Recombinant Carboxyltransferase Responsive to Redox of Pea Plastidic Acetyl-CoA Carboxylase*

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Akiko Kozaki, Koichiro Kamada, Yukio Nagano, Hiro Iguchi, and Yukiko Sasaki

From the Laboratory of Plant Molecular Biology, Graduate School of Agricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

Acetyl-CoA carboxylase regulates the rate of fatty acid synthesis. This enzyme in plants is localized in plastids and is believed to be composed of biotin carboxyl carrier protein, biotin carboxylase, and carboxyltransferase made up of α and β polypeptides, although the enzyme has not been purified yet. Accumulated evidence shows that pea plastidic acetyl-CoA carboxylase is activated by light and the activation is caused by light-dependent reduction of carboxyltransferase, but not of biotin carboxylase, via a redox cascade. To understand the reductive activation of carboxyltransferase at the molecular level here, we obtained the active enzyme composed of decahistidine-tagged (His tag) α and β polypeptides through the expression of the pea plastidic carboxyltransferase gene in Escherichia coli. Gel filtration showed that the molecular size of the recombinant carboxyltransferase is in agreement with that of partially purified carboxyltransferase from pea chloroplasts. The catalytic activity of the recombinant enzyme was similar to that of native carboxyltransferase. These results indicate that the molecular structure and conformation of recombinant carboxyltransferase resemble those of its native counterpart and that native carboxyltransferase is indeed composed of α and β polypeptides. This recombinant enzyme was activated by dithiothreitol, a known reductant of S-S bonds, with a profile similar to that of its native counterpart. The recombinant enzyme was activated by reduced thioredoxin-f, a signal transducer of redox potential in chloroplasts under irradiation. Thus, this enzyme was redox-regulated, like that of the native carboxyltransferase.

ACCase: the eukaryotic form, composed of a large multifunctional polypeptide in cytosol, and the prokaryotic form, composed of four different polypeptides in plastids (1, 2). Gramineae have the eukaryotic form both in cytosol and plastids (3, 4). Plant fatty acids are mainly synthesized in plastids, and the prokaryotic form of ACCase regulates the rate of fatty acid synthesis in most plants (2). In leaves, fatty acid synthesis is modulated by light/dark, presumably by regulation of plastidic ACCase (5). Chloroplasts have a light-signal transduction pathway via a redox cascade, which is used to regulate enzymes of the reductive pentose phosphate cycle (6). Under light, electrons from photosystem I are shuttled through the electron transport chain to ferredoxins and are transferred to thioredoxins by ferredoxin-thioredoxin reductase and thence to target enzymes, reducing disulfide bonds of cysteines in the enzymes and changing their catalytic activities. We previously proposed that such a redox cascade is involved in the light/dark modulation of pea plastidic ACCase and that the reductive activation of the ACCase by thioredoxin links light and fatty acid synthesis (7). This proposition was supported by in vivo evidence that the plastidic ACCase in light is indeed in an active reduced form and that in the dark is in an inactive oxidized form (8). Light and redox sensitivity of the ACCase was also observed for spinach chloroplasts (9).

Pea plastidic ACCase is a multienzyme complex probably composed of biotin carboxyl carrier protein (BCCP), biotin carboxylase, and carboxyltransferase complex made up of two pairs of α and β polypeptides. Of these four polypeptides, β polypeptide is encoded by the plastid genome (10), and the other three polypeptides are encoded by the nuclear genome (2). ACCase catalyzes two different half-reactions, biotin carboxylase (Step 1) and carboxyltransferase reactions (Step 2) to form malonyl-CoA,

\[
\text{BCCP + Mg}^{2+}\text{-ATP + HCO}_3^- \rightleftharpoons \text{BCCP-CO}_2^- + \text{Mg}^{2+}\text{-ADP + Pi}
\]

**Step 1**

\[
\text{BCCP-CO}_2^- + \text{acetyl-CoA} \rightleftharpoons \text{BCCP} + \text{malonyl-CoA}
\]

**Step 2**

Of these two reactions, the carboxyltransferase reaction is redox-regulated (8). The α and β polypeptides in pea have 2 and 11 cysteine residues, respectively. One or more of these cysteines are potential regulatory residues.

Plastidic ACCase as well as its carboxyltransferase have not been purified yet. The molecular mass of pea ACCase has been estimated from gel filtration to be about 700 kDa (10–12), but its molecular composition has not been confirmed. For characterization of ACCase and for identification of regulatory cysteine residues, it is important to express plastidic carboxyltransferase in Escherichia coli. Although cDNA encoding the α polypeptide (11) and DNA encoding the β polypeptide (10) of plastidic carboxyltransferase have been isolated, the recombi-
nant carboxyltransferase has not yet been obtained, probably because of abnormal properties of the two polypeptides.

Enzymes composed of two different subunits are successfully obtained by coexpressing the two polypeptides in E. coli using a plasmid (13–15). By using such a strategy, here we report the expression of active plastidic carboxyltransferase in E. coli. We examined the catalytic and regulatory properties of the recombinant enzyme and compared them with those of the native carboxyltransferase from chloroplasts.

**Experimental Procedures**

Cloning of cDNAs for accA and accD—The cDNAs for the \( \alpha \) and \( \beta \) polypeptides of carboxyltransferase, designated accA and accD cDNAs, respectively, were obtained by reverse transcription polymerase chain reaction (PCR) using RNA from pea (Pisum sativum cv. Alaska) seedling as template. RNA was reverse-transcribed with specific reverse primers for accA, PA03 (5'-CCGGCTAGGTTCCTTAAGAGATTGGCAGTATTACACCA-3') or for accD, PD05 (5'-CCGGTCGACCTTAAGCTAAAGGAAGGAGAACG-3') using superscript RNaseH reverse transcriptase (Life Technologies, Inc.) according to the method described elsewhere (16). PA03 and PD05 have XhoI and BplI restriction sites (underlined), respectively, and stop codons (bold). In addition, PA03 has a ribosome binding site (italic). The 5’- and 3’-strand cDNAs were used for the following PCR amplification. The accA cDNA was amplified using the primers PA03 and PA01 (5'-CTATGAGATGGAGGAGGAA-3'), which were designed to fuse an accA fragment at the N terminal residue of the downstream carboxyltransferase protein (PD05). accD cDNA was amplified using the primers PD05 and PD04 (5'-CCGGTCGACAGTATAAGGAAGAGGACCGATTTACACCA-3'). PA01 is designed to express the \( \alpha \) polypeptide from an accurate N-terminal residue without transit peptide and has the NdeI restriction site (underlined). accD cDNA was amplified using the primers PD05 and PD04 (5'-CCGGTCGACAGTATAAGGAAGAGGACCGATTTACACCA-3'). PCR products were inserted into the EcoRV site of pZErO-2 vector (Invitrogen). The inserted sequences of the resultant plasmids, pZA and pZD, were determined by the sequence analysis using an automatic DNA sequencer (model LIC-400, LI-COR Inc.). The sequence of accD fragment amplified by reverse transcription-PCR was also verified by direct sequencing.

DNA sequences of pea (cv. Alaska) accA differed by five nucleotides from the reported sequences of pea (cv. Miranda) accA (accession number: Z31559). The accA sequence data will appear in the DDBJ/EMBL/GenBank nucleotide sequence data bases under the accession number AB29556.

Construction of Expression Vector—The strategy for construction of expression vector pHisAD is summarized in Fig. 3. For the coexpression of the \( \alpha \) and \( \beta \) polypeptides to produce the carboxyltransferase complex in the cell, accA cDNA and accD cDNA were polycistronically inserted into pET-19b vector containing a decahistidine-tagged (His tag), which allows a fused protein to be purified by a nickel column. The pET-19b vector is designed for fused protein to be cleaved by the protease thrombin. Briefly, the accA fragment was isolated by digestion of pZA with NdeI and XhoI, and the accD cDNA fragment was isolated from pZD by digestion with XhoI and BplI. First, the accC fragment was ligated into XhoI/BplI sites of pET-19b, and then the accA fragment was ligated to XhoI/NdeI sites of pET-19b vector carrying the accD fragment. In the resultant pHisAD, a His tag was designed to be fused to the N terminus of the \( \alpha \) polypeptide.

Expression and Partial Purification of Recombinant Proteins—The \( \beta \) polypeptide and His-tag \( \alpha \) polypeptide of pea carboxyltransferase were expressed in E. coli BL 21(DE3) cells (Novagen) containing pHisAD (Fig. 3). Cells containing the plasmid were grown exponentially in LB medium containing ampicillin (100 \( \mu \)g/ml) at 37°C with shaking (250 rpm). At an \( A_{600} \) of 0.7–0.9, isopropyl \( \beta \)-thiogalactoside was added to a final concentration of 1 mM, and the temperature was lowered to 20°C upon induction. The cultures were incubated for an additional period of 15–20 h at 20°C with shaking (150 rpm). The cells were harvested by centrifugation at 8000 \( \times \)g for 10 min at 4°C and stored at −80°C.

The cell paste from a 2-liter culture was suspended in 200 ml of binding buffer containing 5 mM imidazole, 500 mM NaCl, 0.1% Triton X-100, and 20 mM Tris-HCl (pH 7.9). The eluate was dialyzed against 50 mM Tricine-KOH (pH 8.0) and used for activity measurement. The activity was stable at 4°C for at least 1 month and at −80°C for several months.

Partial Purification of Native Carboxyltransferase from Pea Chloroplasts—From chloroplasts containing 24 mg of chlorophyll, ACCase fractions were prepared before and after thrombin digestion (as described above). The pooled ACCase fractions were separated by anion exchange chromatography (RESOURCE Q (1 ml), Amersham Pharmacia Biotech) with a linear gradient of 0–500 mM NaCl in buffers containing 50 mM Tricine-KOH (pH 8.0) and 1 mM EDTA. At this step, ACCase was eluted from the native carboxylase with BCCP and carboxyltransferase, and these two complexes were eluted at different NaCl concentrations. Carboxyltransferase eluted at about 0.35 M NaCl was again separated by RESOURCE Q to remove contaminating biotin carboxylase with BCCP. The carboxyltransferase was concentrated by ammonium sulfate (50% saturation) precipitation and used as the native carboxyltransferase.

**Gel Filtration**—Both recombinant carboxyltransferase and native carboxyltransferase were precipitated by the addition of ammonium sulfate (50% saturation) and dissolved into 0.07 ml of a buffer containing 50 mM Tricine-KOH (pH 8.0), 1 mM EDTA, and 0.5 mM DTT, and dialyzed. Fifty microliters (20–25 \( \mu \)g of protein for recombinant enzyme or 150 \( \mu \)g of protein for native enzyme) of this dialysate was separated with a Superdex 200 column using a SMART system (Amersham Pharmacia Biotech).

Immunoblotting—Proteins were separated by SDS-PAGE (7.5% (w/v) gel) and transferred to a nitrocellulose membrane. The blots were probed with anti-carboxyltransferase \( \alpha \) or \( \beta \) IgG and then with goat anti-rabbit IgG conjugated to peroxidase (Bio-Rad) diluted 1:3000; the blots were developed with an immunostaining system (Konica) or an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Anti-carboxyltransferase \( \alpha \) polypeptide IgG was prepared against glutathione S-transferase-fused accA protein by immunizing the rabbits. Glutathione S-transferase-fused accA protein was prepared according to the Amersham Pharmacia Biotech protocol. Anti-carboxyltransferase \( \beta \) polypeptide IgG was prepared as described previously (10).

**Immunoprecipitation**—Pea plants (P. sativum cv. Alaska) were grown with a cycle of 16 h of light and 8 h of darkness. Intact chloroplasts were isolated from the leaves of 9-day-old seedlings with a Percoll gradient. The isolated chloroplasts were ruptured by a lysis buffer (500 mM Tricine-KOH (pH 8.0), 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 mM e-amino-n-caproic acid) as described before (10). The lysed chloroplasts were centrifuged for 20 min at 50,000 \( \times \)g. The supernatant (5 ml) obtained from chloroplasts containing 5 mg of chlorophyll was diluted by 3 ml of an immunoprecipitation buffer containing 50 mM Tricine-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 (w/v), and 3.6 \( \mu \)g (50 \( \mu \)g of protein) of anti-carboxyltransferase \( \beta \) IgG was added. The mixture was incubated for 1 h at 25°C and then for 15 h at 4°C. To this mixture, 50 \( \mu \)l of a 5% suspension of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, binding capacity, 20 mg IgG/ml beads) was added and incubated for 1 h at 25°C and then for 1 h at 4°C with gentle rotation. The beads were recovered by brief centrifugation and washed with immunoprecipitation buffer for three times. The proteins bound to the beads were released by treatment with SDS loading buffer for 3 min at 100°C.

Measurement of Carboxyltransferase Activity—Carboxyltransferase activity was measured by the reverse reaction method as described elsewhere (18). The carboxyl transfer from [2-14C]malonyl-CoA to biotin methyl ester was measured. The resultant [14C]acetyl-CoA was evaporated, and the residual [2-14C]malonyl-CoA was determined. A carboxyltransferase fraction in a total of 50 \( \mu \)l of reaction mixture (100 mM Tricine-KOH (pH 8.0), 10 mM biotin methyl ester, 125 \( \mu \)M malonyl-CoA (0.64 kbp), 37.5 \( \mu \)g of bovine serum albumin) was incubated at 30°C. After 0 and 20 min of incubation, 20 \( \mu \)l of the mixture was transferred to 50 \( \mu \)l of Tris-HCl to terminate the reaction. The mixture was heated to dryness at 98°C for 20 min, dissolved in 25 \( \mu \)l of H2O, and applied to Whatman 3MM paper (1 × 1 cm). The difference between radioactivity at time zero and after 20 min of incubation was designated as carboxyltransferase activity. Recombinant spinach thioredoxin-f was a gift from P. Shurmman (Université de Neuchatel, Switzerland). Reduced thioredoxin-f was prepared by incubation with

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RESULTS

Identification of Amino Acid Sequences of α and β Polypeptides—Carboxyltransferase α polypeptide is encoded by a nuclear genome, and its gene is accA. The deduced amino acid sequence from accA cDNA has a transit peptide to enter into chloroplasts at the N terminus. To express the α polypeptide in E. coli, we must determine the accurate N-terminal amino acid sequences of this polypeptide and remove the sequence encoding the transit peptide from the pea accA cDNA. Carboxyltransferase β polypeptide is encoded by the chloroplast genome, and its gene is accD. The alignment of the amino acid sequence deduced from various plant acc genome, and its gene is accD (Fig. 1). The alignment of the amino acid sequences of both α and β polypeptides, we immunoprecipitated carboxyltransferase in the chloroplast extract by anti-carboxyltransferase β polypeptide IgG, separated the precipitates on SDS-PAGE, and analyzed the amino acid sequences of each polypeptide. We found two bands with an apparent molecular mass of 98 and 87 kDa in the region of molecular mass larger than the IgG heavy chain (about 52 kDa). The 98 and the 87 kDa bands were identified to be α and β polypeptides, respectively (Fig. 1A). The N-terminal amino acid sequences of these bands thus determined were aligned with the deduced sequences from the accA cDNA and the accD gene (Fig. 1B).

The cleavage site of the transit peptide of α polypeptide was the 50th amino acid residue, which was in agreement with the site predicted by the method described previously (18). Because pea accA encodes a polypeptide of 875 amino acid residues (11), the α polypeptide is composed of 825 amino acid residues and has a molecular mass of 91,379 Da. A accA cDNA encoding a full-length α polypeptide that does not have a transit peptide was used for constructing the expression plasmid. Of two possible initiation methionines, the first one was found in the immunoprecipitates of the β polypeptide, and the polypeptide, which is composed of 590 amino acid residues (17), starts from the first methionine.

Chloroplast mRNA is sometimes modified by RNA editing (19). To find the RNA editing site of pea accD transcript, we compared the nucleotide sequences of the cDNA with those of the accD gene (Fig. 2A). Only one editing site, cytosine to uracil change, was found at nucleotide position +800. This corresponded to the second nucleotide position of the 267th codon, UCG (serine) codon. The UCG triplet (serine) in the gene was converted to the UUG triplet (leucine) in its mRNA, and the 267th codon for a leucine residue was created by RNA editing. The leucine residue contributes to the conservation of accD protein among most chloroplasts, Cyanobacteria, and E. coli, suggesting that this leucine residue is critical for the protein function.

Because of the presence of an editing site, we used a cDNA, but not a chloroplast DNA, encoding the full-length β polypeptide for constructing the expression plasmid. The molecular mass of the β polypeptide was 87,142 Da, different from that deduced from its DNA, although the apparent molecular mass on SDS-PAGE is 87 kDa (10).

Expression of Carboxyltransferase and Partial Purification—

The cDNAs encoding α and β polypeptides described above were polycistrionically inserted into the PET-19b vector (Fig. 3). The cDNA for the α polypeptide was ligated to the sequences for His tag. For coexpression of the β polypeptide with the α polypeptide, a 12-base pair intercistronic region that contains a ribosomal binding site and an XhoI site was inserted between the genes for the α and the β polypeptides. Thus, an expression plasmid, pHisAD, was constructed.
When bacteria containing pHisAD (Fig. 3) were grown at 37 °C after induction, the gene for α polypeptide was expressed as inclusion bodies, and the gene for β polypeptide was only slightly expressed as inclusion bodies. To obtain a soluble enzyme, we lowered the temperature after the induction to 20 °C and obtained a small amount of soluble carboxyltransferase. The soluble fraction was bound to the nickel column, and carboxyltransferase activity was eluted with 150 mM imidazole. The control experiments using bacteria containing the pET-19b vector only did not show any carboxyltransferase activity. On SDS-PAGE, the active fraction purified with a nickel column gave a major band with a molecular mass of 102 kDa and two minor bands of 72 kDa and 87 kDa (Fig. 4). The α polypeptide fused to His tag is composed of 849 amino acids, and its molecular mass is 94,256 Da. The 102-kDa band reacted with anti-carboxyltransferase polypeptide serum (α) or anti-carboxyltransferase β polypeptide IgG (β).

Estimation of Molecular Size by Gel Filtration—We separated the partially purified recombinant enzyme by gel filtration with a Superdex 200 column in the presence of DTT. Coomassie Brilliant Blue staining of the separated fractions revealed two peaks of 102-kDa His-tag α polypeptide at fractions 12 and 22 and one peak of the 87-kDa β polypeptide at fraction 12 (Fig. 5A). A peak of carboxyltransferase activity was found at fraction 12. Immunoblotting revealed the presence of α and β polypeptides in this fraction. These results indicated that the carboxyltransferase composed of α and β polypeptides existed in fraction 12, and α polypeptide aggregates, probably dimers, were also present in fraction 22. Coomassie Brilliant Blue staining showed that the band intensity of the α polypeptide at fraction 12 was slightly denser than that of the β polypeptide, like that of the native enzyme (Fig. 1), suggesting that the molecular composition of recombinant carboxyltransferase and native counterpart in planta is the same with respect to α and β polypeptides.

To compare the molecular size of the recombinant carboxyltransferase with its native counterpart, we similarly analyzed

![Strategy for cloning accA and accD in expression plasmid vectors. A ribosomal binding site (rbs) was inserted.](image1)

![Expression and partial purification of carboxyltransferase. A, SDS-PAGE of the crude extract from E. coli containing pHisAD and the fraction purified by nickel column. Crude extract from 5 ml of culture were separated on 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue. B, immunoblot of the fraction purified with a nickel column. After separation by SDS-PAGE, the fraction was probed with anti-carboxyltransferase α polypeptide serum (α) or anti-carboxyltransferase β polypeptide IgG (β).](image2)
the partially purified carboxyltransferase fraction (Fig. 5B). The major peak of carboxyltransferase activity and its components were found in fraction 12. These results indicated that the apparent molecular size of the recombinant His-tag carboxyltransferase was similar to that of the native counterpart. Similar and reproducible results were obtained for the recombinant and native enzymes in the absence of DTT (data not shown). The molecular size of the recombinant carboxyltransferase at fraction 12 estimated from the standard protein was 490 kDa.

Characterization of Carboxyltransferase Activity—Because the carboxyltransferase activity in ACCase was sensitive to redox state (8), we examined the sensitivity of the recombinant enzyme to redox state and compared it with that of its native counterpart. In this experiment, the effect of a known reductant of S-S bonds, DTT, was tested (Fig. 6A). In the absence of DTT, the recombinant enzyme was inactive. The addition of monothiol, 2-mercaptoethanol, did not activate the enzyme at a concentration of less than 8 mM. The addition of DTT activated the enzyme, and the activity attained maximum level at about 8 mM. The similar profile was obtained for the native carboxyltransferase, and the requirement for DTT of the two enzymes was the same. These results indicate that the recombinant enzyme is sensitive to redox state, like that of its native counterpart.

Thioredoxins are signal transducers of redox potential in chloroplasts under light (6). Chloroplasts contain 100–200 mM thioredoxin-m and -f. Our previous results showed that thioredoxin-f activated in vitro pea plastid ACCase more efficiently than thioredoxin-m (7). To determine whether thioredoxin-f can activate the recombinant enzyme, we examined the effect of thioredoxin-f at the concentration of 0–160 mM (Fig. 6B).
The addition of spinach thioredoxin-f in reduced form increased the recombinant enzyme activity severalfold compared with that of nonreduced form. Almost the same profiles were obtained for the native enzyme, suggesting that the sensitivity of recombinant enzyme to reduced thioredoxin-f is similar to that of the native enzyme. In the presence of 160 μM reduced thioredoxin, the recombinant enzyme activity was 914 nmol/20 min/mg of protein, and in the presence of 160 μM DTT, it was 59.2 nmol/20 min/mg of protein. The reduced thioredoxin-f activated the enzyme more efficiently than DTT (Fig. 6A), implying that the reduced (dithiol) thioredoxin, having a higher affinity for the carboxyltransferase than DTT, reduced an S-S bond(s) and activated the enzyme.

Comparison of the kinetic parameters for carboxyltransferase-catalyzed transcarboxylation from malonyl-CoA and biotin methyl ester revealed that the recombinant enzyme and its native counterpart had similar $K_m$ values for both substrates: $K_m$ for malonyl-CoA of recombinant and native enzymes were 0.35 and 0.34 mM, respectively, and $K_m$ for biotin methyl ester of recombinant and native enzymes were 1.05 and 1.01 mM, respectively. Both enzymes showed no sensitivity to pH over the range 6.5–8.5 (data not shown). Thus, both the recombinant and native enzymes showed similar catalytic properties. However, the pH profile of carboxyltransferase activity in ACCase (8) was different from that of carboxyltransferase alone. Assembly markedly affected the ionic properties of the carboxyltransferase.

**DISCUSSION**

Plastidic ACCase and its components have not been purified yet because of their labile properties, and we do not know the exact molecular composition of these enzymes. An approach different from conventional purification has been awaited to identify the polypeptide composition. Here we have succeeded in obtaining the recombinant carboxyltransferase for the first time and characterized its properties. We found that the recombinant enzyme composed of His-tag α and β polypeptides has similar molecular size (Fig. 5), redox sensitivity (Fig. 6), and catalytic activity to those of the native counterpart in *planta*. These results indicate that native carboxyltransferase is indeed composed of α and β polypeptides.

Plastidic ACCase resembles to the ACCase in *E. coli*. The sequences of 310 amino acid residues in the N-terminal half of the α polypeptide is conserved in *E. coli* with 81% amino acid similarity (47% identity) (Fig. 7). With respect to the β polypeptide, the sequences of the C-terminal 278 amino acids, excluding reiterated sequence, is conserved in *E. coli* with 78% similarity (39% identity). These conserved domains are probably essential for carboxyltransferase. The close resemblance between pea and *E. coli* enzymes in these domains suggests that subunit structure of the pea enzyme also resembles that of *E. coli* (20), αβ. The molecular size of pea recombinant carboxyltransferase estimated from gel filtration was 490 kDa. The molecular mass of the α polypeptide, the His-tag α polypeptide, and the β polypeptide was 91.4, 94.2, and 67.1 kDa, respectively. The calculated molecular mass of αβ was about 320 kDa, and the observed size was in agreement with the mass of αβ, 480 kDa. Although we can not exclude the possibility of αβ, the subunit structure of the carboxyltransferase is tentatively assumed to be αβ. This assumption accounts for the observed molecular mass of ACCase, about 700 kDa (10). The putative subunit structure of the plastidic ACCase is (BCCP)2 (biotin carboxylase)αβ as in *E. coli* (20). The molecular mass of BCCP and biotin carboxylase was 38 and 53 kDa, respectively. The sum of the calculated molecular mass of (BCCP)2 (biotin carboxylase), 180 kDa, and the apparent molecular mass of αβ, 490 kDa, is 680 kDa and consistent with the observed size of about 700 kDa. The discrepancy in the molecular size between the calculated and the observed values for αβ may be due to the abnormal shape of pea carboxyltransferase. The α polypeptide is a basic protein with a calculated pI of 9.7 and has a basic extra domain of pI 9.7 in C-terminal 473 amino acid residues not conserved in *E. coli*. The β polypeptide is an acidic protein with a pI of 4.3, which has an acidic extra domain with a pI of 3.8 in the N-terminal 226-amino acid residues not conserved in *E. coli*. Probably both domains electrostatically interact to form carboxyltransferase, and the molecular shape of this carboxyltransferase would not be globular and different from standard marker proteins such as thyroglobulin, apoferritin, catalase, and aldolase used in gel filtration experiments. The apparent molecular mass of the α and the β polypeptides on SDS-PAGE was 98 and 87 kDa, respectively, and larger than the calculated values (10). Such abnormal behavior on SDS-PAGE and on gel filtration is probably caused by the intrinsic amino acid sequence of these polypeptides.

The staining level of the carboxyltransferase in Figs. 1 and 5A, showing that the abundance of α-polypeptide exceeded that of β-polypeptide, may not be in agreement with αβ. However, the staining level of each polypeptide is dependent on its amino acid composition and does not always reflect on the composition of the complex. The discrepancy of the staining level is probably caused by the difference of amino acid composition of the two polypeptides.

There are two possibilities for the activation of the recombinant enzyme by DTT: activation by correct refolding of the recombinant protein and activation by redox regulation. The recombinant carboxyltransferase was inactive but was activated by DTT with a similar profile to that of its native counterpart, suggesting that the regulatory cysteines of recombinant enzyme are S-S form and the enzyme is correctly folded like that of the native enzyme. The activation may be caused by reduction of the regulatory S-S bond rather than by correct refolding of recombinant protein by DTT. Monothiol 2-mercaptoethanol that can refold the recombinant protein but is not a strong reductant of S-S bond did not activate the enzyme at a concentration lower than 8 mM, supporting this proposition.

Pea biotin carboxylase similarly expressed in *E. coli* was active in the absence of DTT and insensitive to redox, although there are eight cysteine residues conserved among plants. Thus, the sensitivity of the recombinant carboxyltransferase and biotin carboxylase to redox supports the previous finding that carboxyltransferase but not biotin carboxylase in ACCase is redox-regulated (8).

The recombinant enzyme and its native counterpart showed no sensitivity to pH over the range 6.5–8.5. However, carboxy

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2. A. Kozaki, K. Kamada, Y. Nagano, H. Iguchi, and Y. Sasaki, unpublished data.
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Carboxyltransferase activity in ACCase showed a remarkable sensitivity to pH (8). This indicates that association of carboxyltransferase with biotin carboxylase and BCCP results in a change in the pH preference of carboxyltransferase. When pea ACCase was analyzed by ion exchange chromatography, it dissociated into two components, carboxyltransferase and biotin carboxylase complexed with BCCP (11). This means that ACCase is formed by ionic association of the two components. This association affects the pH preference of carboxyltransferase and results in a marked change in catalytic activity at the physiological pH range, pH 7–8.

Activation of fatty acid synthesis by light is caused partly by activation of the regulatory enzyme ACCase. ACCase is stimulated by the transition of the stroma from pH 7 to pH 8 upon irradiation and by the thiol-reducing agent produced by light (7). The stimulation may consist of at least two features. One is the association of the two components, carboxyltransferase and biotin carboxylase complexed with BCCP, resulting in remarkable changes in pH preference at physiological pH range. The other may be the reduction of S-S bond of cysteine residues in the carboxyltransferase. Further studies on ACCase using recombinant enzyme are necessary to understand this enzyme at the molecular level. A recent report described the carboxyltransferase β-subunit as a candidate for regulation by protein phosphorylation/dephosphorylation (21). The recombinant carboxyltransferase now provides us with ample amounts of enzyme for structure/function studies.

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Akiko Kozaki, Koichiro Kamada, Yukio Nagano, Hiro Iguchi and Yukiko Sasaki

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