Chloroplast-encoded Protein as a Subunit of Acetyl-CoA Carboxylase in Pea Plant*

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The complete sequence of the chloroplast genome has been reported for liverwort, tobacco, rice, and Epifagus virginiana (1-4), and various genes were identified by sequence similarity with proteins of known function. However, there are about 30 open reading frames (ORFs) of unknown function in each genome. Identification of putative gene products and elucidation of their function are required for an understanding of chloroplast biogenesis. We previously reported that an ORF, which we named zfpA because the putative gene product has a zinc finger motif, has significant sequence homology with the dedB gene in Escherichia coli (5), which gene is essential to this bacterium (6). The dedB gene was renamed accD when it was found to encode a subunit of acetyl-CoA carboxylase (7). Hence, the zfpA gene has been also renamed accD, and was predicted to encode a subunit of acetyl-CoA carboxylase (8). This enzyme catalyzes the formation of malonyl-CoA from acetyl-CoA and bicarbonate in the first committed step in de novo fatty acid synthesis. Acetyl-CoA carboxylase from E. coli is a multi-subunit enzyme composed of biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase. AccD protein in E. coli is a β subunit with the molecular mass of 31.5 kDa that associates with α subunits of 35 kDa to form carboxyltransferase (130 kDa), composed of αβ2 (7). In narnmals, this enzyme is composed of one kind of polypeptide containing three functional domains (9). Several acetyl-CoA carboxylases have been purified from plant sources, and these enzymes are not multi-subunit enzymes like those from bacteria, but are single oligomers (10). A bacterial type of enzyme has been found in spinach chloroplasts (11), but the enzyme was not been purified, and there is no evidence that the purified acetyl-CoA carboxylase contains a chloroplast-encoded subunit. In this report we show immunologically evidence that there was accD gene product in pea chloroplasts and that this protein was a subunit of acetyl-CoA carboxylase.

MATERIALS AND METHODS

Construction of pUC18-383 Plasmid—The Scal-StuI fragment of a 17.3-kb DNA fragment (Patl fragment, P8) from pea chloroplasts (12), which contains the COOH-terminal 383 amino acid residues of ORF 590, was inserted into the Smal site of pUC118, and a plasmid was constructed and named pUC118-383.

Expression and Purification of 383 Protein—The chimeric pUC118-383 plasmid was introduced into E. coli JM109, which was treated with isopropyl-β-D-galactoside, inducing 383 protein, which formed inclusion bodies. The inclusion bodies were isolated (13). The solubilized protein had an apparent molecular mass of 52 kDa on SDS-PAGE (14), although its calculated value was 44,197. The protein was separated on two-dimensional gel electrophoresis by isoelectric focusing and SDS-PAGE. The major band on the gel was cut out and electroeluted. SDS in the eluate was crystallized at about 4 °C and removed by centrifugation. The amino-terminal residues of the recombinant 383 protein were sequenced.

Antibody Preparation—Antibodies against the recombinant 383 protein were obtained from rabbits as described elsewhere (15). An oligopeptide (Asp-Arg-Leu-Arp-Ser-Tyr-Glu-Lys-Thr-Gly-Leu-Pro-Glu-Ala) containing the 15 amino acid residues from 399 to 413 inducing 383 protein, was synthesized and conjugated to bovine serum albumin (16). Antibodies against this conjugated protein were obtained from rabbits. These antisera were precipitated with (NH₄)₂SO₄, and the IgG fractions were purified on a DEAE-cellulose column. The IgG was further purified by affinity chromatography on Affi-Gel 10 or 15 (Bio-Rad) conjugated with antigen protein as described in the manufacturer's protocol. IgG against the large subunit of ribulose bisphosphate carboxylase (rbcL protein) was prepared in the same way (17).

Fractionation of Chloroplast Proteins—Pea plants (Pisum sativum cv. Alaska) were grown with a cycle of 12 h of light and 12 h of dark. Intact chloroplasts were isolated with a Percoll gradient from leaves 10 to 12 days old and ruptured by a lysis buffer (50 mM Tris-KOH, pH 8, 1 mM EDTA, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 mM e-amino-n-caproic acid) as described before (18). The lysed chloroplasts were centrifuged for 5 min at 4,000 × g. The supernatant was designated the soluble fraction. The pellet was suspended in high-salt extraction buffer (lysis buffer, 1 M NaCl), stirred for 30 min, and centrifuged for 30 min at 80,000 × g as described elsewhere (18). The supernatant and the pellet were designated the high-salt extract and membrane fraction, respectively. The soluble fraction and high-salt extract were precipi-
tated with ammonium sulfate (75% saturation), and the precipitated proteins were dialyzed against the lysis buffer. The envelope fraction was isolated as described previously (19). The protein concentration was measured with an assay kit (Bio-Rad).

**Immunoblotting**—Proteins were separated by SDS-PAGE and blotted electrophoretically onto a nitrocellulose membrane (20). The blot was hybridized at 55 °C with 23-base oligonucleotide probe (21, 22), containing a six-base sequence, a 23-base sequence of the 5' non-coding sequence of pea chloroplast accD, and a 19-base sequence of the upstream promoter, accD coding sequence, and several non-coding sequences. After hybridization, the blot was washed in 25 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 (w/v), and 1 µg of anti-383 IgG were added to the supernatant, containing a fixed amount of radioactivity (about 2 × 10^6 dpm), 0.1 volume of 2.75% Nonidet P-40, and 16 volumes of a 1% Tween 80 solution containing 5 µg/ml of [35S]methionine (1,000 Ci/mmol) on the surface as described elsewhere (17). The labeled proteins were extracted with GR buffer (330 mM sorbitol, 50 mM Hapes-KOH, pH 8, 1 mM MgCl2, 1 mM MnCl2) and centrifuged at 10,000 × g for 10 min. The supernatant was put on Whatman 3MM paper and the paper was washed with trichloroacetic acid and its radioactivity was counted. The radioactivity incorporated into total soluble proteins was measured (17). To the supernatant, containing a fixed amount of radioactivity (about 2 × 10^6 dpm), 0.1 volume of 2.75% Nonidet P-40, and 16 volumes of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% Nonidet P-40, 10 mM methionine, 1 mM EDTA) were added. To this solution, 2 µl of preimmune IgG (6 µg/µl) was added, and the mixture was incubated for 15 min at 25 °C. Then 12 µl of a 10% suspension of fixed Staphylococcus aureus (binding capacity, 1.3 mg of human IgG/ml, Sigma) was added, and the mixture was incubated for 15 min at 25 °C. This mixture was centrifuged at 10,000 × g for 1 min, and 12 µl of anti-383 IgG (14 µg/µl) was added to the supernatant and the mixture was incubated for 15 min at 25 °C. Then 16 µl of the 10% suspension of fixed S. aureus was added, and the mixture was incubated for 15 min at 25 °C. This mixture was centrifuged at 10,000 × g for 1 min. The pellet was washed four times with immunoprecipitation buffer and suspended in 20 µl of SDS loading buffer (14); the supernatant was heated for 5 min at 95 °C and centrifuged. The supernatant was analyzed by SDS-PAGE followed by fluorography.

**Enzyme Assay**—The acetyl-CoA carboxylase activity of the extract from pea chloroplasts was measured by the method described elsewhere (23). About 0.1–2.5 mg of soluble protein in a total of 200 µl of a reaction mixture (100 mM Tricine-KOH, pH 8, 1 mM ATP, 2.5 mM MgCl2, 30 mM KCl, 1 mM dithiothreitol, 0.2 mM acetyl-CoA, 2 µM [14C]NaHCO3 (10 µCi)) was incubated at 30 °C for 10 min, and the reaction was stopped by the addition of 50 µl of 6 N HCl. Then 50 µl of the reaction mixture was put onto Whatman 3MM paper. The paper was dried, and its radioactivity was counted.

**Immunomicroscopy**—Pea leaf sections were fixed in 0.1 M phosphate buffer, pH 7.3, containing 2.5% glutaraldehyde and 4% paraformaldehyde for 24 h at 4 °C. The sections were washed with the phosphate buffer alone and treated with in a graded series of ethanol concentrations (50–100%) and then propylene oxide to dehydrate them. The sections were then embedded in LR White (London Resin) and sectioned. The sections were placed on nickel grids and floated with the section downward at room temperature on drops of PBS (10 mM phosphate buffer, pH 7.3, 140 mM NaCl) for 20 min and then PBS with 0.1% bovine serum albumin for 20 min. The sections were then floated on PBS containing anti-383 IgG for 24 h at 4 °C, washed with PBS, and then floated on PBS with 0.1% bovine serum albumin containing a 50-fold dilution of protein A-gold (Amersham Corp.), 10 nm of gold particles for 1 h at room temperature. The sections were floated on 2% (w/v) uranyl acetate for 10 min and viewed under electron microscope at 75 kV.

**RESULTS**

**Immunchemical Identification of AccD Protein**—IgG against the recombinant protein (anti-383 IgG) reacted with a protein with a molecular mass of 87 kDa in the soluble fraction and high-salt extract from pea chloroplasts (Fig. 1b). The 87-kDa protein was abundant in the high-salt extract but was not found in the membrane fraction or the envelope fraction. IgG against bovine serum albumin-oligopeptide (anti-15 IgG) similarly reacted with the 87-kDa protein in the soluble and high-salt extract (Fig. 1c). Because the 87-kDa protein reacted with two different antibodies related to the accD protein sequence, the 87-kDa protein is likely to be the gene product of accD. The 87-kDa protein was found in the root extract (Fig. 1d), showing that this protein is a component of root plastids. There are three possible initiation codons in the pea accD protein, the amino acid residues at 1, 12, and 220 (24, 25) and hence there are three possible ORFs.
ORFs 590, 579, and 371, with the calculated molecular mass of 67,116, 65,913, and 41,695, respectively. The 87-kDa had less mobility on SDS-PAGE than any of the three ORFs, but considering that the NH₂-terminal of ORF 590 was very acidic (24), the gene product probably started from the first or the second methionine.

The high-salt extract was analyzed on two-dimensional gel electrophoresis by electrofocusing and SDS-PAGE, and the isoelectric point of accD protein was measured (Fig. 1e). The 87-kDa protein detected immunochemically did not give a clear spot in the stained gel; this protein was a minor component of chloroplasts. The isoelectric point was 4.8, and the protein was acidic, as expected from the amino acid sequence (24, 25).

**Apparent Molecular Mass of AccD Protein Synthesized in Vitro**—The apparent molecular mass of pea accD protein, 87 kDa, was larger than the calculated values, 67.1 or 65.9 kDa. We compared the mobility of protein accD synthesized in vitro with that synthesized in vivo. The in vitro labeled accD proteins obtained were immunoprecipitated with anti-383 IgG and electrophoresed (Fig. 2). The protein synthesized in vitro gave several bands (Fig. 2, lane 2) but its immunoprecipitates exhibited two bands (Fig. 2, lane 4) with mobility similar to that of the in vivo protein (Fig. 2, lane 1). A weak band at 87 kDa and a strong band at 84 kDa probably corresponded to the two polypeptides starting from the first or the second methionine (67.1 and 65.9 kDa). Probably the second initiation codon was more effectively used than the first in this in vitro system. These results suggest that the 87-kDa protein on SDS-PAGE was an accD gene product of 67.1 kDa and that the agreement of apparent molecular mass of the polypeptide synthesized in vitro (87-kDa in lane 4) with that synthesized in vivo (lane 1) excluded the possibility of post-translational modification of this protein, and the shift in molecular mass on SDS-PAGE was attributable to the intrinsic amino acid sequence. The abnormal behavior of the electrophoretic migration is probably caused by acidic property of this polypeptide (Glu, 7.8 mol %; Asp, 11.5 mol %), as reported for several acidic proteins (26).

**Inhibition of Acetyl-CoA Carboxylase Activity by Anti-383 IgG**—Because accD protein was found in the high-salt extract, the acetyl-CoA carboxylase activity in this fraction was measured and compared with that of the other fraction (Fig. 3). The activity in the high-salt extract was higher than that in the soluble fraction. The amount of protein in the soluble fraction obtained from chloroplasts was about 50 times that in the high-salt extract, and the soluble fraction was used later for measurement of acetyl-CoA carboxylase activity.

**Fig. 2. AccD protein synthesized by coupled transcription-translation.** A DNA fragment containing the T7 promoter and the accD sequence was transcribed, and the resultant RNA was translated. The translation products labeled with [³⁵S]methionine (lane 2) were immunoprecipitated by preimmune IgG (lane 3) or anti-383 IgG (lane 4). Proteins (10 µg) from the high-salt extract were probed with anti-383 IgG (lane 1).

To check that accD protein was a subunit of acetyl-CoA carboxylase, the effect of anti-383 IgG on acetyl-CoA carboxylase activity was examined (Fig. 4a). The addition of anti-383 IgG to the solubilized enzyme fraction inhibited acetyl-CoA carboxylase activity about 80%, but the preimmune IgG did not. The reduction of activity corresponded to increasing amounts of 87-kDa protein in the immunoprecipitates (Fig. 4b). These results indicated that accD protein was a subunit of acetyl-CoA carboxylase and probably a subunit of carboxyltransferase as shown in E. coli accD.

**Other Subunits Related to AccD Protein**—Because of the
instability and scarcity of acetyl-CoA carboxylase in pea chloroplasts, it was difficult to purify this enzyme to homogeneity. However, using anti-383-IgG, we partly characterized the enzyme. Acetyl-CoA carboxylase reported elsewhere (27) contains a biotin carboxyl carrier protein or domain containing biotin that is easily detected with streptavidin conjugated with peroxidase. We looked for polypeptides containing biotin among the proteins that precipitated with accD protein.

When soluble proteins from pea leaves were probed with streptavidin, three kinds of polypeptides, of the molecular mass of 35, 63, and 91 kDa, were found on SDS-PAGE (Fig. 5a, lane 1), but only a 35-kDa protein was found in the chloroplast extract (Fig. 5a, lane 2). The 35-kDa protein is a major protein containing biotin in chloroplasts and may be a component of acetyl-CoA carboxylase. When the immunoprecipitates by anti-383 IgG of a chloroplast extract were separated on SDS-PAGE and probed with streptavidin, a 35-kDa polypeptide reacted with the streptavidin (Fig. 5b, lane 7). The polypeptides disappeared when recombinant 383 protein was added to the extract (Fig. 5b, lane 8); that is, the 383 protein competed with the 35-kDa protein that contained biotin. Under the same conditions, the 87-kDa accD protein was precipitated, and the 383 protein also competed with the 87-kDa protein (Fig. 5b, lanes 3–5). These results suggest that accD protein formed a complex with the 35-kDa protein so that the 383 protein competed with the two polypeptides. The 35-kDa protein was presumably a biotin carboxyl carrier protein, and the acetyl-CoA carboxylase was a multi-subunit enzyme.

To find the other polypeptide of acetyl-CoA carboxylase, the proteins in leaves were labeled with [35S]methionine, and the total leaf extract was immunoprecipitated in the presence and absence of a competitor. Two bands, at the molecular masses of 87 and 91 kDa, were immunoprecipitated in addition to several peptides of molecular masses less than 50 kDa (Fig. 5c, lane 9). When recombinant 383 polypeptide was added to the leaf extract before immunoprecipitation, the 87 and 91 kDa bands disappeared, although the polypeptides less than 50 kDa did not (Fig. 5c, lane 10). This result indicates that the recombinant 383 protein competed with the 87- and 91-kDa proteins in the immunoprecipitates but not with the other proteins, and suggests that the 87-kDa accD protein formed a complex with the 91-kDa protein. These two bands were of similar intensity and may be present in equimolar amounts in acetyl-CoA carboxylase. Differences in polypeptides of molecular masses less than 50 kDa in the presence and absence of competitor were difficult to distinguish, and we could not find the 35-kDa protein under those conditions. Other polypeptides less than 50 kDa may have precipitated with accD protein.

Molecular Size of Acetyl-CoA Carboxylase—To examine the apparent molecular size of acetyl-CoA carboxylase, the soluble fraction from pea chloroplasts was analyzed by gel permeation chromatography (Fig. 6). Acetyl-CoA carboxylase activity was found in the region of molecular mass about 700 kDa in which both the 87- and 35-kDa proteins were found (Fig. 6b), and the molecular size of acetyl-CoA carboxylase was about 700 kDa. The 87-kDa protein was also found in the region of lower molecular mass that lacked in the enzyme activity, suggesting that the 87-kDa protein was readily dissociated from the complex.

Effect of Light on the Synthesis of AccD Protein—Expression of most chloroplast genes in pea leaves is strongly induced by light. However, the transcript accumulation of accD is not strongly induced by light (5). To see the effect of light on the synthesis of accD protein, we labeled de novo synthesized protein in pea buds with [35S]methionine during illumination. The labeled proteins were immunoprecipitated by anti-383 IgG or anti-rbcL IgG, and the levels were compared (Fig. 7). RbcL protein is induced by light and was used as a positive marker. During illumination, the synthesis of rbcL protein increased as expected, but that of the 87- and 91-kDa polypeptides did not increase much. The latter two polypeptides were synthesized in etioplasts (Fig. 7, 0 day). Upon illumination, the synthesis increased in the first day and then gradually decreased, but the synthesis of rbcL protein rapidly increased throughout. Thus, the effect of light on rbcL and accD was different. Analogous results were observed for the transcript accumulation of accD and rbcL (data not shown). Activity of acetyl-CoA carboxylase in leaves increases with illumination (28). AccD protein in peas was not induced by light, indicating that the increase in enzyme activity was not due to an increase in enzyme protein.

Localization of AccD Protein—To find accD protein in the cell, we performed immunomicroscopic analysis. A thin fixed section of pea leaves was probed with anti-383 IgG and goat anti-rabbit IgG conjugated with gold particles. Gold particles were found around the thylakoid membrane in the chloroplasts only, but not in the cytoplasm or envelope (Fig. 8). This was further evidence that accD protein was in chloroplast, probably bound loosely to thylakoid membrane.

**DISCUSSION**

The chloroplast accD gene has been found in *E. virginiana* (4), *Synechocystis* (29), two bryophytes (1, 30), a fern (31), and two dicots (2, 5), but the homologues in monocots are either truncated, that is, rice has only a short COOH-terminal domain (3) or totally absent as in wheat (32). The amino acid sequence of the COOH-terminal was moderately conserved among different plants, but that of the NH2-terminal of the two dicots was much longer than the other accD gene, without
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(a) Gel filtration

Fig. 6. Gel filtration of the soluble fraction from chloroplasts. a, soluble proteins (18 mg) were put on a Sephacryl S-300 column (0.9 x 60 cm, Pharmacia) and eluted with lysis buffer. Acetyl-CoA carboxylase activity was measured. b, fractions (5 µl) were electrophoresed and probed with streptavidin or anti-383 IgG, and 35- and 87-kDa proteins were found. Marker proteins were thyroglobulin (669,000), and ribulose bisphosphate carboxylase (550,000).

(b) Immunoblotting

Fig. 7. Effects of light on the synthesis of accD protein. Pea seedlings grown in the dark for 7 days were illuminated with a white fluorescent lamp at about 60 µEm⁻²s⁻¹ for 0–3 days, and leaf proteins were labeled. A fixed amount of radioactivity of the total soluble protein (4 x 10⁶ dpm for accD and 1 x 10⁶ dpm for rbcL) was immunoprecipitated and the values were compared. Exposure time: 2 days for accD and 1 day for rbcL.

Fig. 8. Immunogold electron microscopy of pea leaves. Cy, cytoplasm; Cp, chloroplast. Final magnification: x 24,000.

The homology of the additional sequences. Such variation in this gene raised the question of whether this is a functional gene. Identification of the gene products is needed to answer the question, but only preliminary observations have been reported (25). Our results showed that a protein of an apparent molecular mass of 87 kDa was the gene product of pea accD. Several additional experiments are further evidence.

We showed here that accD protein was a component of acetyl-CoA carboxylase because antibodies to it inhibited the enzyme activity. The protein seems to form a complex with a 35-kDa polypeptide containing biotin and a 91-kDa polypeptide. Several acetyl-CoA carboxylases have been isolated from plant sources. In parsley cells (33), maize leaves (34), rape seed (35), and algae (36), the molecular mass of the native enzymes was estimated to be 420, 500, 550, and 700 kDa, respectively, and the enzymes are composed of single subunits of the molecular mass of 220, 60, 220, and 160 kDa, respectively; each subunit contains biotin. These enzymes are mammalian-type enzymes. These enzymes were not extracted from isolated plastids such as chloroplasts or leucoplasts and are probably cytoplasmic enzymes. The presence of the E. coli type of enzyme in spinach chloroplasts was reported earlier (11), but studies on this enzyme were dismissed because the mammalian-type enzymes were later purified, and the enzyme preparations that yielded several subunits on SDS-PAGE were thought to be degraded by protease (10). Acetyl-CoA carboxylase from E. coli, unlike its counterparts in mammals, dissociates readily into three protein components, all of which are essential for acetyl-CoA carboxylation. Indeed, pea enzyme had the same property; that is, accD protein was readily dissociated from the enzyme complex (Fig. 6). The enzyme dissociated into a complex containing a 35-kDa polypeptide with biotin and a complex containing accD protein during DEAE-cellulose chromatography (data not shown). Our results showed evidence that acetyl-CoA carboxylase in pea chloroplasts was a bacterial-type enzyme. Taking all the results together, there are two kinds of acetyl-CoA carboxylase in plants, the mammalian type in cytoplasm and the bacterial type in chloroplasts, as described earlier (37). De novo fatty acid synthesis in higher plant occurs predominantly, if not exclusively, in the plastids and the bacterial-type enzyme.
participates in the synthesis in plastids. There may be another site of fatty acid synthesis in which mammalian-type enzyme participates.

Recently a mammalian-type enzyme in maize has been shown to be the predominant form of acetyl-CoA carboxylase in both chloroplasts and endosperm (38). Taking this result together with the lack of accD gene in the Gramineae plastid genome, there may be a substantial difference between Gramineae and the other plants.

The genes encoding plant acetyl-CoA carboxylase have not been identified. Our results are the first evidence that the chloroplast-encoded polypeptide is a component of functional acetyl-CoA carboxylase in chloroplasts. All the known genes involved in fatty acid biosynthesis are nuclear encoded, and this is the first report of a fatty acid synthetic gene encoded in plastid genome.

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