Thiol-Disulfide Exchange between Nuclear-encoded and Chloroplast-encoded Subunits of Pea Acetyl-CoA Carboxylase*

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Fatty acid synthesis in pea chloroplasts is regulated by light/dark. The regulatory enzyme acetyl-CoA carboxylase is modulated by light/dark, presumably under redox regulation. Acetyl-CoA carboxylase is a multi-enzyme complex composed of biotin carboxylase and carboxyltransferase (CT). To demonstrate the redox regulation of CT, composed of the nuclear-encoded α and the chloroplast-encoded β subunits, we identified the cysteine residues involved in such regulation. We expressed the recombinant CT in *Escherichia coli* and found that the partly deleted CT was, like the full-length CT, sensitive to a redox state. Site-directed mutagenesis of the deleted CT showed that replacement by alanine of the cysteine residue 207 in the α polypeptide or 442 in the β polypeptide resulted in redox-insensitive CT and broke the intermolecular disulfide bond between the α and β polypeptides. Similar results were confirmed in the full-length CT. These results indicate that the two cysteines in recombinant CT are involved in redox regulation by intermolecular disulfide-dithiol exchange between the α and β subunits. Immunoblots of extract from plants incubated in the light or dark supported that such a disulfide-dithiol exchange is relevant *in vivo*. A covalent bond between a nuclear-encoded polypeptide and a chloroplast-encoded polypeptide probably regulates the enzyme activity in response to light.

The carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) is the first committed step of fatty acid synthesis. ACCase is the enzyme considered most responsible for the primary regulation of fatty acid synthesis. Most plants, with the exception of the grass family (Poaceae), have two types of ACCase: the heteromeric, prokaryotic form in plastids and the homodimeric, eukaryotic form in cytosol (1, 2). The grass family has the homodimeric form in both plastids and cytosol (3, 4). In plants, *de novo* fatty acid biosynthesis takes place in the chloroplasts, and the heteromeric ACCase, except in the grass family, is the regulatory enzyme involved. The heteromeric ACCase is a multi-enzyme complex composed of four subunits, namely biotin carboxylase, the biotin carboxyl carrier protein, and the carboxyltransferase (CT) made up of α and β subunits (1, 2). In these subunits, the β subunit is encoded by the chloroplast genome, and the other three are encoded by the nuclear genome.

In pea leaves, fatty acid synthesis is modulated by light/dark, presumably by regulation of the plastidic ACCase (5). We previously proposed that the redox cascade is involved in the light activation of pea plastidic ACCase (6) and that CT, but not biotin carboxylase, is redox-regulated (7). The activity of several chloroplast enzymes involved in the Calvin cycle is regulated by reversible disulfide-dithiol interchange (8). During photosynthetic electron transport in the light, covalent redox modification mediated by the ferredoxin-thioredoxin signal transduction pathway leads to the reductive light activation of several stromal target enzymes. Thus, the ferredoxin-thioredoxin pathway links light and the Calvin cycle. We have proposed that this pathway also links light and fatty acid synthesis (6). To demonstrate the proposition, it is necessary to identify the target cysteine residues responsive to redox.

Previously we showed that pea recombinant CT expressed in *Escherichia coli* has properties similar to those of authentic CT and is redox-regulated (9). In this study, using the recombinant enzyme, we designed experiments to determine the cysteine residues involved in redox regulation by site-directed mutagenesis and to determine the role of two subunits. There are 2 and 11 cysteines in the α and β polypeptides, respectively. We identified one cysteine in the α subunit and one cysteine in the β subunit involved in redox regulation and found an intermolecular disulfide bond between the nuclear-encoded subunit and the chloroplast-encoded subunit. These results observed in recombinant enzyme were consistent with the results of *in vivo* experiments. Probably such a disulfide bond is redox-regulated in response to light.

**EXPERIMENTAL PROCEDURES**

Cloning of cDNAs for the Deleted accA and accD—RNA editing of the accD transcript encoding the β polypeptide is required for functional CT (9). The accD cDNA, but not the accD gene, was used for cloning. The cDNAs encoding the full-length α and β polypeptides in the pEvo-2 vector (Invitrogen) prepared previously were used as templates. The deleted accD (ΔaccD) cDNA encoding N-terminal 372 amino acid residues starting from the accurate N-terminal sequence lacking a transit peptide (Δα, Fig. 1A) was amplified using forward primer PA01 (5′-CTATAAGGTTGGGAAGAGG-3′) and reverse primer PA02 (5′-GGCTCGAAGTGTCAAGCTTTTTTTCCGTTAGAC-3′). PA01 and PA02 have NdeI and XhoI restriction sites (underlined), respectively. PA03 has a ribosome binding site (italics) and a stop codon (bold). The deleted accD (ΔaccD) cDNA encoding C-terminal 371 amino acid residues (Δβ) starting from amino acid position 220 was amplified using forward primer PD03 (5′-CGCGCTCGAGATGATGGAAAAATTA-GCTGTTTTA-3′) and reverse primer PD02 (5′-CGCGCTAGCTTTAGG-3′).
The the native sequence are given in bold letters. Forward primers (Cys-230 and Cys-233) contain a part of the PD03 sequences (underlined).

| cDNA | Mutation | Forward primer | Reverse primer |
|------|----------|----------------|----------------|
| ΔaccA | Cys-247 | GCCATGGAGGTTGCGATTTGCAATGCTCAATATTC | GTAAATTAGGAGGCTCAATGCTCAATATTC |
|      | Cys-267 | CACAGAGGGCTGTCGATTTGCAATGCTCAATATTC | CAAAGATTCGAGGCTGTCGATTTGCAATGCTCAATATTC |
| ΔaccD | Cys-230 | ATGGGAAATATTGCCTGTATTTGGTTCAGCCG | GGGCACTAGGTCGAGCTCAATATTC |
|      | Cys-233 | ATGGGAAATATTGCCTGTATTTGGTTCAGCGG | CAAAGATTCGAGGCTGTCGATTTGCAATGCTCAATATTC |
|      | Cys-249 | GAATATCGAGGACACGTTGCGATTTGCAATGCTCAATATTC | CTCACCACTAGGTCAGGCAATGCTCAATATTC |
|      | Cys-252 | GCAGGACACTGCGAGGATTTGCAATGCTCAATATTC | CCGGGGACGCTACATATATAGG |
|      | Cys-442 | CCGAGTAGTATGACCGCTGAGTGGTCAGG | CTCACCACTAGGTCAGGCAATGCTCAATATTC |
|      | Cys-466 | CCGGACATTATAGGACACGCTGAGG | CCGGGGACGCTACATATATAGG |

TCAAGAGCAACGAAAAC-3'). PD03 and PD02 have Xhol and Bipl restriction sites, respectively, and a stop codon. The deleted accD cDNA encoding C-terminal 333 amino acid residues from 258 to 590, and lacking a domain Cx1, Cx2, Cx3 in the Δβ, was amplified using forward primer PD04 (5'-CCGTCGAGTATGACCGAGCAGCAGACGACG-3') and reverse primer PD02. PCR was performed for 35 cycles (10 s at 98 °C, 1 min at 55 °C, and 3 min at 72 °C) using Phusion DNA Polymerase (TaKaRa) or for 35 cycles (15 s at 94 °C, 30 s at 55 °C, and 3 min at 72 °C) using KOD Plus (Toyobo). The final PCR products were purified and inserted into the EcoRV site of the pZErO-2 vector. The inserted sequences of the resultant plasmids were determined by the dideoxy chain termination method using an automatic DNA sequencer (model LIC-400, Li-Cor, Inc.).

Site-directed Mutagenesis of Cysteine by Alanine—Site-directed mutagenesis of accA and accD cDNAs was introduced by PCR. The accA and accD cDNAs in the pZErO-2 vector (10) were used as templates for the first and second PCR reactions, except for mutagenesis at Cys-230 or Cys-233. In the first PCR, the forward primer, PA01, and reverse primer PD01 (5'-CCGTCGAGTATGACCGAGCAGCAGACGACG-3') were used. The second PCR was performed using PA01 and PA02 for accA cDNA mutation and using PD03 and PD02 for accD cDNA mutation. To prepare a mutation at Cys-230 or Cys-233 in the full-length enzyme and analyzed on immunoblots probed with either the anti-α polypeptide or the anti-β polypeptide IgG.

RESULTS

Cysteine Residues in Pea α and β Polypeptides

Pea CT and its recombinant enzyme were redox-regulated in vitro (6, 10), suggesting the presence of redox-sensitive cysteines in the α and β polypeptides consisting of 825 and 590 amino acid residues, respectively. Fig. 1A shows schematically the position of cysteines deduced from pea accA and accD cDNAs and the conserved domain in E. coli. N-terminal 310 amino acids of the α polypeptide were conserved in E. coli with 81% similarity (47% identity), and C-terminal 278 amino acids of the β polypeptide, excluding the reiterated sequence, were conserved in E. coli with 78% similarity (39%
Fig. 1. Cysteine residues in the α and β polypeptides. A, schematic representation of the full-length (α and β) and deleted (Δα, Δβ, and Δαβ) polypeptides. The numbers are the amino acid positions of each full-length polypeptide. The positions of cysteines are marked by asterisks. B, sequence alignments of the regions designated a, b, and c in A. The numbers are the amino acid positions of the full-length pea polypeptides. Black arrowhead, cysteine residues in pea; white arrowhead, possible initiation methionine in Δβ and Δαβ; asterisk, cysteines in \( \text{CX}_{5}\text{CX}_{2-12}\text{CX}_{2}\text{C} \). Amino acid residues identical in one-half or more of the pea sequences are shaded black, and conservative substitutions are shaded gray.

The other domains absent from E. coli counterparts are not always conserved among plant proteins.

There are two cysteines at positions 247 and 267 in the pea α polypeptide. Alignment shows that the sequences around these cysteines are highly conserved (Fig. 2B); Cys-247 is found in higher plants, and Cys-267 is found in all sequences available. In pea β polypeptides, there are 11 cysteines. Five of them in the N-terminal domain are not conserved among plant proteins (data not shown), but six cysteines in the C-terminal domain are considerably conserved. Four of them, at positions 230, 233, 249, and 252, are found in all the counterparts available and form a motif, \( \text{CX}_{5}\text{CX}_{2-12}\text{CX}_{2}\text{C} \). Cysteine at position 442 is found only in pea, and cysteine at 466 is found in all photosynthetic organisms available. Taking into account the redox-insensitive properties of E. coli CT, such a sequence comparison does not directly predict the redox-sensitive cysteines in both the α and β polypeptides.

**Requirements of the Deleted CT for DTT**

**Expression**—There are 13 possible cysteines involved in redox regulation (Fig. 1A), and we attempted to reduce them. To investigate whether the domains not conserved in E. coli, namely, the C-terminal half in the α subunit and the N-terminal half in the β subunit, are necessary for redox regulation, we prepared enzymes in which these sequences were deleted, using an expression plasmid, pHisΔΔD (Fig. 2A), and examined the redox sensitivity.

The eluate partially purified by a nickel column showed redox-sensitive CT activity. The specific activity was 0.366 unit. The eluate exhibited three major bands of 52, 45, and 44 kDa by Coomassie Brilliant Blue staining (Fig. 2B). A band of 44 kDa reacted with antibodies against the α polypeptide, agreed with the calculated molecular mass, 44.3 kDa, and was identified as His tag Δα. Both 52- and 45-kDa bands reacted with antibodies against the β polypeptide. Determination of the N-terminal sequence of the 52- and 45-kDa bands revealed that the 52-kDa band corresponded to the Δβ protein starting from the predicted first methionine at position 220 (Fig. 1B). The N-terminal sequence of the 45-kDa polypeptide started from serine at position 259, suggesting that the third methionine at position 258 was recognized as another starting point. The calculated molecular masses of the Δβ and a polypeptide starting from serine 259 (Δβ*) were 41.7 and 37 kDa, respectively, and the observed size was about 8–10 kDa larger than the calculated value. Such a shift in molecular mass on SDS-PAGE was observed for the full-length β subunit and was attributable to the intrinsic amino acid sequence (11). Usually the 52-kDa band was denser than that of the 45 kDa, and Δβ was the major product.

To estimate the molecular size of the deleted CT, the partially purified eluate was separated by gel filtration in the presence of DTT. The peak CT activity appeared in fraction 16. Coomassie Brilliant Blue staining and immunoblotting of separated fractions revealed a peak of His tag Δα, Δβ, and Δαβ polypeptides at fraction 16. A peak composed of only the His tag Δα polypeptide was not found, unlike the expression of full-length polypeptides (10), suggesting that most of the expressed His tag Δα formed a complex with Δβ or Δβ*.

**Active Form and Domain Required for Catalytic Activity**—Two forms of the deleted CT were obtained by expression of pHisΔΔD. To determine the form(s) having CT activity, we constructed another plasmid to express Δαβ protein starting from the methionine at position 258. The eluate obtained by expression of this plasmid showed no CT activity. This result indicates that the measurable activity by expression of pHisΔΔD is the complex of His tag Δα with Δβ but not that of Δβ* polypeptides. The deleted sequences from 220 to 257, which contain a \( \text{CX}_{5}\text{CX}_{2-12}\text{CX}_{2}\text{C} \) motif conserved among all the β polypeptides, are required for catalytic activity but not for association with the Δα polypeptide. This is not a case in which a zinc finger motif is usually required for complex formation. It is likely that the \( \text{CX}_{5}\text{CX}_{2-12}\text{CX}_{2}\text{C} \) motif is important for catalytic activity.

**Redox Sensitivity**—We examined the sensitivity of the deleted CT partially purified to the redox state and compared it with that of the full-length CT. In this experiment, the effect of a known reductant of S–S bonds, DTT, was tested. DTT activated the enzyme in a profile similar to that of the full-length enzyme (Fig. 2D). The monothiol 2-mercaptoethanol did not activate the enzyme efficiently. These results indicate that the deletion mutant was sensitive to the redox state, like the full-length CT (9), and that the redox-sensitive cysteines were
located in the Δα and Δβ polypeptides. Thus, the deleted sequences, the C-terminal half of the α polypeptide, and the N-terminal half of the β polypeptide are not necessary for redox regulation and may have some other functions. The number of possible cysteines was reduced from 13 to 8.

Requirements of Site-directed Mutants for DTT

To identify the redox-sensitive cysteines in the deleted CT, a set of mutants, each with a single substitution of cysteine to alanine, was constructed, and the requirement of each mutant CT for DTT was tested. There were no significant differences in the efficiency of expression and purification of all mutants compared with the deleted wild-type enzyme. However, solubility differed with different mutant enzymes. Most mutant enzymes were precipitated under low ionic strength or by freezing. Therefore, each enzyme activity was determined using freshly eluted fraction from nickel column.

The relative specific activity of mutants in either the presence or absence of 8 mM DTT was determined (Table II). The deleted CT is redox-sensitive, and if a cysteine involved in redox regulation is replaced, the resultant mutant enzyme is expected to be converted to a redox-insensitive form. We surveyed such an enzyme active in the absence of DTT. In the presence of DTT, the mutant proteins retained varying levels of activity. Substitutions of Cys-267 in Δα and Cys-466 in Δβ had modest effects, retaining more than 50% of the wild-type activity. Other mutant enzymes were more impaired, retaining only 0–30% of the wild-type activity, suggesting that alanine replacements of cysteine residues does not satisfy the structural requirements at these sites for enzyme folding and the substrate binding. In particular, Cys-233 or -252 in a CXXC motif is probably required for catalytic activity. In the absence of DTT, only two mutants, the substitution of Cys-267 in the Δα and the substitution of Cys-442 in the Δβ polypeptides, were active and their activities were almost equal to those occurring in the presence of DTT. These results indicate...
that Cys-267 in Δα and Cys-442 in Δβ are required for redox regulation.

To confirm the role of the two cysteines at the same position in the full-length CT, we generated mutant enzymes with a substitution of cysteine by alanine and measured their activity (Table II). Although the CT activity was low, measurable activity was found in the absence of DTT, indicating that Cys-267 in the α subunit or Cys-442 in the β subunit is required for redox sensitivity and that the redox sensitivity of the full-length enzyme is probably mediated exclusively through these two cysteines. The molecular size of the deleted CT is about half that of the full-length CT, and the specific activity of the deleted CT corrected for the difference of molecular size was comparable with that of the full-length. The extra sequences, the C-terminal region of the α polypeptide and the N-terminal region of the β polypeptide (Fig. 1A), do not play an important role for exhibition of the enzyme activity. The low activity of these mutants suggests that the replacement of the cysteine by alanine may bring about inappropriate conformation for catalytic activity, probably by interacting with the extra sequences of the full-length CT. The interaction, which does not occur in the wild-type full-length enzyme, is somehow harmful for the expression of the full activity by preventing efficient substrate binding or subunit-subunit interaction.

### Table II

| Position of cysteine replaced by alanine | Relative activity ( – DTT / + DTT ) |
|----------------------------------------|-------------------------------------|
| Deleted CT                             |                                    |
| None (wild type)                       | 0 / 100                             |
| α 247                                  | 0 / 32                              |
| α 267                                  | 62 / 77                             |
| β 230                                  | 0 / 8                               |
| β 233                                  | 0 / 3.7                             |
| β 249                                  | 0 / 18                              |
| β 252                                  | 0 / 0                               |
| β 442                                  | 6 / 6                               |
| β 466                                  | 0 / 51                              |
| Full-length CT                         |                                    |
| None (wild type)                       | 0 / 100                             |
| α 267                                  | 2.4 / 5.7                           |
| β 442                                  | 1.2 / 2.4                           |

**Intermolecular Disulfide Bond**

Each subunit had only one cysteine responsible to redox, suggesting that an intermolecular but not an intramolecular disulfide bond is formed under nonreducing conditions. The molecular composition of CT is probably αβ2, and there are possible intermolecular disulfide bonds between homodimers or heterodimers. To determine which type of S–S bond was formed, SDS-PAGE analysis of several recombinant enzymes was performed in the presence or absence of the reducing agent 2-mercaptoethanol. To prevent rearrangement of disulfide bonds during treatment with a sample loading buffer, SH groups were alkylated with iodoacetic acid. The modified proteins were denatured and separated by SDS-PAGE in the presence or absence of 2-mercaptoethanol and probed with antibodies against the α or β polypeptides (Fig. 3).

When the deleted wild-type enzyme was analyzed, the band of His tag Δα (44 kDa) observed in the presence of 2-mercaptoethanol was shifted to about 125 kDa in its absence (Fig. 3a). The bands of Δβ (52 kDa) and Δβ* (45 kDa) were also shifted to about 125 kDa. These results suggest that both the His tag Δα and Δβ polypeptides formed intermolecular S–S bonds, probably between His tag Δα and Δβ or Δβ*.

In the absence of DTT, both anti-α and anti-β polypeptide IgG reacted with the two shifted bands at ~200 kDa, supporting the idea that the α polypeptide formed an S–S bond with Δβ or Δβ* under nonreducing conditions, although the apparent molecular size of the shifted bands was again larger than the sum of the two polypeptides. These results support the idea that the shifted bands are heterodimers of His tags α and Δβ or Δβ* and

![Fig. 3. SDS-PAGE analysis of alkylated CT under reducing or nonreducing conditions.](http://www.jbc.org/)
suggest that the S–S bond is formed between Cys-267 in α and Cys-442 in Δβ or Δβ*.

To confirm the presence of the S–S bond in full-length wild-type CT, we analyzed the CT under nonreducing conditions (Fig. 3c). The shifted bands of about 230 kDa were observed in both α and β polypeptides, supporting the idea that the S–S bond was formed in full-length CT as expected. The shifted minor bands probably resulted from the β polypeptide, starting from the second and third methionine of the β polypeptide. The remaining α polypeptide was the polypeptide expressed in excess not associated with the β polypeptide (10).

If the disulfide bond is formed between Cys-267 in the Δα and Cys-442 in the Δβ or Δβ*, mutation of these cysteines breaks the disulfide bond and results in the loss of the shifted band under nonreducing conditions. We analyzed the mutants and found that the molecular size of the polypeptides is the same in the presence and absence of 2-mercaptoethanol. We lost the shifted heterodimer bands of His tags Δα and Δβ or Δβ* under nonreducing conditions (Fig. 3, d and e). The results indicate that each cysteine is necessary for an intermolecular S–S bond and that a critical disulfide bond is formed between Cys-267 in the α subunit and Cys-442 in the β subunit.

Occurrence of Disulfide Form of the Enzyme in Vivo

We have shown previously that ACCase from light-adapted plants is in a much more reduced form than that from dark-adapted plants and that light-dependent reduction of ACCase occurs in vivo (7). To ensure that the results observed in the recombinant enzyme (Fig. 3) were applied in vivo, we examined disulfide bridge formation in the enzyme by immunoblots of extracts from plants in light or dark (Fig. 4). The shifted bands of about 230 kDa were observed in both α and β polypeptides in the absence of 2-mercaptoethanol, suggesting that both the α and β polypeptides formed a S–S bond in vivo. The disulfide form of about 230 kDa was more abundant in the dark-adapted plants than in the light-adapted plants; the reverse was found for thiol forms of about 98 kDa for α polypeptide and 90 kDa for β polypeptide, suggesting that a light-dependent reduction of the disulfide bond indeed occurred in vivo. Probably the thiol-disulfide exchange observed in vivo is relevant in vivo, although we cannot completely exclude the possibility that a different set of cysteine residues is involved in the S–S bond in vivo.

**DISCUSSION**

The experiments reported here show that the two cysteines, Cys-267 in the Δα polypeptide and Cys-442 in the Δβ polypeptide, are required for redox regulation of the recombinant CT. The observation appears to be relevant in vivo, and the two cysteines are likely involved in the reductive activation of CT, and therefore in that of ACCase, by disulfide-dithiol interchange. In the light, electrons from photosystem I are shuttled through the electron transport chain to ferredoxin and are transferred to thioredoxins by ferredoxin-thioredoxin reductase and then to ACCase, reducing the disulfide bonds in the CT and resulting in the light activation of fatty acid synthesis.

All of the polypeptides encoded by the plastid genome form a functional complex with polypeptides encoded by the nuclear genome. For example, ribulose-bisphosphate carboxylase, RNA polymerase, Clp protease, and ATP synthase are composed of chloroplast-encoded and nuclear-encoded polypeptides (12). In these enzymes, the catalytic or structural polypeptides are encoded by the chloroplast genome, and the regulatory polypeptides are encoded by the nuclear genome. The same may be true for pea CT, although further experiments are needed to characterize the precise function of the two subunits. The chloroplast-encoded α polypeptide has catalytic and regulatory functions, and the nuclear-encoded α polypeptide has a regulatory function. To date, we do not know the molecular interaction between catalytic and regulatory polypeptides. A new finding obtained in our experiments indicates that molecular interaction of the two polypeptides is characterized as follows: the nuclear-encoded polypeptide forms a covalent bond with the chloroplast-encoded polypeptide and thereby changes enzyme activity.

There are several chloroplast enzymes activated by thioredoxin (13), including fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, phosphoribulokinase, ATP synthase, NADP-dependent malate dehydrogenase, glucose-6-phosphate dehydrogenase, and ribulose-bisphosphate-carboxylase-activase. The cysteine residues involved in redox regulation are identified by site-directed mutagenesis, and some consensus motifs among plants have been found for each enzyme. However, a consensus motif among enzymes has not yet been found. A comparison of the primary sequences of these enzymes with redox-insensitive cytosolic isoforms or with the redox-insensitive counterpart from different organisms reveals that the redox-sensitive cysteines are located on extra loops or extensions (14). Light-regulated NADP-dependent malate dehydrogenase possesses sequence extensions at both the N- and C-terminal ends compared with the constitutive active counterpart and two cysteines at the N terminus and two cysteines at the C terminus are involved in redox regulation (15, 16). Chloroplast fructose bisphosphatase possesses an insertion containing three cysteines compared with the constitutive active cytosolic enzyme and two of them are involved in redox regulation (17, 18). In contrast to these enzymes, different features are found for pea ACCase. First, the molecular structure of pea plastid ACCase differs considerably from redox-insensitive cytosolic ACCase (1, 2), and we cannot propose a possible origin of redox-sensitive cysteines, although there are some similarities in their CT domains. Second, the regulatory cysteine in the α polypeptide is not located in the plant-specific extra domains but in domains conserved among organisms, reflecting the differences from other chloroplast enzymes in terms of the evolutionary origin of redox sensitivity. Third, intermolecular disulfide bonds among different polypeptides are involved in redox regulation, whereas intramolecular disulfide bonds are involved in the other chloroplast enzymes.

Available sequence data show that the Cys-267 in the α polypeptide is conserved among organisms but the Cys-442 in the β polypeptide is found only in pea. Other organisms have a serine at this position. Replacement of Cys-442 by serine resulted in CT with very low activity (data not shown) suggesting that Cys-442 is important for pea CT. At present, we cannot propose a possible consensus sequence of CT for redox regula-

![Fig. 4. SDS-PAGE analysis of enzymes extracted from dark- or light-adapted plants.](http://www.jbc.org/)

**Fig. 4.** SDS-PAGE analysis of enzymes extracted from dark- or light-adapted plants. 8 (light) or 10 (dark) μg of protein from chloroplast extract was alkylated, separated on 5% SDS-PAGE, and probed with anti-CT α or anti-CT β IgGs as described in the legend for Fig. 3. D, extract from dark-adapted plants; L, extract from light-adapted plants.
tion. There is a possibility that this type of redox regulation is unique to the pea, although spinach (19) and tobacco ACCases\(^2\) are redox-regulated, and another set of cysteines may be responsible in other species. In the case of sedoheptulose bisphosphatase, the redox-sensitive cysteines of *Chlamydomonas reinhardtii* were predicted to be different from those of plants (20).

Replacement of Cys-247 or -267 in the \(\alpha\) polypeptide by serine resulted in mutants of low activity, less than 3% of the deleted wild-type (data not shown), whereas the corresponding replacements by alanine residues allowed the more active CT, about 30–80% of the wild-type (Table II). Serine residues at these positions are not compatible with the formation of a functional complex of CT activity, but alanine replacement of Cys-247 or Cys-267 satisfies the structural requirement at these sites for CT folding and stability. Similar results were obtained for replacement of the cysteine by serine in the \(\beta\) polypeptide. Serine is a hydrophilic amino acid, and alanine, like cysteine, is hydrophobic. It is likely that the hydrophobicity of cysteine plays an important role in the functional folding of CT. Such results were reported for rhodopsin (21) and small soluble proteins such as trypsin inhibitor (22) and lysozyme of CT. Such results were reported for rhodopsin (21) and small soluble proteins such as trypsin inhibitor (22) and lysozyme (23).

A conserved motif, \(\text{CX}_2\text{CX}_{12-15}\text{CX}_2\text{C}\), forms a putative zinc finger motif. The deletion of this domain suggests that this motif is required for catalytic activity, probably binding metal ions. A preliminary experiment to identify the metal ion by flame reaction showed the presence of Zn but not of Fe.\(^3\) Further experiments are needed in order to understand the precise roles of the \(\alpha\) and \(\beta\) subunits.

\(^2\) Y. Madoka, and Y. Sasaki, unpublished observation.

\(^3\) A. Kozaki, K. Mayumi, and Y. Sasaki, unpublished observation.

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Thiol-Disulfide Exchange between Nuclear-encoded and Chloroplast-encoded Subunits of Pea Acetyl-CoA Carboxylase

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