained for thiolase (Fig. 7). Moreover, following illumination (open circles, bottom panels), ACOX disappeared more rapidly than thiolase. In general, however, the two patterns seem to be very similar.

**DISCUSSION**

In the present study, we report the cDNA sequence of a pumpkin glyoxysomal long chain ACOX in addition to the cDNA sequence of a previously reported *Phalaenopsis* ACOX (21).

The present results clearly show that the protein encoded by this gene is a plant long chain ACOX. The deduced amino acid sequences of pumpkin and *Phalaenopsis* cDNA sequences have an identity of 76%, indicating that the *Phalaenopsis* cDNA also codes for a long chain ACOX. Comparing other ACOXs, the best identity (30%) is obtained for the rat pristanoyl-CoA oxidase, which acts on 2-methyl-branched CoA-esters and straight long chain acyl-CoAs (22). Mammalian peroxisomes contain three ACOX isozymes that are not capable of oxidizing acyl-chain CoA esters of less than 8 carbons. In mammalian cells, the β-oxidation of short chain fatty acids is accomplished in mitochondria, in which acyl-CoA dehydrogenases act instead of ACOXs. Three peroxisomal mammalian ACOXs have been identified: PRISCOX (30% identity), palmitoyl-CoA oxidase...
(28% identity), which reacts with CoA esters of very long, long, and medium chain fatty acids (28), and trihydroxycoprostanoyl-CoA oxidase (29% identity), which oxidizes the CoA esters of the bile acid intermediates dihydroxycoprostanic acid and trihydroxycoprostanic acid (29). On the contrary, plant peroxisomes seem to contain ACOXs that are active on short, medium, and long chain acyl-CoAs (11) and are able to perform a complete \( \beta \)-oxidation of fatty acids to acetyl-CoA (2). Three plant ACOX isoforms have previously been purified and characterized. One is from cucumber cotyledons that is active on long and medium chain acyl-CoAs and that is a homodimer with subunits of 72 kDa (10). The other two are from maize and are active on medium and short chain acyl-CoAs, respectively (11). The former is a monomeric enzyme of 62 kDa, and the latter is a homotetrameric enzyme of 15 kDa. Three different genes seem to code for the three ACOX isoforms, as they have different subunit molecular weights (11). The report by Hooks et al. (11) was the first to imply the presence of a short chain ACOX in eukaryotic cells. Mammalian ACOX isoforms, nevertheless, show slightly different substrate preferences and seem to have very similar subunit molecular weights of about 75 kDa. Therefore, only the plant long chain ACOX should share common ancestral genes with the mammalian ACOXs.

In the present study, we were able to correlate the sequence of the isolated ACOX clone with a long chain specific ACOX by applying an antiserum against the expressed ACOX/histidine-tagged fusion protein. This antiserum recognized only long chain and medium chain ACOX activity and not short chain ACOX activity when pumpkin enzymes were separated by hydrophobic interaction chromatography (Fig. 4). The immunoreactive band corresponded to a molecular mass of 73 kDa in accordance with the calculated molecular mass of mature pumpkin long chain ACOX (72,414 Da) and with the previous report of 72 kDa for the cucumber long chain ACOX (10). The levels of ACOX mRNA do not seem to be greatly controlled by light. The ACOX protein that built up during the initial 5 days of germination disappeared during the transition from glyoxysomes to leaf peroxisomes upon exposure of the seedlings to light. Similar patterns have previously been observed for malate synthase (8) and citrate synthase (9). The appearance and disappearance of the mRNAs preceded the change in the ACOX protein during the microbody transition. Thus, the ACOX levels seem to be determined at both the

Fig. 6. Developmental changes in the level of mRNAs for pumpkin ACOX (A) and thiolase (B). Top panels, Northern blots of total RNA from one cotyledon of dark-grown seedlings. RNA was blotted on a nylon membrane, and then the membrane was allowed to hybridize with specific probes. Middle panels, Northern blots of RNA of seedlings after being transferred to continuous illumination 5 days after the onset of germination. Bottom panels, quantification of spot intensities of dark-grown (●) and light-grown (○) seedlings.

Fig. 7. Developmental changes in the level of proteins for pumpkin ACOX (A) and thiolase (B). Top panels, Western blots of 0.05% of total homogenate from 10 cotyledons of dark-grown seedlings. Total homogenate was blotted on a nylon membrane, and then the membrane was allowed to hybridize with specific probes. Middle panels, Western blots of total homogenate of seedlings after being transferred to continuous illumination 5 days after the onset of germination. Bottom panels, quantification of spot intensities of dark-grown (●) and light-grown (○) seedlings.
translational and posttranslational levels.

It is worth noting that pumpkin glyoxysomal long chain ACOX proteins are synthesized as larger precursors containing a cleavable amino-terminal presequence, namely PTS2 (27, 30), as in the case for some other plant peroxisomal proteins, such as malate dehydrogenase (23, 24), citrate synthase (9), and thiolase (7, 25). In all cloned mammalian ACOXs, a carboxyl-terminal signal (PTS1) is present, but there is no PTS2 signal (31). This indicates that the plant ACOX import mechanism differs from the mammalian one. It has been suggested that ACOX is a key enzyme of \( \beta \)-oxidation because it can control and regulate the flux of acyl-CoAs at the first step of the \( \beta \)-oxidation spiral (32). Particularly, the long chain acyl-CoA oxidase may represent a regulatory point considering the fact that most fatty acids of plant storage lipids are long chain molecules. In conclusion, this type of control mechanism could tightly regulate the long chain ACOX (as the first step of the \( \beta \)-oxidation cascade), or it could be involved in a coordinate or differential regulation of the expression of the three ACOX enzymes in plant tissues (11). To verify such a hypothesis, the cloning and an expression analysis of the two other ACOXs will be necessary.

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Hiroshi Hayashi, Luigi De Bellis, Katsushi Yamaguchi, Akira Kato, Makoto Hayashi and Mikio Nishimura

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