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Evolutionary conserved light regulation of Calvin cycle activity by NADPH-mediated reversible phosphoribulokinase/CP12/glyceraldehyde-3-phosphate dehydrogenase complex dissociation

(photosynthesis/bidirectional dehydrogenase/cyanobacteria/green algae/chloroplast)

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ABSTRACT **For higher plant chloroplasts, two key enzymes of the Calvin cycle, phosphoribulokinase (EC 2.7.1.19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.13), have recently been shown to be oligomerized onto the nonenzymatic peptide CP12. Enzymatic activity depends on complex dissociation, mediated by NADPH. The discovery of genes for CP12 in mosses, green algae, and cyanobacteria, together with the analysis of equivalent multiprotein complexes of *Chlamydomonas* and *Synechocystis* suggests that light regulation of Calvin cycle activity via NADPH-mediated reversible phosphoribulokinase/CP12/GAPDH complex dissociation is conserved in all photosynthetic organisms, prokaryotes and eukaryotes. *In vitro* complex reconstitution assays with heterologously expressed *Synechocystis* wild-type and mutagenized CP12 demonstrate a conserved subunit composition, stoichiometry, and topology in this complex. Further finding of genes, coding for chimeric proteins, carrying CP12 or parts of it as genetic fusions, indicates that evolution has used the peptide loops of CP12 as universal modules to keep various enzymatic activities under the control of NADP(H). These fusion events occurred at least twice in evolution. First was the fusion of the duplicated genes for CP12 and the ORF4 protein of *Anabaena variabilis* to the chimeric gene for the heterocyst-specific expressed ORF3 protein, most probably involved in N_2 fixation. A second gene fusion, which led to the higher plant chloroplast-specific GAPDH subunit, GAPB, has taken place during the transition from water- to land plants.**

The various biochemical reactions involved in photosynthesis have been grouped into two stages. First, the light-driven linear flow of electrons and hydrogen through membrane-bound multiprotein complexes leads to the reduction of the ferredoxin/thioredoxin system and in addition to the production of the highly energized metabolites ATP and NADPH. In turn, these products are necessary for the second step, i.e., the energy-consuming reductive conversion of CO_2 into carbohydrates, which are used for starch synthesis in the light. These latter reactions follow a cyclic sequence, named the Calvin cycle. In the dark the cycle must be shut off to avoid substrate concurrence with other biosynthetic pathways. Over decades it was established that light/dark regulation of Calvin cycle activity is mediated by reduction of the various involved enzymes by thioredoxin *f* in the light and spontaneous oxidation in the dark (1). Recently, for higher plants, a small nuclear encoded chloroplast protein, CP12, which was proposed to form two intramolecular peptide loops via disulfide bonds between neighboring cysteine residues (2), was shown to be oligomerized together with phosphoribulokinase (PRK, see *Abstract*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, see *Abstract*) in a stable 600-kDa heterooligomeric protein complex. The stoichiometry of this complex was proposed to be two N-terminally dimerized CP12 peptides, each carrying one PRK dimer on its N-terminal and one GAPDH heterotetramer (A_2B_2) on its C-terminal peptide loop. *In vitro*, this complex dissociates in the presence of NADP(H). PRK activity was shown to be inhibited by NAD and NADP, but was significantly activated in the presence of NADPH. PRK as well as GAPDH activity was therefore suggested to depend not only on reduction of the catalytic sites of the enzymes by thioredoxin *f* but finally on PRK/CP12/GAPDH complex dissociation and further allosteric activation of at least PRK by NADPH. These data lead to the hypothesis that higher plant chloroplasts contain a pool of thioredoxin-preactivated, but complexed and therefore inactive, PRK and GAPDH. Furthermore, Calvin cycle activity depends on complex dissociation, controlled by the ratio of NADPH to NADP, which is directly linked to the light-driven electron flux in the thylakoid membranes (3). To prove this hypothesis and to verify whether this would be a general mode for light regulation of Calvin cycle activity in all photosynthetic organisms, we analyzed how cyanobacteria, the evolutionary ancestors of chloroplasts in eukaryotes, and green algae regulate Calvin cycle activity. Besides that, the possible role of the peptide loops as universal genetic modules in the evolution of NADP(H)-mediated regulation of additional enzyme activities will be discussed.

MATERIALS AND METHODS

Materials. *Synechocystis* (PCC 6803) and *Chlamydomonas reinhardtii* (cw15) were grown photo-autotrophically under a 16/8 hr light/dark regime in liquid BG11 (4) and high salt medium (5), respectively, with 5% CO_2 in the air during the light phase. After 4 days, exponentially growing cells were harvested by centrifugation, resuspended in 100 mM Tris·HCl (pH 7.0)/75 mM KCl/1 mM EDTA/10% (vol/vol) glycerol (column buffer) and immediately broken in a French press. Cell debris was removed by ultracentrifugation (80,000 × *g*, twice for 10 min each) and the cleared supernatants were subjected to size exclusion chromatography, performed on a preequilibrated TSK-G3000SW column (see below). Polyclonal antisera used for immunochemical protein identification were generated in rabbits [for spinach chloroplast

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Abbreviations: PRK, phosphoribulokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; His-tag, histidine-tag.
Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AJ005284 and AJ005285).
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GAPDH (GAPA and GAPB) and *Synechocystis* CP12] or in chicken (for spinach CP12), by using for immunization histidine-tagged (His-tagged) proteins overexpressed in *Escherichia coli* and purified by metal ion affinity (see below). The antiserum against spinach PRK was a gift from K.-H. Suess (J. P. K. Gatersleben, Germany).

CP12 Gene Cloning. Cloning of the *Synechocystis* CP12 was done by the use of specific DNA primers, derived from the genomic DNA sequence (D90908) in a standard PCR assay using genomic DNA as a template. *C. reinhardtii* and *Ceratodon purpureus* PCR fragments were amplified out of λ cDNA libraries by vector and heterologous CP12 primers, used for earlier performed pea CP12 mutagenesis. Both DNA strands of each clone were completely sequenced.

***In Vitro* Mutagenesis of Recombinant *Synechocystis* CP12.** For each mutagenesis two specific cDNA oligonucleotides were designed, each containing a single nucleotide exchange in the codon for the cysteine residue to be changed to serine. Two separate PCR assays were performed in parallel, using the cloned gene for *Synechocystis* CP12 as a template. Each of the assays contained one of the CP12 mutagenizing primers and one countercurrent 5′- or 3′-end oligonucleotide, respectively, both containing an appropriate restriction site for later ligation into the expression vector. After this first round of amplification, aliquots of these two PCR assays were together used as the template for a second assay, using only the two outer primers with the aim to amplify the hybridization product of the two PCR fragments of the first reactions. The products of the second reactions were purified by standard phenol/chloroform treatment and, after ethanol precipitation, subjected to restriction enzyme assays to create the appropriate sites for cloning of the mutagenized CP12 coding regions in-frame with an N-terminal His-tag of the expression vector, pET14b (see below).

Protein Overexpression in *E. coli*. For overexpression the PCR-amplified and completely sequenced relevant ORFs were ligated in-frame with an N-terminal His-tag coding sequence of the expression vector pET14b and cloned in *E. coli* strain BL21 DE3 (Novagen, Madison, WI). Induced *E. coli* cells were broken in a French press, cell debris was removed by centrifugation, and overexpressed proteins were purified out of the soluble supernatant by metal ion affinity chromatography on Talon resin, following the manufacturer's protocol (CLONTECH).

Proof for Intramolecular Disulfide Bonds. Slot blots of His-tagged proteins overexpressed in *E. coli* and purified by metal ion affinity were performed with the slot blot apparatus of Pharmacia, and the blotted proteins were analyzed for disulfide bonds by the use of the DIG (digoxigenin) Protein Detection Kit (Boehringer Mannheim).

***In Vitro* Protein Complex Reconstitution Assays.** Soluble supernatants (1 ml) of broken *Synechocystis* cells (see above) or lysed isolated intact pea chloroplasts (1) were incubated with 20 mM DTT for 1 hr at 30°C. After removal of DTT by Sephacryl-G25 size exclusion chromatography, ≈20 μg of overexpressed and affinity-purified His-tagged CP12 (or of the indicated mutagenized CP12 proteins) and 0.1 mM NAD were added. Reconstitution assays were incubated for 48 hr at 4°C, diluted by 1 vol of 20 mM Tris·HCl, pH 8.0/100 mM NaCl (binding buffer), and chromatographed on separate metal ion affinity columns, each containing 200 μl of Talon resin. Columns were washed with 20 vol (8 ml) of binding buffer and the last milliliter (last wash) was collected. Bound protein complexes were eluted with 1 ml of binding buffer, containing either 100 mM imidazole or 2.5 mM NADPH. Proteins in the last washes and eluates were precipitated by 10% trichloroacetic acid (1 hr in an ice bath), sedimented by centrifugation, and resuspended in loading buffer for SDS/PAGE (6). Equal aliquots were run through the gel, and proteins were visualized by silver staining of the gel (7) or, after transfer onto nitrocellulose, immunochemically analyzed (8) by using the antisera described above.

Miscellaneous Procedures. DNA cloning, sequencing, and PCR techniques followed standard procedures. Size exclusion chromatography of soluble protein extracts, performed on a TSK-G3000SW column, as well as PRK activity assays and thin-layer chromatography, were as briefly described (3).

RESULTS AND DISCUSSION

CP12 Is a Conserved Protein in Photosynthetic Prokaryotes and Eukaryotes. The discovery of a CP12 isologue encoding ORF in the genomic sequence of the cyanobacterium, *Synechocystis*, suggests that NADPH-mediated light regulation of Calvin cycle activity occurred already in cyanobacteria. In addition, cloning of cDNA sequences for CP12 proteins of the green alga *C. reinhardtii* and also of the moss *Ceratodon purpureus* indicates that this mechanism may be conserved in all photosynthetic organisms (Fig. 1*A*). To investigate whether the cysteine residues of the *Synechocystis* CP12 also form intramolecular peptide loops via disulfide bonds, the CP12-encoding ORF of *Synechocystis* and, for a control, a cDNA fragment encoding the mature CP12 of spinach, were cloned

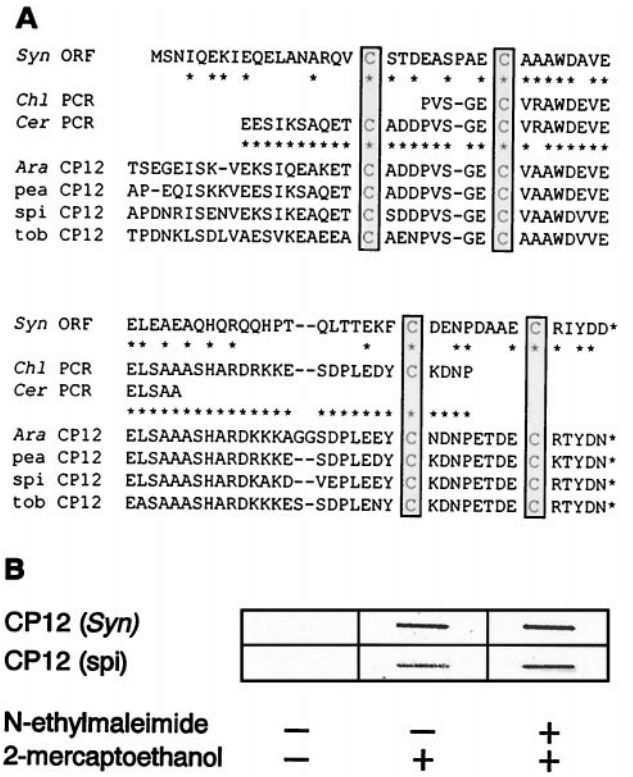

FIG. 1. CP12 is an evolutionarily conserved protein of photosynthetic organisms. (*A*) Known primary structures of CP12 proteins of higher plants, derived from cDNA translations, were aligned with the translation of an ORF in the genomic DNA sequence of *Synechocystis* (strain PCC6803) and cloned PCR products out of cDNA libraries, made from RNA of *C. reinhardtii* (unpublished) and *Ce. purpureus*. Conserved cysteine residues are boxed. Amino acid residues of the *Synechocystis* CP12 as well as of *Chlamydomonas* and *Ceratodon* PCR product translations, which are identical with at least one residue of the higher plant CP12 peptides, are marked by asterisks. (*B*) The conserved cysteine residues of CP12 form peptide loops via disulfide bonds. Equal amounts of His-tagged *Synechocystis* and spinach CP12, overexpressed in *E. coli* and purified by metal ion affinity, were slot blotted onto nitrocellulose. The filters were treated with alkylating and/or reducing reagents and subsequently analyzed for the presence of reduced thiol groups by a colorimetric assay following the protocol for the DIG Protein Detection Kit (Boehringer Mannheim).

in fusion with an N-terminal His-tag and overexpressed in *E. coli*. Equal amounts of the metal ion affinity-purified proteins were slot blotted onto nitrocellulose and analyzed for the presence of disulfide bonds between the thiol groups of neighboring cysteine residues (Fig. 1*B*). As demonstrated, free thiol groups could, in contrast to the approach with the untreated proteins, be detected only after incubation of the blotted proteins with the reducing agent, 2-mercaptoethanol. Pretreatment of the blotted proteins with the alkylating agent *N*-ethylmaleimide failed to prevent the reduction of the cysteine SH groups during the subsequent incubation with 2-mercaptoethanol, demonstrating that all thiol groups present in the overexpressed proteins must have been oxidized to disulfide bonds before reduction with 2-mercaptoethanol. Besides the decreased size of the N-terminal peptide loop by the loss of one internal amino acid residue in the "eukaryotic" CP12 peptides (see Fig. 1*A*), the double loop structure of CP12 as given in ref. 3 seems to be conserved from cyanobacteria up to higher plants and is therefore expected to fulfill similar functions.

Conserved NADP(H)-Dependent PRK/CP12/GAPDH-Complex Dissociation and PRK Activity. After size exclusion chromatography of soluble protein extracts of *Synechocystis* and *Chlamydomonas,* SDS/PAGE and immunoblot analysis revealed that CP12 forms apparent 550-kDa-protein complexes with PRK and GAPDH in both organisms (Fig. 2*A*). Treatment of prefractionated complexes with NADPH and rechromatography of the assays on the same column demonstrated that NADPH causes complete dissociation of PRK and GAPDH from CP12. In contrast to NADP, which, although to a lower extent, also leads to complex dissociation, the complexes remained stable during incubations with an equal volume of water (Fig. 2*A*) or the nonphosphorylated dinucleotides NAD and NADH (not shown), indicating that the negatively charged phosphate groups in NADP and NADPH may disturb electrostatic protein/protein interactions between the negatively charged residues in the peptide loops of CP12 and positively charged epitopes of the two enzymes PRK and GAPDH. Complete dissociation of the PRK/CP12/GAPDH complexes is also achieved by incubation of the pooled complex fractions with the nonphysiological, strong reducing agent DTT, most probably because of destruction of the peptide loops in CP12 by reduction of the disulfide bonds between the neighbor cysteine residues. This treatment, often used to simulate light, i.e., reduced thioredoxin, *in vivo* (9), even led to the dissociation of the GAPDH tetramer of *Synechocystis* to monomers.

To investigate whether dissociation of the PRK/CP12/GAPDH complex is necessary for enzyme activity, aliquots of

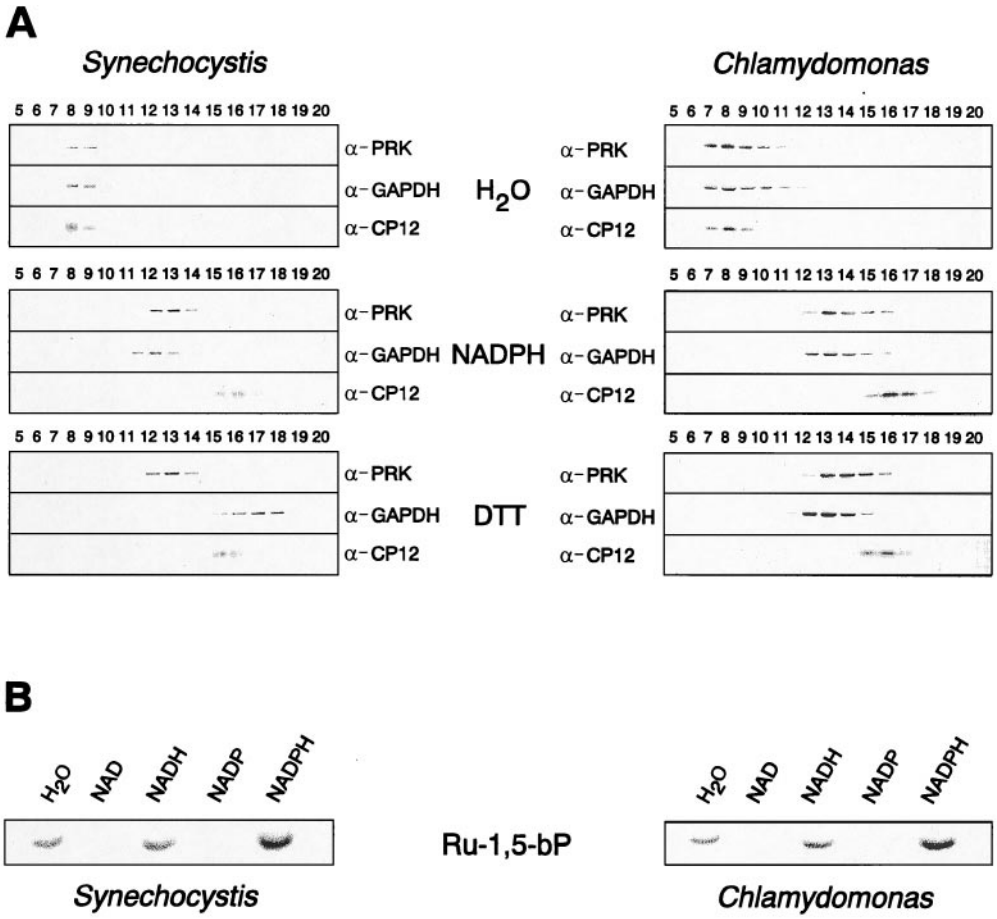

FIG. 2. Identification and characterization of PRK/CP12/GAPDH complexes in *Synechocystis* and *C. reinhardtii*. (*A*) Isolated complexes remain stable, but dissociate in the presence of NADPH or DTT, respectively. Soluble supernatants of lysed *Synechocystis* and *Chlamydomonas* cells were subjected to size-exclusion chromatography. The pooled complex fractions 7–9, which contained the complex, were treated either with NADPH (2.5 mM, 30 min, 4°C) or with the reducing agent DTT (20 mM, 30 min, 30°C). For a control, they were incubated with the same volume of water (30 min, 4°C). All assay mixtures were rechromatographed on the same size-exclusion column, and the collected fractions were determined by immunoblot analysis using antisera (α) to PRK, GAPDH, and CP12. Lanes are numbered according to the collected column fractions. (*B*) PRK activity depends on dissociation of the PRK/CP12/GAPDH complex and NADPH. Pooled complex fractions 7 and 8 from size-exclusion chromatography (see above) were assayed for ribulose 5-phosphate phosphorylation activity dependent on the indicated nicotinamide-adenine dinucleotides (2.5 mM each). Formation of radiolabeled ribulose 1,5-bisphosphate (Ru-1,5-bP) was visualized after thin-layer chromatography and exposure of the plates to x-ray films.

the pooled complex fractions from size exclusion chromatography were preincubated in parallel with the four different nicotinamide-adenine dinucleotides and subsequently assayed for PRK activity (Fig. 2*B*). Both the water control and the assay with NADH showed little phosphorylation activity, most probably due to weak complex dissociation, caused by dilution in the assay. A significant increase in the formation of ribulose 1,5-bisphosphate was observed only in the presence of NADPH. In contrast, NADP as well as NAD exhibited a strong inhibitory effect on PRK activity. These data clearly demonstrate that in both analyzed organisms, *Synechocystis* and *Chlamydomonas*, enzyme activity depends, as was recently proposed for higher plants (3), on NADPH-mediated PRK/CP12/GAPDH complex dissociation and further allosteric activation of at least PRK by NADPH.

Conserved PRK/CP12/GAPDH Complex Subunit Composition, Arrangement, and Stoichiometry. To analyze the physical subunit arrangement of the *Synechocystis* PRK/CP12/GAPDH complex, *in vitro* complex reconstitution assays were performed (Fig. 3*A*). A soluble supernatant of lysed *Synechocystis* cells was preincubated with the strong reducing agent DTT, to dissociate the authentic PRK/CP12/GAPDH complexes by opening the peptide loops of CP12. After removal of DTT by gel filtration, heterologously expressed His-tagged CP12 of S*ynechocystis* was added and assays were incubated for protein complex reconstitution. Proteins, oligomerized onto the recombinant CP12, were then copurified by metal ion affinity chromatography on Talon columns. SDS/PAGE and silver staining of the proteins eluted by imidazole revealed, in addition to the two bands at ≈16 and 18 kDa, both representing recombinant CP12, only one single prominent (double) band at ≈36 kDa (Fig. 3*A*). Using NADPH as eluent instead of imidazole also resulted in the elution of only one protein (double) band at ≈36 kDa. In the latter case, recombinant CP12 remained bound to the column. Western blot analysis of identical gel lanes demonstrated that in both cases, these 36-kDa doublets contain both of the expected proteins, PRK and GAPDH (data not shown). The oligomerization of only PRK and GAPDH to the recombinant CP12 protein *in vitro* strongly sustains the results obtained by size exclusion chromatography approaches (see Fig. 2*A*), that the proposed PRK/CP12/GAPDH complex is indeed composed only of CP12, PRK, and GAPDH. In addition, the finding that NADPH causes dissociation of both enzymes from the immobilized reconstituted complex indicates that copurification of PRK and GAPDH with recombinant CP12 by metal ion affinity chromatography is not artificial, but reflects correct PRK/CP12/GAPDH complex assembly *in vitro*. The 16- and 18-kDa forms of CP12 detected after SDS/PAGE most likely represent molecules reduced to a different extent, which in turn influences their structure and running behavior on SDS/PAGE.

For further analysis, heterologously expressed His-tagged CP12 of *Synechocystis* (wild type), as well as mutagenized His-tagged CP12 peptides, which either are unable to form the N-terminal peptide loop, due to a Cys-1 to Ser substitution, or which are unable to form the C-terminal peptide loop, caused by a introduced Cys-4 to Ser mutation, were added to aliquots of the DTT-reduced cytosolic supernatant of lysed *Synechocystis* cells and incubated for protein complex reconstitution. As demonstrated (Fig. 3*A*), recombinant wild-type CP12 oligomerized with both PRK and GAPDH. In contrast, the Cys-1 to Ser mutagenized CP12 peptides were unable to bind PRK, but assembled with GAPDH, whereas the Cys-4 to Ser mutagenized CP12 peptides did not oligomerize with GAPDH, but bound PRK. Control reconstitution assays without the addition of any His-tagged CP12 peptides failed in the detection of both enzymes, PRK and GAPDH, after chromatography (not shown). These results are in good agreement with those, obtained for a higher plant CP12, where a Cys-1 for Ser

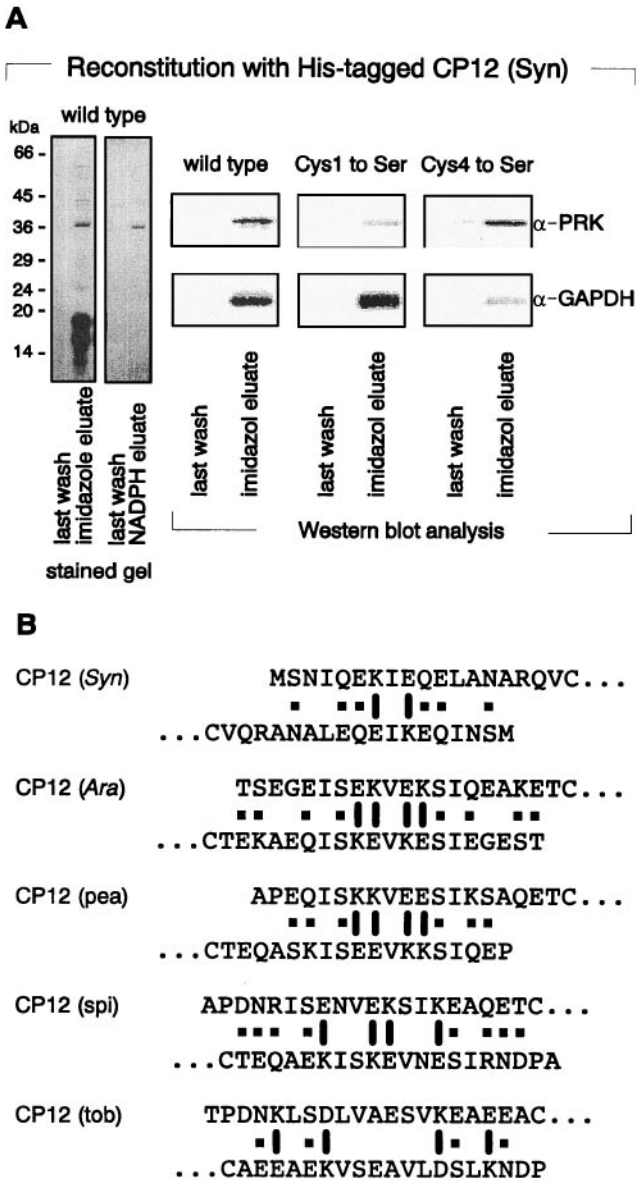

FIG. 3. Subunit composition and topology of the PRK/CP12/GAPDH complex of *Synechocystis*. (*A*) PRK binds to the N-terminal, GAPDH to the C-terminal peptide loop of CP12. Equal amounts of His-tagged, overexpressed and metal ion affinity purified CP12 of *Synechocystis*, as well as of the indicated cysteine for serine mutagenized and in *E. coli* overexpressed proteins, were added to aliquots of a DTT-reduced, soluble supernatant of lysed *Synechocystis* cells for *in vitro* complex reconstitution. After incubation, assay mixtures were chromatographed on metal ion columns. Bound proteins were eluted by either 100 mM imidazole or 2.5 mM NADPH in binding buffer, respectively, and subsequently characterized after SDS/PAGE by protein silver staining or Western blot analysis with monospecific antisera against PRK and GAPDH, as indicated. (*B*) CP12 might dimerize via charged amino acid residues located in its N terminus. N-terminal amino acid sequences up to the first cysteine of the so far known CP12 peptides of the indicated species are positioned in an antiparallel order. The resulting palindromic distribution of charged amino acid residues, thought to form salt bridges, is indicated by barrels. Amino acid residue pairs with the potential to form additional hydrogen bonds are marked by ■.

exchange in pea CP12 prevents interaction with PRK in the yeast two-hybrid system (3). In addition, the high homology of the C-terminal peptide loop of CP12 to the C-terminal extension of higher plant GAPDH subunit GAPB (see below), which was shown to be necessary for the oligomerization of

four A_2B_2 heterotetramers to the "regulatory" hexadecameric A_8B_8 form (10), also sustains the proposed topology for CP12/GAPDH interaction. As a consequence of these arguments, it is concluded that PRK binds to the N-terminal, and GAPDH to the C-terminal peptide loop of CP12. These results further indicate that no direct interaction between PRK and GAPDH is necessary to oligomerize with CP12. Nevertheless, interaction between GAPDH and PRK, when assembled to CP12, cannot be excluded and may contribute to complex stability.

The molecular masses of the PRK/CP12/GAPDH complexes of *Synechocystis* and *Chlamydomonas* were both estimated by size exclusion chromatography to be ≈550 kDa. For higher plants (pea and spinach) complex masses were determined by the same method to be ≈600 kDa (3). No proteins, other than PRK and GAPDH, which have joint maxima together with CP12, were detected in the complex fractions of all organisms analyzed so far, nor copurified with recombinant CP12 in the *in vitro* complex reconstitution assays. Complex dissociation by NADPH (see Fig. 2*A*) ended up in apparent molecular masses of ≈80 kDa for dimeric PRK and 150 kDa for tetrameric GAPDH, as determined by size exclusion chromatography. From these facts, it is deduced that CP12 can form homodimers. Subunit stoichiometry of the complex would then be two CP12 molecules (2 × 8 kDa), two PRK dimers (4 × 36 kDa), and two GAPDH tetramers (8 × 36 kDa), leading to a calculated molecular mass of ≈450 kDa. The difference between the apparent (550 kDa) and the calculated molecular mass of the complex (450 kDa) may reflect the influence of its three-dimensional structure on its behavior in size exclusion chromatography. Comparative analysis of the known amino acid sequences of CP12 peptides of different species led to the identification of the N-terminal 20 amino acid residue stretch as a potential dimerization domain (Fig. 3*B*). Aligning each of these epitopes with itself in an antiparallel orientation exhibits a palindromic distribution of oppositely charged amino acid residues, which may render possible electrostatic interactions by the formation of salt bridges and hydrogen bonds. A simplified model, demonstrating this subunit arrangement and stoichiometry in the conserved PRK/CP12/GAPDH complex, was recently given in ref. 3.

***Synechocystis* CP12 Can Replace Pea CP12 in the Pea PRK/CP12/GAPDH Complex.** Further complex reconstitution assays with overexpressed mature CP12 of pea in stromal extracts of lysed pea chloroplasts resulted in efficient oligomerization of PRK to the recombinant CP12. In contrast, GAPDH bound less efficiently and in a nonstoichiometric relation of its subunits, GAPA and GAPB (Fig. 4 *Left*). This may reflect the potential of higher plant chloroplast A_2B_2 heterotetrameric GAPDH to oligomerize *in vitro* in the presence of NAD to the A_8B_8 hexadecameric form (11) and, in addition, demonstrates that chloroplast GAPDH A_4 homotetramers can also oligomerize with CP12. Incubation of the overexpressed CP12 of *Synechocystis* in the DTT-treated prereduced pea stroma ended up in a stoichiometric binding of PRK and both GAPDH subunits, GAPA and GAPB, onto the recombinant CP12 (Fig. 4 *Right*). In this assay, the high affinity of *Synechocystis* CP12 to the A_2B_2 heterotetrameric GAPDH of pea may have prevented self-oligomerization of GAPDH to the "regulatory" A_8B_8-hexadecameric form. Compared with pea CP12, the higher affinity of the *Synechocystis* CP12 to the A_2B_2 heterotetrameric GAPDH of pea also indicates that, during the evolution of higher plants, a well regulated process of coevolution between CP12 and GAPDH must have taken place, to direct the preferred formation of either the PRK/CP12/GAPDH complex or the hexadecameric A_8B_8 form of GAPDH. The potential physiological function of the higher plant chloroplast A_8B_8 hexadecameric form of GAPDH, so far demonstrated only *in vitro*, remains to be established.

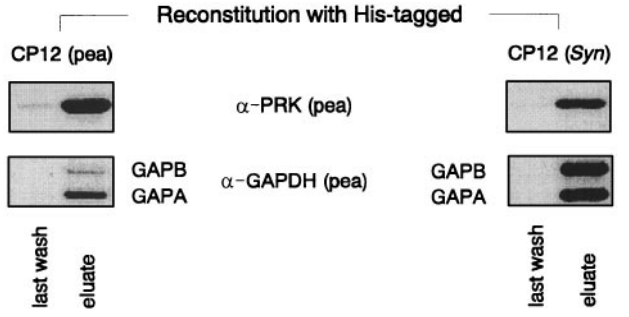

FIG. 4. CP12 of *Synechocystis* can substitute for pea CP12 in the pea PRK/CP12/GAPDH complex. *In vitro* protein complex reconstitution assays were performed in DTT-reduced stroma preparations of isolated intact pea chloroplasts, adding either recombinant, His-tagged mature pea CP12 or *Synechocystis* CP12. After incubation, assays were chromatographed to metal ion affinity columns. Eluted proteins were, after SDS/PAGE, characterized by Western blot analysis using the indicated antisera.

Evolution of the Heterocyst-Specific ORF3 Protein of *Anabaena variabilis* and Higher Plant Chloroplast GAPDH Subunit GAPB. Database searches for CP12 genes or proteins together with experimental data (see Fig. 1) revealed that they are present exclusively in photosynthetic organisms and that the tertiary structure of the CP12 proteins remained conserved in the evolution from cyanobacteria up to chloroplasts of higher plants. The evolutionary ancestor of the CP12 protein of *Synechocystis* remains unknown. The discovery of sequences for chimeric proteins, which obviously originate from genetic fusions of genes for still-existing proteins with sequences encoding either the complete or the C-terminal part of CP12, may indicate that evolution has used the peptide loops of CP12 to get also other enzyme activities adjustable by NADP(H)-mediated reversible protein complex dissociation (Fig. 5 *A* and *B*).

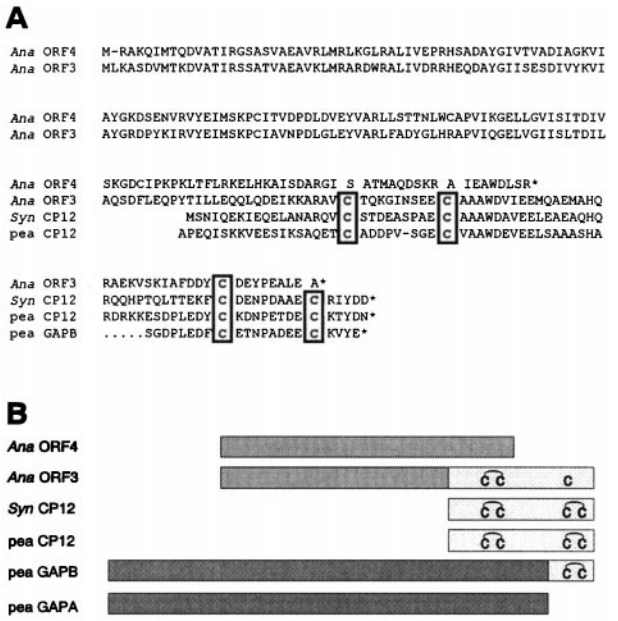

FIG. 5. Evolution of *A. variabilis'* heterocyst-specific ORF3 protein and higher plant chloroplasts' GAPDH subunit GAPB. (*A*) Amino acid sequences of the hypothetical proteins, ORF4 and ORF3, from *A. variabilis*, most probably involved in heterocyst-specific N_2 fixation, and the sequence of the C-terminal extension of pea chloroplast GAPDH subunit GAPB, involved in photosynthetic CO_2 fixation, are aligned with the peptide sequences of CP12 from *Synechocystis* and pea. Peptide loop-forming conserved cysteine residues are boxed. (*B*) Graphical illustration of the proposed gene fusion events. Homologous sequences given in *A* are presented by identically shadowed boxes. CP12 cysteine residues are indicated by a single or double c.

The ORF3 protein, encoded in the operon for the subunits of the NADP(H)-dependent bidirectional hydrogenase of *A. variabilis* (12) probably has resulted from the genetic fusion of the coding region for the ORF4 protein (13) and CP12. This may indicate that the ORF3 gene product plays a significant role in the regulation of this heterocyst-specific hydrogenase activity by NADP(H)-mediated reversible protein complex dissociation.

The high degree of homology between higher plant GAPDH subunits GAPA and GAPB (14) and the significant homology between the C-terminal extension of subunit GAPB and the C-terminal peptide loop of CP12 (2), together with the finding of a CP12 gene already in *Synechocystis*, strongly suggest that higher plant chloroplast GAPDH subunit GAPB has evolved by gene duplication of subunit GAPA and subsequent genetic fusion with the C-terminal part of a duplicated CP12 gene. In respect to photosynthesis, cyanobacteria, algae, and mosses contain (so far as is known) only GAPA subunits, which form homotetrameric A_4 GAPDH complexes, which are unable to oligomerize to higher aggregates. As demonstrated above, these GAPA tetramers are assembled, together with PRK dimers and CP12 proteins, into 550-kDa PRK/CP12/GAPDH complexes, which dissociate in the presence of NADPH. For the development of higher land plants, which consist also of nonphotosynthetic tissues, that are to be provided with energized metabolites, it might had been necessary to increase the production of photosynthetic assimilates by increasing the amount of photosynthetic GAPDH, and to get this additional activity also linked to the light-driven electron flux in the thylakoids. As mentioned above, the evolution of subunit GAPB has enabled the formation of A_2B_2 heterotetramers, which, due to the C-terminal extension of subunit GAPB, can, as demonstrated *in vitro*, form less or nonactive A_8B_8 hexadecamers. These complexes were shown to be dissociated, as is described for the PRK/CP12/GAPDH complex, in the presence of DTT and NADP(H), but also by its substrate, 1,3-bisphosphoglycerate (15).

Conclusions. The discovery of genes for CP12, already in the cyanobacterium *Synechocystis*, as well as in the green alga *C. reinhardtii*, the moss *Ce. purpureus*, and various higher plants, together with the demonstration of conserved PRK/CP12/GAPDH complex composition and function, suggests that light regulation of Calvin cycle activity via NADPH-mediated dissociation of PRK/CP12/GAPDH complexes is conserved in all photosynthetic organisms. The elucidation of this regulatory mechanism may offer new ideas for the engineering of plants with improved efficiency in photosynthesis. The detection of chimeric proteins, containing CP12, or parts of it, as genetic fusions has shown that the peptide loops of CP12 have been used as evolutionary conserved modules for ''natural genetic engineering,'' allowing control of different enzymatic activities by NADP(H). Further analysis of the molecular interactions in this mechanism, approved for more than three billion years, might also provide novel ideas for *in vitro* protein design and modeling for the creation of regulated biotechnological processes in the future.

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