J. Biochem. 132, 291–300 (2002)

Molecular Cloning and Characterization of a Group II Chaperonin δ-Subunit from Soybean[1]

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Received May 24, 2002; accepted June 4, 2002

Molecular characterization of plant group II chaperonin (CCT, c-cpn, or TriC) still remains elusive. By PCR-based cloning techniques using soybeans, we have made a successful attempt to clone a δ-subunit homologue of CCT (CCTδ). This subunit is responsible for the binding of an *in vivo* substrate, α-actin, by assisting the correct folding of the cytoskeletal protein in mouse, and the occurrence of the subunit homologue in plant CCT was unclear. As the cloning strategy, a putative amino acid segment, NH_2-Gly-Gly-Gly-Ala-Pro-Glu-COOH, which is tightly conserved in all known animal and yeast CCTδ subunits, was chosen for designing a degenerate primer of the PCR-cloning. The resultant 1881-bp cDNA was found to have an open-reading frame of 533 amino acids with a calculated molecular mass of 57,677 Da and to share about 58–65% identity overall at the amino acid level with the corresponding subunits known to date. Using antibodies raised against *Escherichia coli*-produced soybean insoluble CCTδ as a monitoring tool, we purified soybean CCT from the extract of its immature seeds. STEM images demonstrated that the molecular shape of soybean CCT is a double eight-membered ring, which resembles the known group II chaperonins. The CCT also reactivated a denatured firefly luciferase with a significant, but limited level of the native enzymic activity in an *in vitro* system. Northern blot analysis showed that soybean CCTδ gene, which is intronless and composed of a small family, was only expressed at a very early stage of seed development of soybean.

Key words: a double eight-membered ring structure, group II chaperonin, Northern blot analysis, plant CCTδ subunit, protein folding activity.

Plant embryogenesis and seed development involve complex physiological stages in which numerous genes are expressed in a tissue-specific and developmentally-controlled manner. In soybeans, the rapid pace of cell division and cell expansion after embryo formation suggests the remarkable expression of genes encoding cytoskeletal proteins such as tubulin and actin during an early stage of seed development. It is likely that correct folding, assembly, transport, and localization of the newly synthesized proteins within the cellular compartments of the seed are well-organized and highly regulated events.

It is now clear that the proper folding of cellular proteins is facilitated by chaperonins, a particular class of chaperones and multimeric toroidal complexes, which direct the chemical energy derived from ATP hydrolysis into the promotion of the folding process. Chaperonins have been characterized from bacteria (chaperonin GroEL), mitochondria (60-kDa heat-shock protein, Hsp60) and chloroplasts (rubisco-binding protein, RBP) (*1, 2*). These three families of chaperonins have a similar molecular structure consisting of 14 homologous 60-kDa subunits that are arranged in two heptameric rings stacked on top of each other in inverted orientation and are classified as group (class or type) I chaperonins (*3, 4*). Later, another type of chaperonin was discovered in the cytosol of animals and yeast (*1, 3–6*). This group II chaperonin appears to have eight heterogeneous subunits of approximately 60 kDa that form a double eight-membered ring structure (*1, 3*). Based on this structure, the chaperonin has been called TCP-1 ring complex (TriC) (*1, 4*). Since distinct genes encoding these subunits have been characterized, this chaperonin family is also named chaperonin containing t-complex polypeptide 1 (CCT) (*3, 7, 8*).

Whereas group I chaperonins like GroEL are promiscuous, assisting in the folding of many other proteins, only a small number of proteins such as tubulin and actin have

[1] This work was supported by a grant from the Basic Research Core System of the Science and Technology Agency of Japan (to C.F.). The nucleotide sequences reported in this paper have been submitted to the DDBJ /EMBL/GenBank with the accession numbers AB 004233 and AB 003589.

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Abbreviations: c-cpn, cytosolic chaperonin; CCT, chaperonin containing t-complex polypeptide-1; CCTδ CCT δ-subunit; DAF, day(s) after flowering; GuHCl, guanidine chloride; IgG, Immunoglobulin G; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenyl methylsulfonyl fluoride; rCCTδ, recombinant CCTδ; STEM, scanning transmission electron microscope/microscopy.

been described as natural substrates of CCT (*9*).

To date, only three complete sets of eight heterogeneous subunits α, β, γ, δ, ε, ζ, η, and θ (also termed CCT-1 to CCT-8, respectively) have been identified in human, mouse and yeast (*10–12*). Individual CCT subunit sequences have also been deduced from DNA sequences of puffer fish, *Fugu rubripes* (*13*); fruitfly, *Drosophila melanogaster* (*14*); nematode, *Caenorhabditis elegans* (*15*); protozoan, *Tetrahymena pyriformis* (*16*); and a host of other organisms (*3, 4, 12*).

As the sequences of the eight CCT subunit types are most divergent from each other, the substrate-specificity may be due to the divergence of the subunits. Indeed, Llorca *et al.* (*8*) showed that α-actin binds to murine CCT using two specific and distinct interactions: the small domain of actin binds to CCTδ and the large domain to CCTβ or CCTε, indicating that the binding of α-actin to CCT is both subunit-specific and geometry-dependent. These results suggest that eukaryotic CCT has a different mechanism of substrate-recognition from prokaryotic GroEL.

Information on plant group II chaperonins is scarce (*2, 17*). A cDNA encoding *Arabidopsis thaliana* CCTα-homologue has been cloned by using mouse CCT cDNAs as a probe (*11*). The presence of the α- and ε-subunits of CCT was detected in soluble fractions of protein extracts from maize with antibodies raised against homologous subunits from yeast and oat (*18*). However, only two types of plant CCT subunits, CCTα and CCTε, have hitherto been cloned, sequenced and characterized (*19–21*). Therefore, the questions of how plant CCT genes are regulated, whether the δ-subunit homologue is involved in plant CCT, and which proteins are mediated by plant CCT during seed development have yet to be answered.

Against this background, we confirmed that the δ-subunit homologue is involved in plant CCT complex. In addition, we successfully purified soybean CCT complex from the immature seeds and assayed its refolding activity by using denatured luciferase.

MATERIALS AND METHODS

Materials—Soybean (*Glycine max*), var. Bonminori, in which seed maturation occurs about 60 days after flowering (DAF), was used in this experiment. Seeds were harvested from an early stage (*ca.* 13 DAF) to a late stage (*ca.* 58 DAF) of seed development at specified intervals. Stems (hypocotyls) were collected from seedlings grown during 2 to 8 d after imbibition and young leaves were collected from 20-day-old plant body. These materials were separately frozen in liquid nitrogen and stored at –80°C until use. For preparation of total DNA, the seedlings (5–8 days old) were used. [α-^{32}P]dCTP (3,000 Ci/mmol) and [α-^{32}P]dATP (5,000 Ci/mmol) were purchased from Amersham (UK). Taq-DNA polymerases for PCR were obtained from Takara (Kyoto). Restriction endonucleases and other enzymes for molecular cloning were obtained from Nippon Gene and Takara. Oligonucleotides were custom-synthesized and purified by Takara and Sawady Technology (Tokyo). All other chemicals used were purchased from Wako Chemicals (Osaka).

RNA Purification, cDNA Synthesis and Genomic DNA Preparation—Total RNAs were prepared from the plant materials by the phenol/SDS method except for the seeds collected at the late stages of development (*22*). As major seed storage protein glycinin is largely stored in seeds up to the late stages (48 and 58 DAF) of seed development (*22, 23*), total RNAs from the seeds collected as above were prepared by the method of Chirgwin (*24*). Poly (A)$^+$RNAs were isolated from the total RNAs by using oligo(dT)-magnetic beads (PolyATtract® mRNA Isolation System, Promega). Poly (A)$^+$RNAs derived from 18, 38, and 58 DAF were employed in the synthesis of the total cDNA with avian myeloblastosis virus reverse transcriptase (Takara) and a 33-mer oligo(dT)-containing *Bam*HI site [5′-GCGGATCC(dT)$_{25}$-3′].

Genomic DNA was extracted from the seedlings using the cetylmethylammonium bromide (CTAB) method of Rogers and Bendich (*25*).

PCR Cloning of Soybean CCTδ cDNA—Based on a conserved hexapeptide region of known animal and yeast CCT δ-subunits, Gly-Gly-Gly-Ala-Pro-Glu, a degenerate primer CHA-DP (sense); 5′-GGIGGIGGIGC(G/A/T/C)CC(G/A/T/C)-GA-3′ (I, deoxyinosine) was synthesized and used for PCR cloning of the 3′-end fragment of soybean CCTδ-like cDNA. PCR samples were subjected to the following amplification cycle: denaturation at 95°C (48 s), annealing at 56°C (48 s), extension at 72°C (1 min) and 32 cycles with the final extension at 72°C (8 min). After purifying the resulting PCR product electrophoretically, it was subcloned into pCR™ 2.1 vector (Invitrogen, The Netherlands) and sequenced. After confirming the sequence, 5′-nucleotide region of soybean CCTδ-like cDNA was obtained by the 5′-RACE technique (5′-RACE system, version 2.0, Life Technologies, USA), using a pair of nested primers: CHA-1 (antisense), 5′-GCATTTATCTCACCCTGTGCATGACGATTCC-3′; and CHA-2 (antisense), 5′-GCCAGAGTATAGGGAATAACTTCAAGCG-3′; Finally, a continuous cDNA sequence covering the entire translated region of the soybean CCTδ-like transcript was amplified by use of a pair of primers: CHA-3; 5′-ATGTCGGCAATCGCGGCTCCCC-3′ (sense); and CHA-4, 5′-CTACCTCACAGTTACAATATCATC-3′ (antisense). DNA sequencing was performed by the dideoxynucleotide method (*26*) using a DSQ-1000 automated DNA sequencer (Shimadzu, Kyoto) and a Thermo Sequenase Cycler Sequencing kit (Amersham, UK) according to the manufacturer's protocols. Computational analyses of DNA sequences were performed by the GENETYX programs (Software Development, Tokyo).

Screening of Soybean Genomic Library for CCTδ Gene—A soybean genomic library in the lambda FIX® II vector, constructed by Stratagene (USA), was screened for the CCTδ-encoding gene using the [α-^{32}P]dCTP labeled 1.6-kb cDNA fragment as probe (Takara DNA labeling kit). Nitrocellulose filters (Schleicher & Schuell, Germany) containing recombinant phage plaques were hybridized to the probe at 42°C overnight and then exposed to X-ray film (Konica, Tokyo) with an intensifying screen (DuPont NEN, USA). After purification, approximately 10 μg of phage DNA was digested by restriction enzymes and subjected to Southern blot analysis. A recombinant DNA fragment generated by *Xba*I-cleavage was subcloned into a pUC19 vector and sequenced.

Amplification of Soybean β-Tubulin and Actin cDNA Sequences—Based on the published genomic DNA sequences encoding soybean β-tubulin (*27*) and actin (*28*), two pairs of specific primers were designed and used for PCR amplification of the complete coding fragments of the corresponding

cDNAs. The PCR products were ligated to the pCR™2.1 vector, and positive clones were confirmed by sequencing.

Northern Hybridization—Samples of Poly(A)$^+$RNA (0.5 μg) were electrophoresed on 1% (w/v) agarose gel after denaturation with glyoxal and dimethyl sulfoxide and then blotted onto nylon membranes, Zeta-Probe (Bio-Rad, USA). The membranes were baked at 80°C under vacuum for 2 h, then hybridized separately with the ^{32}P-labeled probes of CCTδ, β-tubulin, and actin cDNAs. To assay the substantial amount of mRNAs contained in each mRNA preparation used, we measured Cerenkov's counts of ^{32}P-incorporated cDNAs synthesized as described previously (*29*). As a standard, we used 4.7×10^5 cpm of the synthesized cDNAs, as equivalent to 1 μg of mRNAs.

Genomic Southern Analysis—Aliquots containing 10 μg of genomic DNA were digested with appropriate restriction enzymes, fractionated on 0.8% (w/v) agarose gel, transferred onto a Zeta-Probe membrane using a Vacugene 2016 vacuum blotting unit (LKB, Sweden), baked in vacuum, and allowed to hybridize with the cDNA probe mentioned above. After hybridization, the membrane was washed (at 65°C in 0.1× SSC, 0.1% SDS) for 30 min and then exposed to an X-ray film at –80°C for 3–5 d.

Construction of a Bacterial Expression Plasmid and Purification of Recombinant CCTδ (rCCTδ)—A pair of oligonucleotide primers containing *Nde*I and *Bam*HI sites was used to generate a cDNA fragment covering the entire coding region (bases 8–1610). This fragment was then subcloned into pET-21a(+) vector (Novagen, USA). The *E. coli* strain BL21(DE3) harboring the expression plasmid pECTδ 922 was grown in LB medium supplemented with ampicillin (50 μg·ml^{-1}). When the absorbance of the culture at 600 nm reached 0.8, IPTG was added to the culture medium at a final concentration of 1 mM and the incubation continued for a further 12 h. The washed cells were suspended in an aliquot of 200 mM acetate (pH 5.5) containing 0.1 mg·ml^{-1} lysozyme, 1 mM EDTA, and 0.1 M NaCl, then disrupted in an ultrasonicator (Nihonseiki Seisakusho) as previously (*29*). The overexpressed 58-kDa protein was located in the cell debris fraction. After washing six times with 0.1 M sodium phosphate buffer (pH 7.0), the resultant cell debris was suspended in a SDS sample buffer and boiled for 10 m. After centrifugation, the supernatant was loaded on a semi-preparative SDS-gel. The target protein band of 58 kDa was separated on the gel, excised after partial CBB staining, then electroeluted using a dialyzing tube in 0.1 M sodium phosphate (pH 7.0) at 30 mA for 2 h in a cold room. To confirm whether the purified 58-kDa protein is the translated product of the expression plasmid pECTδ922, NH_2-terminal sequencing was performed by using an ABI protein sequencer (model 477A) coupled with an ABI phenylthiohydantoin amino acid analyzer (model 120A).

Preparation and Purification of Anti-rCCTδ Immunoglobulin G—Before the immunization schedule, the homogeneity of the obtained rCCTδ was confirmed by SDS-PAGE analysis and determination of the NH_2-terminal sequence, which was NH_2-M-S-A-I-A-A-P-Q-H-R-S-S-K. Polyclonal antibodies against the purified rCCTδ were raised in random-bred female albino rabbits by injecting about 200 μg of the recombinant protein in 1 ml of Freund's complete adjuvant (Yatron, Tokyo) hypodermically, according to the immunization schedule described previously (*29*). The resultant antisera were judged to be monospecific to soybean CCTδ using boiled sample-buffer extract of *E. coli* cells harboring pECTδ922.The antisera showing a band corresponding to CCTδ and reactivity at a dilution in the range of 1:1,000 to 1:1,500 on Western blot analyses (Bio-Rad HRP Immunoblotting kit, USA) were combined, and the immunoglobulin G (IgG) fraction was separated according to the procedure of protein A-adsorbent (*30*).

Purification of Intact Soybean CCT Complex—About 300 g of immature soybean seeds (18 DAF) were ground in liquid nitrogen, then homogenized in 0.6 liter of ice-cold extraction buffer (20 mM Tris-HCl, pH 7.2, 10 mM KCl, 20 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 0.5 mM 1,10-phenanthroline, 0.3 μM aprotinin, 1 μM leupeptin, and 2 μM pepstatin A). All further purification steps were carried out at 4°C. The homogenate was centrifuged at 40,000 rpm for 1 h in a Hitachi 55P-72 ultracentrifuge with RPS40T rotor (Hitachi, Tokyo). The resultant supernatant (total cell extract) was dialyzed overnight against a solution containing 40% (w/v) ammonium sulphate. After centrifugation at 5,000 rpm for 10 m, the precipitate was resuspended in 2 ml of the above extraction buffer and dialyzed against the extraction buffer overnight. The CCT complex was separated by 10–40% sucrose gradient centrifugation in the extraction buffer at 28,000 rpm for 18 h. The fractions containing CCT complex, as assessed by Western blotting using anti-rCCTδ IgG, were pooled, dialyzed against extraction buffer containing 5% glycerol, and then applied onto an ATP-agarose (5 ml) column (Sigma, USA). After passing three times and then washing the column with the extraction buffer exhaustively to remove unbound proteins, proteins adsorbed to the column were eluted by the extraction buffer supplemented with 10 mM ATP (Oriental Yeast, Tokyo). The elutants were collected, concentrated by Centricut (Krabou, Osaka; 50-kDa cut-off), snap-frozen in liquid nitrogen and stored at –80°C until use. During the concentration step, the combined eluate was diluted several times with the extraction buffer in order to remove free ATP from the CCT-containing solution. The CCT preparation obtained was analyzed by both native and SDS-PAGE using Daiichi multigel 2/15 and 10/20, respectively (Daiichi, Tokyo), and the occurrence of δ-subunit of the CCT was confirmed by Western blotting. For the preparation of the total extract, immature seeds (about 30 g per stage) specified at 13, 18, 28, 38, 48, and 58 days after flowering (DAF), were collected and used. Stems (hypocotyls) were collected from seedlings grown in the dark at specified intervals ranging from 2 to 8 d after imbibition. About 300g of each seedling was used for the preparation of CCT complex. In the case of leaves, about 300g of leaves of 20-day-old plants were employed. All the procedures used for the isolation of CCT complex from seedlings and juvenile plants were the same as in the case of developing seeds, except that the extraction volume was reduced to 1/2 of that used for immature seeds. Protein was assayed by the BCA method (*31*) using a Protein Assay kit (Sigma) and crystalline bovine albumin (Seikagaku Kogyo, Tokyo) as standard. Native PAGE and SDS-PAGE were performed as described previously (*32*).

Scanning Transmission Electron Microscopy (STEM)—The purified CCT complex (about 60 μg) dissolved in the buffer mentioned above was adsorbed onto glow discharged–activated carbon-coated grids and negatively stained with 2% uranyl acetate. Electron micrographs were recorded at 100 kV using a STEM (H 7500, Hitachi).

Luciferase Reactivation Assay—Firefly (*Phocinus pyralis*) luciferase, which was produced in *E. coli* and purified to homogeneity, was obtained from Wako Chemicals (Osaka). The enzyme was treated with a denaturing buffer (pH 7.2) containing 6 M GuHCl, 2 mM DTT, and 30 mM Tris-HCl, at 25°C for 2 h, then diluted 100-fold by the addition of a refolding buffer (pH 7.2) containing 50 mM KCl, 30 mM HEPES, and 2 mM DTT. As standard refolding mixture, 10 μl of the above dilutant, containing 0.5 ng of denatured luciferase, was adjusted to a volume of 50 μl by addition of the refolding buffer in the presence or in the absence of factors such as 10 ng of CCT complex, 5 mM $MgCl_2$, 2 mM ATP, and 1.0 mg/ml BSA. To examine the effects of factors on renaturation of the denatured luciferase, the above refolding mixture was incubated with individual factor or with mixed factors at 25°C for 1 h, and then 10 μl of the resultant solution was added to 50 μl of luciferase activity assay solution provided by the manufacturer (PicaGene®, Toyo-Ink, Tokyo). The activity was determined by use of a Packard 1900 TR liquid scintillation counter. Refolding yield was given as the percentage of specific luciferase activity relative to a sample of native firefly luciferase assayed at 25°C.

To clarify whether the activity obtained after refolding of denatured luciferase in the presence of soybean CCT was actually due to the action of the CCT, an inhibition test was performed employing anti-rCCTδ IgG. For this experiment, purified CCT preparation (100 ng) was mixed with anti-rCCTδ IgG (62 μg) dissolved in 40 μl of the refolding buffer, incubated for 30 m at 25°C, then centrifuged at 20,000 rpm for 30 m. The denatured luciferase (0.5 ng) dissolved in 10 μl of the same buffer was then added to the resultant supernantant. To assay the refolding activity of the antibody-treated CCT complex, the resulting mixture was incubated at 25°C for 1 h. Luciferase activity was assayed by the methed described above. In negative control experiments, anti-rCCTδ IgG was replaced by IgG (62 μg) from a non-immunized rabbit.

To investigate whether group I chaperonin also refolds firefly luciferase, we chose GroE as a model. Purified recombinant GroEL and GroES were purchased from Takara. All the procedures for the refolding and assay of remaining activity were done as described in case of CCT complex.

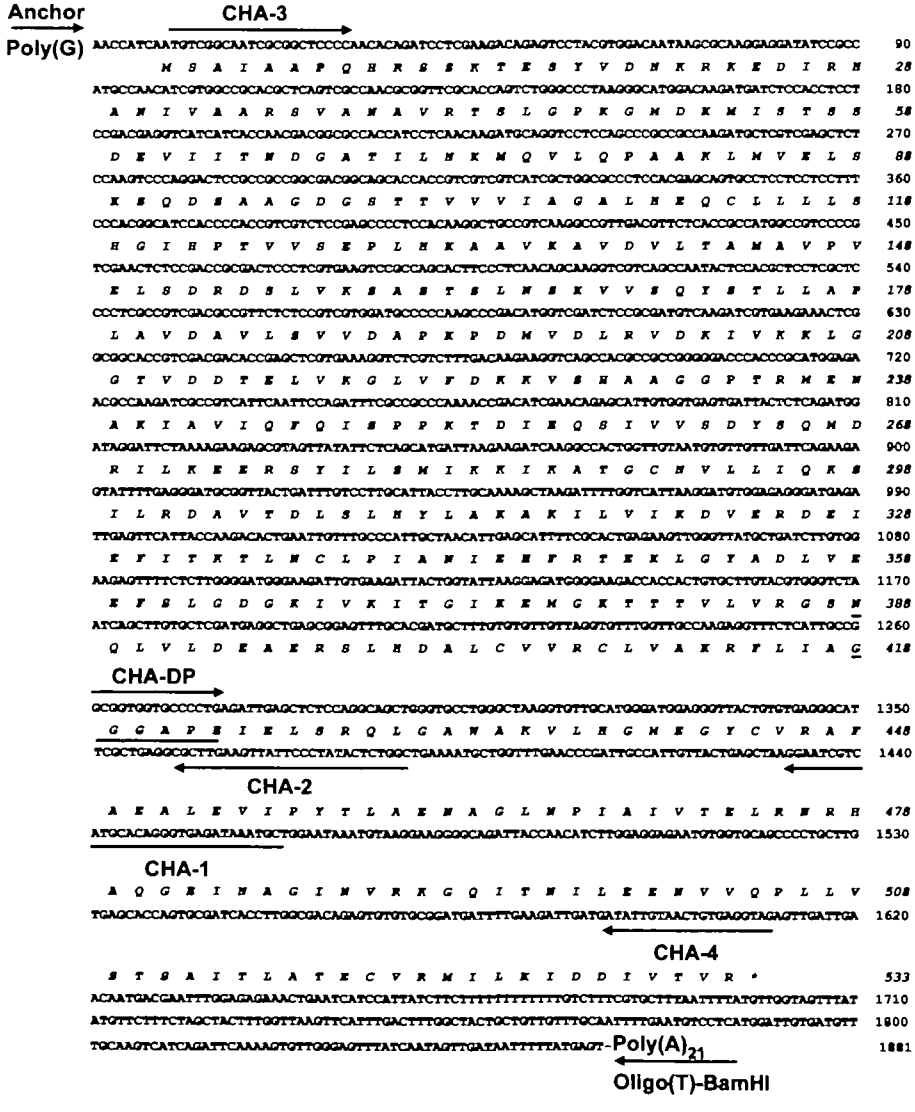

Fig. 1. **Nucleotide sequence of a CCTδ-subunit ds-cDNA insert of pCCTδ 718.** The complete nucleotide sequence for the coding strand of the cloned ds-cDNA is shown with the predicted amino acid sequence for the primary translation product of a CCTδ-subunit mRNA. The numbers on the right side indicate the nucleotide and the amino acid positions, respectively. The residues upon which the degenerate primer CHA-DP was based are underlined. The primer positions with the corresponding names are shown by the arrows above (sense) or below (antisense) the nucleotide sequence.

RESULTS AND DISCUSSION

Cloning and Characterization of Soybean CCT δ-Subunit cDNA—Because we could not speculate on to determine the optimal seed-developmental stage at which a high level expression of mRNA species encoding the CCT δ-subunit–like protein occurred, three separated total cDNA pools were prepared by using mRNAs derived from 18 DAF, 38 DAF, and 58 DAF seeds. A trial-and-error experiment, in which the annealing temperature varied between 46°C and 70°C employing a pair of 16-fold degenerate primers (CHA-DP) and 5′-oligo-(dT)–containing *Bam*HI site, was carried out through PCR amplification of above cDNA pools. The 18 DAF cDNA pool produced one faint band of about 0.6-kb, but the other two pools did not. Upon sequencing the above 0.6 kb insert was shown to extend as far as the poly(A) segment, and contained an open-reading frame encoding a sequence having significant homology with other CCT δ-subunit family members. Alignment of the amino acid residues with maximum homology revealed that 56–62% of the amino acid positions of soybean homologue were identical to other CCT δ-subunit proteins. This significant homology strongly suggested that the clone obtained was a CCT δ-homologue. To get a clone covering the 5′-terminal nucleotide sequence of the CCTδ cDNA by PCR technique, both CHA-2 and the 5′-RACE "abridged anchor primer" were employed. The resultant PCR product (about 1.4 kb long) was found to contain a coding frame corresponding to the nucleotide positions 1 to 1387, and an overlap of some 100 nucleotides compared to that of 0.6 kb PCR fragment with no mismatches. As the final step, the primer pair CHA-3 and CHA-4, based upon the nucleotide sequences obtained from the 0.6 kb and 1.4 kb PCR products, was used to isolate the continuous DNA fragment covering the entire translated region of soybean CCT δ-subunit cDNA. The sequence of this clone, named pCCTδ718, completely matched the corresponding regions of the above-mentioned intermediary cDNA fragments. The overall 1,881-bp nucleotide sequence of soybean CCTδ-subunit cDNA is shown in Fig. 1. The single open-reading frame encodes a polypeptide of 533 amino acids with a calculated molecular mass of 57,677 Da. At the 5′-terminus, a nontranslated region of 8 nucleotides was found preceding the AUG translation start codon, but the possibility that the soybean CCT δ-subunit mRNA may contain additional noncoding nucleotides at the extreme 5′-end was not further investigated. On the other hand, following the UAG translation stop codon is a 3′-terminal nontranslated region of 250 nucleotides, adjacent to a poly (A)-tail. When the deduced amino acid sequence was compared with those of known CCTδ subunit cDNAs, its overall identity was 65% (human), 64% (*Fugu* fish), 62% (mouse), 59% (nematode), and 58% (yeast), respectively. Although sequence differences are scattered throughout the CCTδ subunit molecules, several consensus segments with at least six consecutive identical amino acid residues, one of which was used for the construction of CHA-DP degenerate primer as shown in Fig. 1, were observed in every δ-subunit homologue (data not shown). The hydropathic analysis showed that the hydrophobic segment located in the NH_2 terminal part of the putative CCTδ-subunit protein was extremely short (equivalent to only 4–5 amino acid residues including the initial methionine), indicating that this protein family has no signal peptide, so that it may be synthesized on membrane-free polysomes.

Organization of Soybean CCTδ Gene—Southern blot analysis revealed that at least two major DNA fragments were hybridized with the cDNA probe in each restriction digestion (Fig. 2A), suggesting the existence of a small CCTδ family gene in the soybean genome.

From a positive recombinant phage, a 4.2-kb *Xba*I-fragment covering the entire soybean CCTδ-subunit gene was subcloned, and the resulting plasmid pCHA-310 was completely sequenced. As represented schematically in Fig. 2B, a soybean CCTδ subunit gene including a 2,019-bp 5′- and a 584-bp 3′-flanking region coupled with a 1,602 bp coding region, was found in the genomic fragment.

The nucleotide sequence of the cDNA and that of the corresponding region in the genomic clone were completely identical. This implies that the soybean CCTδ-subunit mRNA species cloned here is the transcript of this gene. The results also showed that this gene involves no intron, like the corresponding gene from yeast (*10*) but unlike that

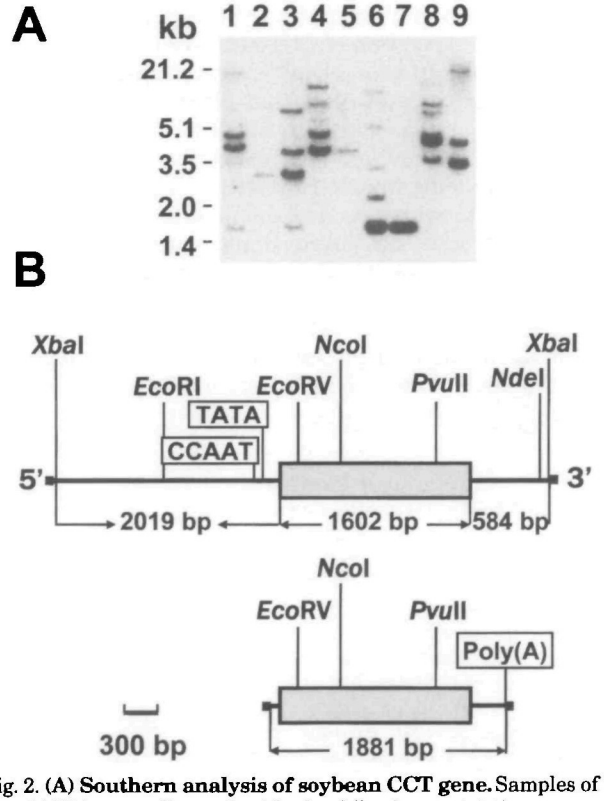

Fig. 2. **(A) Southern analysis of soybean CCT gene.** Samples of 10 μg of DNA were digested with the following restriction enzymes: *Eco*RI (lane 1), *Xba*I + *Eco*RI (lane 3), *Xba*I (lane 4), *Xba*I + *Nco*I (lane 6), *Nco*I (lane 8) and *Pvu*II (lane 9). The positive controls were purified fragments from the CCTδ cDNA insert of pCCTδ718: *Xba*I + *Eco*RI-fragment (lane 2), *Xba*I-fragment (lane 5), and *Xba*I + *Nco*I-fragment (lane 7). The size markers indicated on the left. The DNA fragments were separated on a 1% (w/v) agarose gel, blotted onto a nitrocellulose membrane and hybridized with the ^{32}P-labeled CCTδ cDNA as probe. **(B) Schematic representation of soybean CCTδ gene and its cDNA with selected restriction sites.** The filled boxes correspond to the coding region, while the bold lines show the 5′- and the 3′-untranslated regions. The numbers indicate the sizes of DNA fragments in bp. Putative CAT- and TATA-boxes of the gene are shown by the solid boxes. Poly(A)-tail is also shown by a solid box.

from *Fugu* fish, which is interrupted by 12 introns (*13*). To date, we have only been able to compare the transcribed region of the above three CCTδ genes with individual cDNA sequences which have relatively high homology (around 60%) with one another. When compared to known CCT genes encoding other subunits such as CCTα (4 introns) of nematode (*15*), CCTη (4 introns) of protozoan (*16*), CCTα (5 introns) of mouse (*33, 34*), the number of introns involved in a CCT gene is quite flexible and may vary with both source and kind of subunit.

Expression Patterns of the CCTδ Transcript and Its Translation Product During Soybean Seed Development—As shown in Fig. 3A, it seems that the expression pattern of mRNA encoding CCTδ subunit in immature seeds during seed development of soybean is deeply rooted in a manner regulated spatially and temporally. The highest level of the gene expression was found at a very early stage of development (13–18 DAF). Thereafter, it gradually decreased and almost disappeared at an early middle stage (28 DAF). It is of importance to note that the expression pattern coincided well with those of β-tubulin and actin mRNAs through seed development. Leaves and stems (hypocotyls) derived from young soybean plant also showed a high level of expression of CCTδ transcript together with those of the above cytoskeletal protein transcripts. These results support the possibility that plant CCT complex, in which δ-subunit homologue is involved, also assists the folding of tubulin and actin as well as the cases of leaves and stems of young plants. However, it is known that the level of mRNA expression and the quantity of its translation product are not always correlatable. Therefore, changes in the level of CCTδ subunit during seed development were examined by use of antibodies against rCCTδ. In Fig. 3B, Western blot analysis using anti-rCCTδ IgG showed up a single band corresponding to an apparent molecular mass of about 58 kDa on an SDS-gel when total cell extracts (about 8 μg protein/slot) prepared from immature seeds at early stages of seed development were used. Extracts from young stems and leaves showed the same pattern, but no band was observed when extracts from middle (38 DAF) and late (48 and 58 DAF) stages of development were used. Thus, the present data showed that CCTδ production closely correlates with the expression of the corresponding mRNA species (Fig. 3), suggesting that the expressed mRNAs encoding CCTδ may be immediately used for the protein synthesis on membrane-free polysomes, and that the half-life of δ-subunit molecules synthesized is relatively short. In mouse, on the other hand, it was demonstrated that the expression levels of the eight different subunits are tightly co-regulated to maintain a constant ratio of these subunits, which constitute the murine CCT hexadecamer complex with a fixed subunit arrangement (*35*). It is known that tubulin and actin, the major constituents of cytoskeletal microtubules and microfilaments in eukaryotic cells, are involved in cell division or cell expansion (*37*). It has also been demonstrated that CCT of animals and yeast assists the correct folding of tubulin and actin both *in vivo* and *in vitro* (*3–6*). Assuming that the expression of mRNAs encoding tubulin and actin also correlates with the production of the corresponding proteins in the early and early middle stages of seed development, it is likely that the coordinated response of CCTδ is largely exploited to assist the correct folding of these cytoskeletal proteins in the early stages of seed development. Previous experiments demonstrated that mRNA-expression of glycinin, a major seed storage protein of soybean, becomes predominant in cotyledonary tissue of developing seeds at around 28 DAF and is followed by correlative accumulation of the protein in protein bodies of the tissue (*23, 29*). Based upon the foregoing, it is possible that tubulin and actin are synthesized with the correct folding by the aid of CCT complex and subsequently polymerized

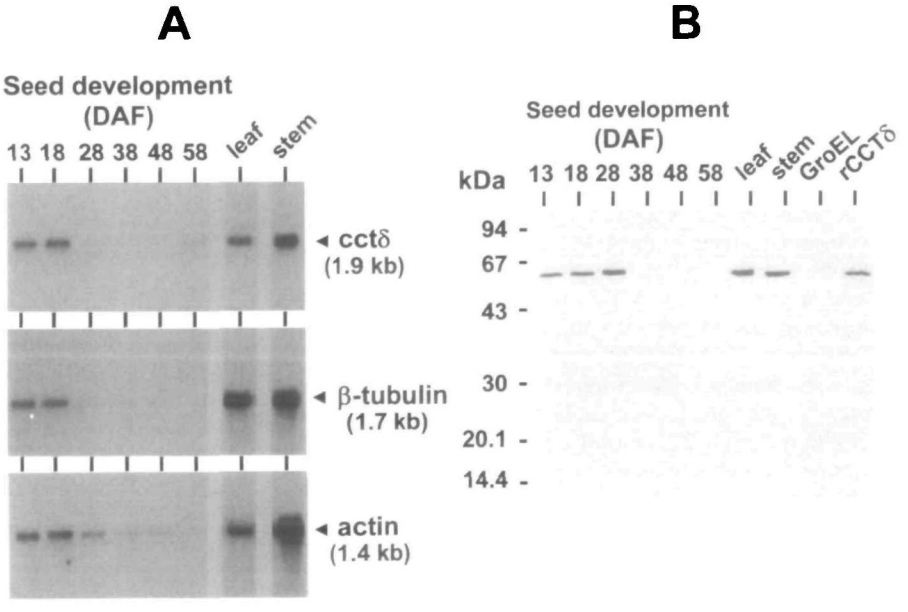

Fig. 3. **(A) Comparative Northern analysis of CCTδ, β-tubulin and actin.** Samples (0.5 μg) of poly(A)$^+$ RNAs from developing seeds, harvested at six different stages, 13, 18, 28, 38, 48, and 58 days after flowering (DAF), as well as from young leaves and stems. The mRNA-blotted membrane was used repeatedly for hybridizing with the CCTδ, β-tubulin or actin cDNAs, after removing previous signals. **(B) An immunodetection of CCT complex involved in "total extract" fraction obtained from immature seeds during seed development.** Total extracts, each of which contains 8 μg protein in the slot, were prepared from developing seeds at specified intervals as expressed as DAF. As a positive control, rCCTδ (20 ng protein) was employed and for a negative control, rGroEL (20 ng) was loaded. Samples from leaves and stems were loaded with threefold protein amounts on each slot and run. After blotting onto the membrane, anti-rCCTδ IgG (124 μg protein) was reacted with the membrane. The left lane shows the positions of molecular markers (Pharmacia Biotech AB), which were excised as a strip after blotting and then visualized by CBB stain separately.

in the proper manner inside immature storage cells, preceding a developmental stage when glycinin production is maximum. Thus, soybean cotyledonary cells are able to accumulate an enormous amount of the ergastic substance, causing the extreme cell-expansion during seed development.

Purification and Characterization of Soybean CCT Complex from the Immature Seeds—To characterize the CCT complex further, we attempted to isolate it from immature seeds, stem and young leaves of soybean. In order to detect the fraction where the complex containing δ-subunit was concentrated, Western blot analysis was done, together with SDS-PAGE visualized by CBB-stain. However, when the stems and leaves were employed, protein amounts of the chaperonin fraction just after eluting the absorbed materials from an ATP-column were only about 1/4 of the amount from immature seed (data not shown). Especially in case of leaf tissue, some of the STEM images demonstrated contamination by a group I chaperonin–like complex consisting of typical two heptameric rings, supposing that the chaperonin fraction from leaves was composed of CCT complex and a group I chaperonin, probably rubisco-binding protein from chloroplasts (data not shown). Therefore, we focused on the purification of soybean CCT complex from immature seeds. As shown in Fig. 4A, the chaperonin-containing fractions from the initial three purification steps gave the predominant single band similar to the position of rCCTδ-migration on a SDS-gel visualized by Western blot analysis, whereas there was no clear band equivalent to the position of rCCTδ by CBB-stain. Nevertheless, the final affinity purification step showed the predominant single band with an apparent molecular mass of about 980,000 Da, visualized by CBB on a native electrophoretic gel with a linear gradient of acrylamide ranging from 2 to 15% (Fig. 4B, lane CBB). To confirm whether this single band of nearly 1,000 kDa on the native gel corresponds to the δ-subunit-containing CCT complex, Western blot analysis was done. The CBB-stained protein band matched the band visualized by anti-rCCTδ IgG. To get further information concerning protein constituent of CCT complex, SDS-PAGE analysis was done under denaturing conditions. As demonstrated in Fig. 4C, several closely migrating polypeptides with apparent molecular masses of 58-67,000 Da emerged from the CCT complex. These results suggested that soybean CCT complex is composed of heterogeneous subunits. Moreover, Western blot analysis showed that the above CCT complex contains δ-subunit homologue (Fig. 4C, lanes rCCTδ and WB). To observe the shape of soybean CCT, STEM studies were done. As shown in Fig. 5, the CCT complex in almost all cases showed a double octameric ring structure, resembling the known group II chaperonins. This observation provides strong support for the conclusion that the macromolecular material purified here is a member of the chaperonin family, which can be judged as group II. This is the first reported STEM-image of plant CCT. At the final step of the purification process, the yield of CCT complex from immature soybean seeds harvested at 18 DAF was about 87 μg per 300 g seeds (wet weight). Due to the low yield of the CCT complex from soybeans, further analyses such as the isolation and characterization of some CCT-forming subunits were not done. To date, subunit homologues of plant CCT complex have been detected immunologically from various vegetative tissues of maize (*18*), oat (*20*), and cucumber (*21*). However, purification and functional analyses of these CCT complexes have not yet been reported.

Soybean CCT Complex Mediates the Refolding of Denatured Firefly Luciferase—In view of the limited yield of the

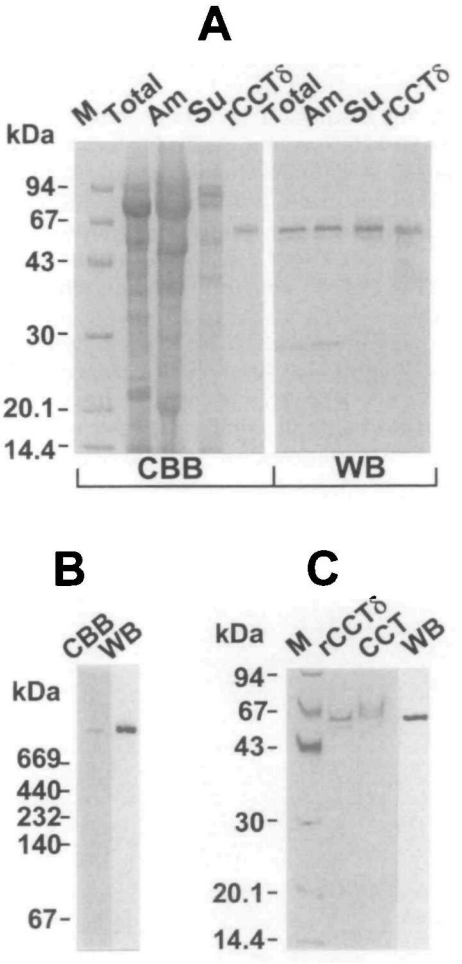

Fig. 4. **Purification of soybean CCT complex and the existence of CCTd subunit.** (A) Polyacrylamide gel electrophoretic analysis showing protein components at the initial three steps of the purification process. CBB, protein bands visualized by Coomassie Brilliant Blue; WB, protein bands revealed by anti-rCCTδ IgG; lane M, molecular marker proteins (Pharmacia Biotech AB). The left lane shows the position on the gel and the corresponding molecular mass of individual marker; lane Total, total protein extract from 18-DAF seeds; lane Am, a protein fraction obtained by 40% (mass/vol) saturated ammonium sulphate precipitation; lane Su, a CCT-enriched fraction obtained by a 10–40% (w/v) sucrose gradient centrifugation; lane rCCTδ, rCCTδ as a positive control. (B) Native PAGE pattern of the eluate from ATP-agarose affinity column. CBB, protein band revealed by Coomassie Brilliant Blue; WB, protein band visualized by anti-rCCTδ IgG. The left lane shows the positions of high molecular markers (Pharmacia Biotech AB). (C) SDS-PAGE pattern of the eluate from the ATP-agarose column. M, molecular markers (Pharmacia Biotech AB). The migrating positions of individual proteins are shown as the apparent molecular masses in the left lane. rCCTδ, rCCTδ as a positive control; CCT, subunits of CCT complex revealed by CBB stain; WB, δ subunit which was separated from CCT complex and visualized by anti-rCCTδ IgG. The gel-mobility of the δ subunit revealed by the immunostain was measured by the positions of CBB-stained markers, which were run and blotted together, followed by the excision of the lane from the same membrane used in WB

CCT from soybeans and the restricted substrate range of known CCTs from animals and yeast, how might we assay the refolding ability of the obtained CCT? When radiolabeled tublin and actin, which are thought to be native and preferential substrates of group II chaperonin, were used, binding of denatured cytoskeletal proteins to CCT was only analyzed by autoradiography on native PAGE (*36*). Taking account of high sensitivity, quantitativeness and rapidity, a chemiluminescence-based luciferase assay system was tested because it has been shown that denatured luciferase could regain the activity up to 60% of its initial level by the action of bovine testis CCT (*37*). Thus, refolding yield of the monomeric 60-kDa enzyme firefly luciferase facilitated by the soybean CCT complex was examined. At extreme dilutions of the denaturant and denatured luciferase in the refolding buffer, luciferase did not regain any activity (data not shown), as was also the case upon separate addition of Mg^{2+} and ATP/ Mg^{2+} to the dilute enzyme solution (Fig. 6A). However, the addition of BSA at the concentration of 1.0 mg/ml had very limited effect on the refolding yield. These results may suggest that firefly luciferase was strongly susceptible to irreversible change of the native conformation during denaturation and refolding. In the refolding experiments using CCT complex, we usually added the chaperonin in 12-fold molar excess over firefly luciferase in the refolding mixture presence or absence of factors. In calculation of the molar ratio of CCT complex to luciferase, we used 980 kDa as the apparent molecular mass of CCT complex based upon the molecular shape of two octameric subunit rings of around 60 kDa. Through refolding experiments, the CCT complex reactivated denatured luciferase up to 12.1% of its initial activity in the presence of ATP and Mg^{2+}. Limited but significant refolding yield (~2.1%) was observed upon incubation in the presence of Mg^{2+}, but in the absence of ATP [Fig. 6A (d)]. This may be due to the presence of ATP molecules that bound specifically on CCT complex during the purification process. ATP-dependent refolding of luciferase was only observed in the refolding buffer containing Mg^{2+} and CCT complex. Even in the presence of CCT complex and ATP, no reactivation was observed without the addition of Mg^{2+} (Fig. 6A, f). This may reflect the fact that both ATP and Mg^{2+} are required for releasing refolded luciferase from the cavity of CCT complex (*37*).To confirm whether reactivation of luciferase was due to the action of CCT complex containing the δ-subunit, the inhibition of CCT activity was examined by using anti-rCCTδ IgG. A remarkable repression of CCT activity (about 90% of the initial activity eliminated.) was observed in the

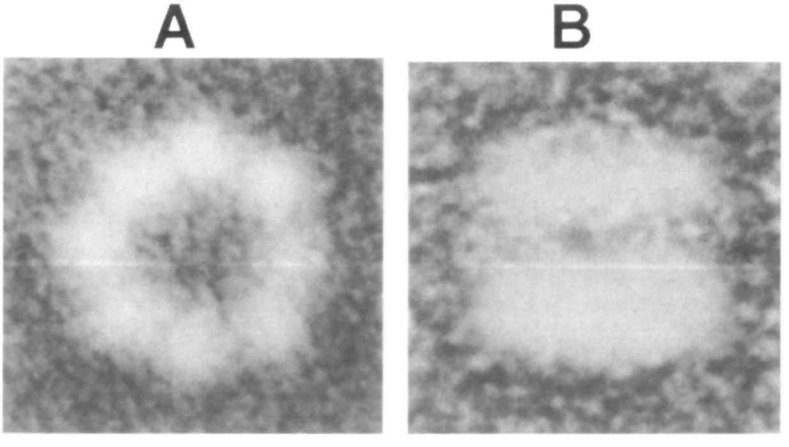

Fig. 5. **Molecular shape observation of soybean CCT complex purified from ATP-agarose column by STEM.** A, top view; B, side view; Average image of top (A) and side (B) views of soybean CCT provides putative CCT-conformation consisting of the eight-membered ring and the double-torus structure.

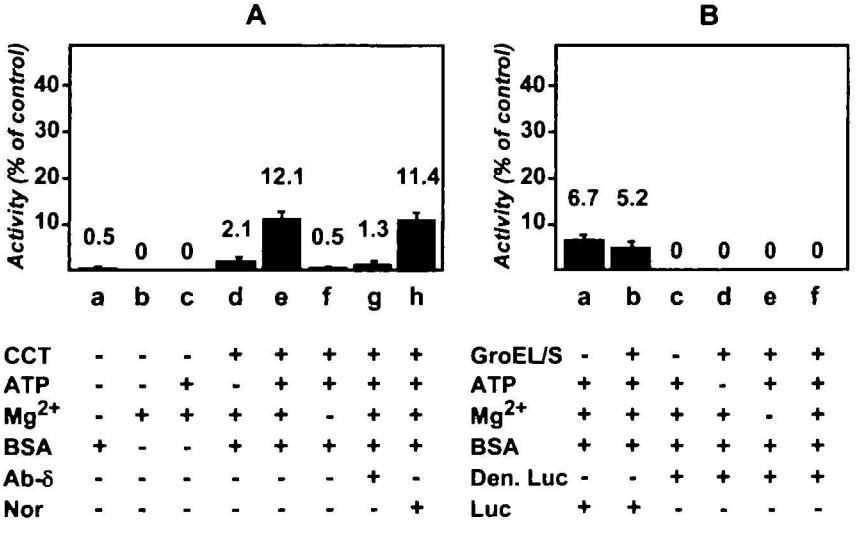

Fig. 6. **Refolding yield of denatured firefly luciferase in the presence of various factors, such as ATP, Mg^{2+}, BSA, and chaperonin.** A, soybean CCT used for refolding assay. (a) effect of BSA on a spontaneous refolding; (b) Mg^{2+}-effect in the absence of CCT; (c) ATP-Mg^{2+}-effect in the absence of CCT; (d) ATP-dependency in the presence of CCT; (e) complete refolding mixture; (f) Mg^{2+}-requirement; (g) inhibition test by anti-rCCTδ IgG; (h) a negative control experiment. B, GroEL/ES used for refolding assay. (a) effect of the incubation on native luciferase stability in the absence of GroEL/ES; (b) effect of the incubation on native luciferase stability in the presence of GroEL/ES; (c) refolding effect on denatured luciferase in the absence of GroEL/ES; (d) ATP-dependent refolding activity; (e) Mg^{2+} requirement; (f) refolding effect of GroEL/ES on denatured luciferase in the presence of ATP, Mg^{2+} and BSA.

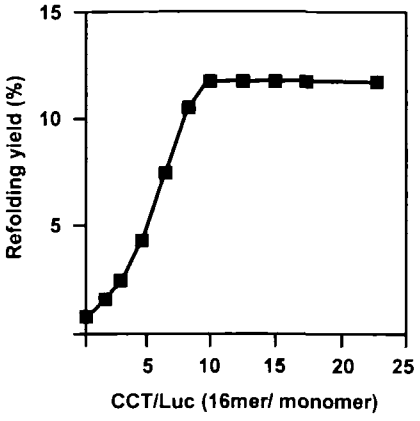

Fig. 7. **Dependency of the refolding yield of luciferase on the concentration of CCT complex from soybean.** The values represent the refolding yield after a 1-h refolding incubation. The *abscissa* indicates the molar ratio of CCT complex to the luciferase monomer in the refolding mixture.

presence of BSA, ATP, and Mg^{2+} (Fig. 6A, g), whereas the negative control experiment showed that less than 6% of the initial luciferase activity was only inhibited nonspecifically by a non-immunized serum-derived IgG as above (Fig. 6A, h).These results strongly suggest that refolding yield of luciferase is due to the chaperonin action of CCT complex containing the δ-subunit.

To investigate whether group I chaperonin may also assist the refolding of luciferase, GroE (14-mer GroEL/7-mer GroES), which has a wide substrate spectrum, was employed for the refolding experiment under the condition of a 12-fold molar excess of GroE complex relative to the monomer concentration of luciferase. As shown in Fig. 6B, GroE complex did not assist the refolding of luciferase at all under conditions that allow significant refolding yield by the action of the CCT complex. Even though native luciferase was incubated in the refolding buffer containing ATP, Mg^{2+}, BSA and GroE for 1 h at 25°C, the enzyme drastically reduced the chemiluminescence activity up to around 6% of its initial level (Fig. 6B, b). This may suggest that GroE could not trap firefly luciferase into the inner space, due to the rather high molecular mass of the protein used as substrate (60 kDa), and the native luciferase may be inactivated during the incubation. From the evidence, it is possible to speculate that a chloroplast-derived group I chaperonin such as rubisco-binding protein, by which rubisco large subunit (about 53 kDa) is folded correctly, may also not assist the renaturation of firefly luciferase.

To examine whether the refolding yield of luciferase depends upon the concentration of CCT complex in the refolding mixture, refolding experiments in the presence of various concentrations of CCT complex were done. As shown in Fig. 7, the increase in the refolding yield gave a saturable tendency. When the concentration of CCT complex was increased, the refolding yield of firefly luciferase also increased rapidly, and then reached plateau at 12.1% of its initial level, when CCT-complex concentrations became greater than around 10-fold molar excess over the luciferase monomer. Even in the presence of a 23-fold excess of CCT complex, the refolding yield of the luciferase was not improved, but inhibitory effects on the level were also not observed. These observations may strongly indicate that the refolding conditions used here were suitable. Gebauer *et al.* (*38*) reported that the refolding yield of firefly luciferase by CCT complex purified from rabbit reticulocytes was about 30% of the initial level under conditions used. Recently, it has been shown that group II chaperonins require several cofactors (*4, 8*), and that CCTs cooperate with a cochaperone called GimC (*G*enes *i*nvolved in *m*icrotubule biogenesis *C*omplex) or prefoldin (*7, 39*). In addition, particular domains of unfolded substrate were shown to bind specifically to some of the eight differing subunits of CCT complex, where δ-subunit appeared to play an important role in the binding process (*40–43*).

In conclusion, identification of the heterogeneous subunits involved in the CCT complex of soybean, and cloning and characterization of the corresponding genes demand elaborate investigations, aimed at the elucidation of mechanisms involved in the protein folding events in a plant system.

We gratefully acknowledge H. Yoshida and Y. Itoh from Hitachi Scientific Instrument Center (Nissei Sangyo Co., Ltd.) for performing a scanning transmission electron microscopical observation of soybean CCT complex. C.F. is also indebted to Michiyo Yokoto for preparation of the manuscript.

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