

The Small Protein CP12: A Protein Linker for Supramolecular Complex Assembly

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ABSTRACT: CP12 is an 8.5-kDa nuclear-encoded chloroplast protein, isolated from higher plants. It forms part of a core complex of two dimers of phosphoribulokinase (PRK), two tetramers of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and CP12. The role of CP12 in this complex assembly has not been determined. To address this question, we cloned a cDNA encoding the mature CP12 from the green alga *Chlamydomonas reinhardtii* and expressed it in *Escherichia coli*. Sequence alignments show that it is very similar to other CP12s, with four conserved cysteine residues forming two disulfide bridges in the oxidized CP12. On the basis of reconstitution assays and surface plasmon resonance binding studies, we show that oxidized, but not reduced, CP12 acts as a linker in the assembly of the complex, and we propose a model in which CP12 associates with GAPDH, causing its conformation to change. This GAPDH/CP12 complex binds PRK to form a half-complex (one unit). This unit probably dimerizes due partially to interactions between the enzymes of each unit. Reduced CP12 being unable to reconstitute the complex, we studied the structures of oxidized and reduced CP12 by NMR and circular dichroism to determine whether reduction induced structural transitions. Oxidized CP12 is mainly composed of α helix and coil segments, and is extremely flexible, while reduced CP12 is mainly unstructured. Remarkably, CP12 has similar physicochemical properties to those of "intrinsically unstructured proteins" that are also involved in regulating macromolecular complexes, or in their assembly. CP12s are thus one of the few protein families of intrinsically unstructured proteins specific to plants.

In recent years, the use of very sensitive analytical techniques has revealed the presence of small proteins in macromolecular complexes. Their presence raises questions about their role in the formation and regulation of higher order structures (1–3).

CP12 is a small, 8.5-kDa nuclear-encoded chloroplast protein that has been isolated and cloned from a few eukaryotic and prokaryotic photosynthetic organisms (4–6). Many expressed sequence tags (ESTs) encoding putative CP12 proteins have been found in a wide variety of photosynthetic organisms in the past few years (7, 8). CP12 has been isolated as part of a core complex composed of CP12, phosphoribulokinase (PRK), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).¹ These enzymes are regulated by light and are involved in CO₂ assimilation in photosynthetic organisms (Benson-Calvin cycle). Interestingly, CP12 shares sequence homologies with the C-terminal extension of GapB, a GAPDH subunit in the chloroplasts of higher plants (4). This C-terminal end is responsible for the

oligomerization of the higher plant GAPDH (A₂B₂) into a hexadecamer (A₃B₃), which is regulated by metabolites such as 1,3-biphosphoglyceric acid (9, 10). In green algae, GAPDH is present as an A₄ homotetramer which has not been shown to oligomerize to form a regulatory GAPDH.

Our group isolated a PRK/GAPDH complex, composed of two dimers of PRK and two homotetramers A₄ of GAPDH, from the green alga *Chlamydomonas reinhardtii* (11). Recent cryo-electron microscopy studies suggested that other components are part of this complex (12). As Wedel et al (5) have shown that CP12 occurs with GAPDH and PRK in *C. reinhardtii*, this small protein was a good candidate. Recent mass spectrometry and kinetic studies on native GAPDH (isolated from the green alga) showed that it was a complex of GAPDH and CP12 (13), confirming that the complex described earlier by Avilan et al (11) was the same as the one isolated by Wedel et al (5). The activities of the enzymes involved in this complex are regulated in vitro by metabolites such as NADP(H) (5, 6, 14) and thioredoxins or the nonphysiological reducing agent DTT (15).

A partial sequence of CP12 in *C. reinhardtii* was obtained by PCR (5) (accession number in TrEMBL: 065775), but the 5' and 3' ends corresponding to the N- and C-terminal parts of the mature protein were missing. We have therefore cloned the entire cDNA of algal CP12 and developed an expression system in *Escherichia coli*.

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¹ Abbreviations: GAPDH: glyceraldehyde 3-phosphate dehydrogenase, PRK: phosphoribulokinase, CD: circular dichroism, NMR: nuclear magnetic resonance, SPR: surface plasmon resonance; IUP: intrinsically unstructured protein.

Previous studies on recombinant GAPDH (devoid of CP12) and native GAPDH (associated with CP12) demonstrated that the association of CP12 with GAPDH was responsible for changes in the kinetic parameters of GAPDH, probably due to conformation changes. These results already defined a new function for CP12, different from the regulatory one described earlier (5, 6). Nevertheless, since the highly homologous C-terminal extension of gapB is involved in the oligomerization of higher plant GAPDH, and given that CP12 is also part of the PRK/CP12/GAPDH complex, it may also play a role in the assembly of the complex. We have therefore studied its role under oxidized and reduced state in the assembly of the PRK/CP12/GAPDH complex from *C. reinhardtii*. We used surface plasmon resonance binding experiments to determine how this complex might be assembled and for the first time, we propose a model for its assembly pathway.

Since oxidized and reduced CP12 do not have the same behavior regarding complex formation, and since no structural data were available for any of the cloned CP12 proteins, we analyzed the structure of oxidized and reduced CP12 in solution by NMR spectroscopy and circular dichroism (CD). We also linked these results with the role of CP12 in the assembly of the PRK/CP12/GAPDH complex.

EXPERIMENTAL PROCEDURES

cDNA Library Construction. *C. reinhardtii* (cw15) was grown photoautotrophically for 4 days under a 16/8 h light/dark regime in high salt medium (16) with 5% CO₂ in the air during the light phase. The exponentially growing cells were then harvested 2.5 (A) and 6 h (B) after light setting. Nucleic acids were isolated by three rounds of phenol/chloroform and a final chloroform extraction. The total nucleic acids were precipitated with potassium acetate/2-propanol and separated by LiCl precipitation. The polyadenylated RNA was purified by chromatography on oligo-dT cellulose (Pharmacia, Freiburg) and cDNA was synthesized using a Pharmacia (Freiburg) kit. Double-stranded adapter-linked cDNAs were ligated into the dephosphorylated arms of λ gt11 and packed into Gigapack II Gold phage extracts (Stratagene, La Jolla). The resulting phages were incubated with *E. coli* strain Y1088 for 20 min at room temperature, plated on BBL-agar, and the plates incubated at 37 °C overnight. The resulting plaques were overlaid with SM to elute the phages. The elutes were centrifuged and the cleared phage-containing supernatants were adjusted to 7% DMSO. Aliquots of the two libraries (A and B) were stored at -80 °C. The titers of the libraries were about 10⁸ pfu/mL, containing about 90% recombinant phages.

Cloning of *C. Reinhardtii* CP12. Primers were designed from ESTs available at NCBI Genbank (AV626097 and AV634344) (17). They were used to amplify directly CP12 (forward primer: 5'CTAGCGGCCAGCCCCGTGTG3' and reverse primer: 5'GAATTAGGAGCATCTGCGCCG3') from the *C. reinhardtii* cDNA library mentioned above. The PCR product (248 bp) was cloned in pGEM-T (Promega) and sequenced. The primers against the partial sequence of *C. reinhardtii* published in ref 5 were also used (forward: 5'CCGGTTAGTGGAGAATGCG3' and reverse: 5'GGT-TATCCCTTACAGTAATCC3').

Overexpression and Purification of *C. reinhardtii* CP12. The N-terminal sequence of native CP12 was obtained from

C. reinhardtii purified PRK/CP12/GAPDH complex in solution (Edman method). The N-terminal sequences of PRK and GAPDH are known to be DKDKT and EKKIR, respectively. The remaining amino acid residues were thus attributed to CP12. Primers containing the *Nde*I and *Xho*I restriction sites were used to amplify and clone mature CP12 cDNA in frame with the N-terminal histidine tag of the pET16b expression vector (Novagen) (forward primer 5'CATATGAGCGGCCAGCCCCG3' and reverse primer 5'GAGCTCAATTAGGAGCAT CTGC3'). A cleavage site by factor Xa is present between the histidine tag and mature CP12.

C. reinhardtii CP12 cDNA was expressed in freshly transformed *E. coli* BL21(DE3)pLysS. Bacteria were grown in 2YT medium with 100 μ g/mL ampicillin and 34 μ g/mL chloramphenicol at 37 °C until the OD₆₀₀ was 0.5 to 0.6. Cultures were cooled on ice and CP12 synthesis was induced with 1 mM isopropyl- β ,D-thiogalactopyranoside (IPTG). Protein synthesis took place at 30 °C overnight.

Bacteria were collected by centrifugation and suspended in Ni-NTA buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazol, pH 8.0). Cells were broken by sonication and centrifuged at 27 000g for 20 min. The supernatant contained recombinant CP12 bearing a histidine tag (His-CP12). Recombinant His-CP12 was purified by metal ion affinity chromatography on Ni-NTA agarose (Qiagen) (1.2 \times 8 cm). The column was equilibrated in Ni-NTA buffer. Contaminants were first eluted out with 100 mM imidazol. Apparently homogeneous His-CP12 was eluted with 200 mM imidazol and stored at -20 °C.

The His-CP12 used for in vitro complex reconstitution was digested with factor Xa (Novagen) (1u for 33 μ g of CP12). The sample was applied to a gel filtration Sephadex G50 (1 \times 50 cm) column in 50 mM NaH₂PO₄, 50 mM NaCl, pH 7.5 to separate CP12 from factor Xa and the histidine tag. Purified CP12 was stored at -20 °C.

The PRK/CP12/GAPDH complex was purified from *C. reinhardtii* WM3⁻ as in ref 11. PRK and GAPDH may also be purified separately to obtain the native isolated enzymes (11). Recombinant GAPDH was purified as in ref 13.

Redox State of CP12. The free SH groups in CP12 were quantified by alkylation of the reactive cysteine residues with iodoacetamide. Iodoacetamide was prepared as in ref 18. Oxidized and reduced CP12 were alkylated with 100 mM iodoacetamide for 30 min at room temperature and in the dark. Samples were desalted on a Zip-tip C₁₈ and analyzed by mass spectrometry (MALDI-TOF). Alkylation of one cysteine residue increased the mass by 57.27 Da. Reduced CP12 was obtained by incubating the protein with 30 mM DTT for 1 h at 30 °C.

In Vitro Complex Reconstitution. Recombinant GAPDH and native PRK were dialyzed separately against reconstitution buffer (30 mM Tris, 100 mM NaCl, 2 mM EDTA, 0.1 mM NAD, pH 7.9) supplemented with 5 mM Cys or 1 mM DTT on a YM30 centricon (Amicon, Millipore). Oxidized or reduced CP12 was added in a molar ratio of 1 monomer of CP12 to 1 tetramer of GAPDH and 1 dimer of PRK (1:1:1) to reconstitute the complex. The proteins were dialyzed again in the same buffer and concentrated to 50 μ L on a YM10 centricon (Amicon - Millipore). 10% glycerol was added and the proteins were incubated for 1 h at 30 °C and kept at 4 °C overnight or longer.

Surface Plasmon Resonance (SPR) Binding Experiments. Oxidized CP12 or His-CP12 (ligand) was immobilized on CM5 sensor chips in HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) P20, pH 7.4; BIAcore) by amine coupling using the amine coupling kit supplied by BIAcore. The aim of 50 RU (resonance unit) was reached. 785 RU of reduced PRK were immobilized on a CM5 chip by SH coupling.

A range of protein (analyte) concentrations were dialyzed against buffer HBS-EP and 0.1 mM NAD and used to determine the dissociation constants with a BIACORE 2000 instrument. In each interaction run, the analyte was injected for 3 min to record the association phase to the flow cells, after which buffer was injected for 3 min to record the dissociation phase. The association and dissociation phases were used to derive the dissociation and association rates (k_d and k_a , respectively). The equilibrium-dissociating constant (K_d) is given by $K_d = k_d/k_a$. Global fittings of the exponential curves (sensorgrams) giving both k_a and k_d values and the response at equilibrium (R_{eq}) in RU were performed using the BIAeval 3.1 program using a model equivalent to the 1:1 Langmuir model for adsorption to a surface.

Circular Dichroism Studies on His-CP12. Spectra were recorded on a Jasco-810 dichrograph and analyzed with the programs Dicroprot (<http://dicroprot-pbil.ibcp.fr/>) and CDPro (<http://lamar.colostate.edu/~sreeram/CDPro/main.html>). All CD spectra are averages of four accumulations from 260 to 180 nm with a scan speed of 10 nm/min. His-CP12 was dialyzed against phosphate buffer (10 mM Na₂HPO₄–NaH₂PO₄, pH 6.0). Measurements were performed in 0.2 cm quartz cuvettes in a final volume of 300 μ L.

NMR Studies of His-CP12. Homonuclear NMR experiments were recorded on His-tagged CP12 and CP12 in 50 mM NaH₂PO₄, 50 mM NaCl, pH 6.0, 10% (v/v) ²H₂O. NMR heteronuclear studies were carried out on His-CP12 synthesized in M9 minimal medium containing 1 g/L of [¹⁵N]-ammonium chloride (Isotec) as the sole nitrogen source. The protein was purified on Ni–NTA agarose. The samples were dialyzed against NMR buffer (50 mM NaH₂PO₄, 50 mM NaCl, pH 6.0) and concentrated to 0.9 mM in a final volume of 450 μ L. A 50 μ L aliquot of ²H₂O (10% v/v) was added and the sample was degassed with argon before recording.

2D homonuclear TOCSY spectra, NOESY spectra, 2D ¹H–¹⁵N HSQC spectra (19), and heteronuclear nOe {¹H}–¹⁵N spectra (20) were recorded at 27 °C on Varian Inova 400 and 600 MHz spectrometers equipped with a triple-resonance (¹H, ¹³C, ¹⁵N) probe, including shielded z-gradients. Spectra were processed using FELIX version 97.0 (Accelrys). Chemical shifts were measured with reference to the H₂O signal taken at 27 °C relative to DSS. ¹⁵N chemical shifts used the 1H/X frequency ratios as indirect references, as reported in ref 21.

Mass Spectrometry Studies. MALDI-TOF mass spectra were obtained on a Voyager DE Pro mass spectrometer (Applied Biosystems). Samples were prepared as in ref 13.

Miscellaneous Procedures. Antibodies against recombinant CP12 and GAPDH from *C. reinhardtii* were obtained after rabbits were given three injections of the purified proteins. Protein concentrations were determined with the Bio-Rad protein dye reagent, using bovine serum albumin as standard. Native gels were performed on a Phast System apparatus, with 4–15% gradient gels (Pharmacia).

Proteins were transferred and revealed using GAPDH, CP12, or PRK antibodies.

RESULTS

C. reinhardtii CP12 is Similar to Higher Plant CP12. PCR on the cDNA library constructed from *C. reinhardtii* cw15 strain gave a sequence identical to that of the EST AV634344. Genomic DNA from the WM3[–] strain of *C. reinhardtii* could only be amplified with the primers corresponding to the sequence of the EST. N-terminal sequencing of CP12 within the PRK/CP12/GAPDH complex showed that the first amino acid residues of mature CP12 embedded in the complex were SGQPA. This sequence is identical to part of one translation frame of our PCR product. *C. reinhardtii* CP12 is highly homologous to other CP12s (Figure 1).

Recombinant CP12 is Mainly Oxidized and Contains two Disulfide Bridges. Mass spectrometry of purified recombinant CP12 with free thiol groups alkylated by iodoacetamide, showed a main peak at m/z 8743, corresponding to the unalkylated form of CP12. Given the small size of iodoacetamide, the absence of alkylation may be linked to the presence of two disulfide bridges. There were also two very weak peaks at m/z 8800 and 8856, corresponding to partially reduced forms (data not shown).

CP12 was reduced with 30 mM DTT at 30 °C for 1 h and the four thiol groups were alkylated by iodoacetamide. Thus, all thiol groups were accessible after reduction (data not shown).

Role of CP12 as a Protein-Linker in the Assembly of the PRK/CP12/GAPDH Complex. CP12 has been isolated from a few eukaryotic and prokaryotic photosynthetic organisms as part of multi-enzyme complexes and the function of this small protein in complex assembly is unknown.

The complex can be reconstituted in vitro using native isolated PRK and GAPDH (purified from the green alga). This reconstituted complex had the same kinetic properties as the native one purified from *C. reinhardtii* (11). Yet, no complex could ever be reconstituted in vitro using isolated native PRK and recombinant GAPDH. The main difference was the replacement of native isolated GAPDH, which is in fact a complex of GAPDH and CP12, by recombinant GAPDH (devoid of CP12). Reconstitution assays were performed with native isolated PRK, recombinant GAPDH, and recombinant oxidized CP12. The addition of the small protein ensured the assembly of the complex (Figure 2). Thus, CP12 acts as a linker that is essential for complex assembly.

CP12 may also be obtained under a reduced state after treatment with a strong reducing agent such as DTT. We therefore checked whether reduced CP12 could still reconstitute the complex in vitro. Native PRK, recombinant GAPDH, and reduced CP12 were concentrated together and the complex reconstituted in the presence of 5 mM Cys (Figure 2), but not in the presence of 1 mM DTT. Two possibilities arose. The simplest was that reduced CP12 could be reoxidized prior to complex assembly when the mild reducing agent was used, but not in the presence of a stronger reducing agent such as DTT. The other possibility was that reduced CP12 could reconstitute the complex with formation of its disulfide bridges at the contact of either GAPDH or PRK in the presence of Cys. This would be prevented by

Arabi1	1	KATSEG-----EISEKVEKSIQEAKETCA-DDPVS	SGECVA
Arabi2	1	KAAPEG-----GISDVVEKSIKEAQETCA-GDPVS	SGECVA
Pisum	1	-AAPE-----QISKKVEESI KSAQETCA-DDPVS	SGECVA
Spinacia	1	--APDN-----RISENVEKSIKEAQETCS-DDPVS	SGECVA
Nicot	1	SATPDN-----KLSDDLVAESVKEAEEACA-ENPVS	SGECVA
Arabi3	1	TKMREE-----KLSEMIIEEKVKEATEVCE-AEEMSE	EECRV
Chlamy	1	SGQPAV-----DLNKKVQDAVKEAEDACA-KG-TS	ADCAV
Nosto1	1	MTTTLN-----SFETAGAANLEEAITEAITEARTT	CELNGSDSANCAV
Nosto2	1	MTMTFNVAPSGANSNDNNGTTNLEKAILAAIAEA	ARTTCEQNGDGSFNCAV
Synec	1	MS--N-----IQEKIEQELANARQVCSTDEAS	PAECA
Thermo	1	MS--N-----LEKQIEQAREEAHKICDTEGATS	GQCAA
Tricho	1	MT--NN-----DIEEKIEQERQIARQACDISGANS	KECAA
Arabi1	35	AWDEVEEELSAAASHARDKKKAGG--SDPLEEY	CNDNPETDECRTYDN
Arabi2	35	AWDEVEEELSAAASHARDKKKADG--SDPLEEY	CKDNPETNECRTYDN
Pisum	33	AWDEVEEELSAAASHARDRKKE---SDPLEDY	CKDNPETDECKTYDN
Spinacia	33	AWDVVEEELSAAASHARDKAKD---VEPLEEY	CKDNPETDECRTYDN
Nicot	35	AWDVVEEELSAAASHARDKKKES---SDPLENY	CKDNPETDECRTYDN
Arabi3	35	AWDEVEEVSQARADLRIKLKLNLN--QDPLES	FCQENPETDECRIYED
Chlamy	34	AWDTVEEELSAAVSHKKDAVKADVTLTDP	LEAFCKDAPDADECRTYED
Nosto1	44	AWDIVVEELQAEKSDQQQ-AKAK---KNSLENY	CDRHPASVECLIYDV
Nosto2	50	AWDIVVEELQAEKSHQQQ-AQKR---KSSLES	FCDLHPEALECLIYDV
Synec	32	AWDAVEEELAEAAHQQR-QHPT---QTTLEK	FCDENPDAECRIYDD
Thermo	32	AWDALEELQAEAAHQRAEQQDH---KTSFQ	QYCDNDPDAAECRIYDD
Tricho	34	AWDAVEEELQAEASHQKE--QKP---KSSFE	IYCENNPAAECRIIDDN

FIGURE 1: Sequence of *C. reinhardtii* CP12 aligned with those of CP12s from other photosynthetic organisms and hypothetical sequences. Alignment was performed with ClustalW. Hypothetical sequences were obtained by BlastP with *C. reinhardtii* sequence as template. Arabi1, 2, and 3: *Arabidopsis thaliana* putative CP12s AAL32917, NP_191800, AAM62589, respectively; Pisum: *Pisum sativum*; Spina: *Spinacia oleracea*; Nicot: *Nicotiana tabacum*; Chlamy: *Chlamydomonas reinhardtii*; Nosto1 and 2: *Nostoc punctiforme* (ZP_00106092) and *Nostoc* sp. (NP_484948) (8), respectively; Synech: *Synechocystis* PCC6803; Thermo: *Thermosynechococcus elongatus* BP-1 (NP_682610) (7); Tricho: *Trichodesmium erythraeum* IMS101 (ZP_00071116). All accession numbers are from NCBI.

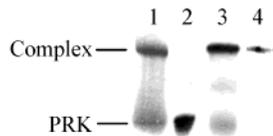


FIGURE 2: In vitro reconstitution assays with oxidized and reduced CP12. Proteins were transferred to a nitrocellulose membrane and revealed with antibodies raised against PRK. Lane 1: complex purified from *C. reinhardtii* (control; 2.4 μ g). Lane 2: in vitro reconstitution using native isolated PRK and recombinant GAPDH in a molar ratio of one dimer of PRK for one tetramer of GAPDH with 0.15 nmol of each (\approx 2 μ g). Lane 3: CP12 was added to the reconstitution mixture at a molar ratio of one monomer of CP12 for one dimer of PRK and one tetramer of GAPDH, using 0.15 nmol of each (\approx 2 μ g). Lane 4: Complex reconstitution assay with 5 mM Cys using reduced CP12, in the same molar ratio with 0.06 nmol of each (0.5 μ g).

DTT because of its higher reducing power. To test these possibilities, we incubated reduced CP12 with the reconstitution buffer supplemented with 5 mM Cys or 1 mM DTT overnight and alkylated CP12. 5 mM Cys did not prevent the formation of the two disulfide bridges (no alkylation), whereas in the presence of 1 mM DTT the four thiol groups were alkylated (data not shown).

Influence of CP12 on the Affinity of GAPDH for PRK. SPR studies with immobilized reduced PRK (ligand), and different concentrations of native GAPDH (GAPDH/CP12) (analyte) have shown that the dissociation constant (K_d) of the native GAPDH/PRK association is 62 nM (22). We determined the dissociation constants of different subcomplexes by SPR to find how oxidized CP12 acts in complex assembly. The K_d was higher (190 nM) when recombinant GAPDH (devoid of CP12) was used as analyte on immobilized PRK (ligand) than when native GAPDH was associated with PRK (Figure 3A).

The K_d was extremely low (0.44 nM) when oxidized His-CP12 was used as ligand and recombinant GAPDH as analyte (Figure 3B). The curves were fitted to a pseudo 1:1 Langmuir model. The estimated K_d was much higher when PRK was used as analyte (1.3 μ M) (Figure 3C). Repetition of the experiments with oxidized CP12 showed that the tag did not affect the specificity of interaction.

Wedel et al proposed a model in which the formation of the complex involved the dimerization of two monomers of CP12 (6). We tested this hypothesis by measuring the K_d of oxidized CP12 on itself. The value obtained was high (51 μ M). The values obtained for the K_d s are summarized in Table 1.

Structural Transitions of CP12 upon Reduction. To determine whether the specific role of oxidized CP12 in complex assembly, compared to reduced CP12, could be linked to structural transitions and not only to the presence of free thiol groups, we investigated the structure in solution of oxidized and reduced His-CP12 by CD and NMR.

CD spectra for oxidized CP12 showed two minima, at 222 and 205 nm, indicating helical structures (Figure 4). Yet, the maximum at 192 nm was smaller than expected for an all α -helix protein, probably because of the presence of random coils (minimum at 198 nm). The estimation of protein secondary structure indicated that CP12 is composed of α -helix with some random coil. Recombinant reduced CP12 was also analyzed by CD and NMR to determine the effect of reducing the two disulfide bridges on the structure of the protein. The CD spectra showed that CP12 underwent a dramatic conformational change after reduction and became even more disordered. The α -helix content decreased, while those of random coils and undetermined structures increased (Figure 4).

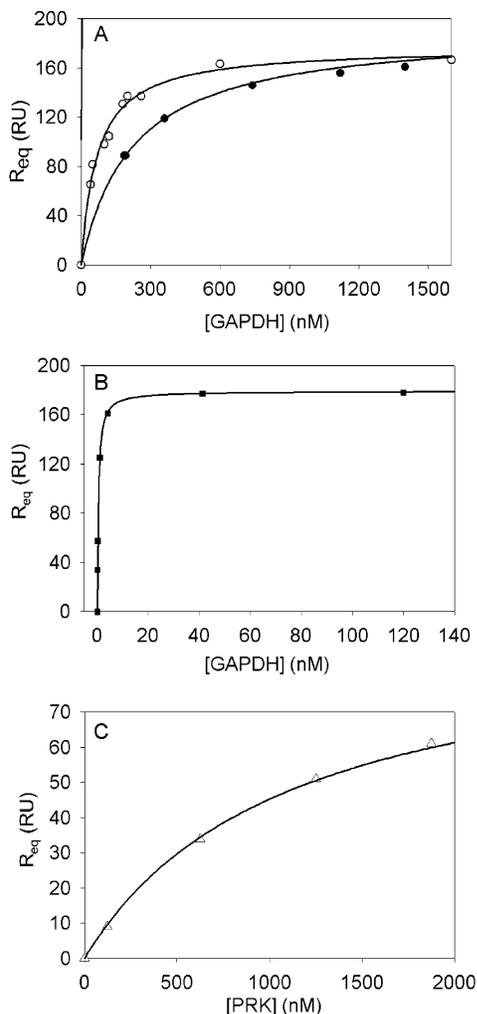


FIGURE 3: SPR binding experiments. The response at equilibrium R_{eq} (in resonance unit (RU)) was reported as a function of the analyte concentration. The experimental points were fitted to a hyperbola: $R_{eq} = (R_{max}[GAPDH]) / (K_d + [GAPDH])$. Scatchard plots ($R_{eq}/[GAPDH]$ versus R_{eq}) were also used and the slope gave the same estimations (data not shown). (A) PRK was used as a ligand and native GAPDH (○) or recombinant GAPDH (●) were used as analyte. (B and C) Oxidized His-CP12 was immobilized (ligand) and recombinant GAPDH (B) or native PRK (C) were used as analyte. The errors on the estimated parameters were less than 10% in all cases.

Table 1: Dissociation Constants Obtained by Surface Plasmon Resonance in Bold, the Lowest K_d s Obtained

analyte	CP12 _{ox} (M)	PRK (M)
CP12 _{ox}	0.51×10^{-4}	nd
GAPDH _{rec.}	0.44×10^{-9}	0.19×10^{-6}
PRK	0.13×10^{-5}	nd
GAPDH _{nat.}	nd	0.62×10^{-7}

To gain further insight into the structural transition of CP12 upon reduction and the mobility of reduced CP12, we also examined the effects of reduction by NMR. Figure 5 shows the HSQC spectrum of an oxidized sample of ^{15}N -CP12. Most of the resonances are localized in the range 8.5–8 ppm. The resonances of CP12 have not been assigned due to the superimposition of these signals and the absence of any nOe transfer in homonuclear NOESY experiments (data not shown). However, a first general conclusion on CP12 properties could be drawn from the spectra. Only about a

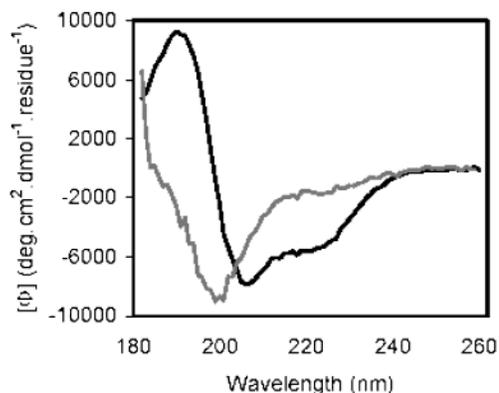


FIGURE 4: CD studies showing the structural changes of His-CP12 upon reduction. Spectra were obtained with $8 \mu\text{M}$ of oxidized His-CP12 (black line) or reduced His-CP12 (grey line) in phosphate buffer pH 6.0.

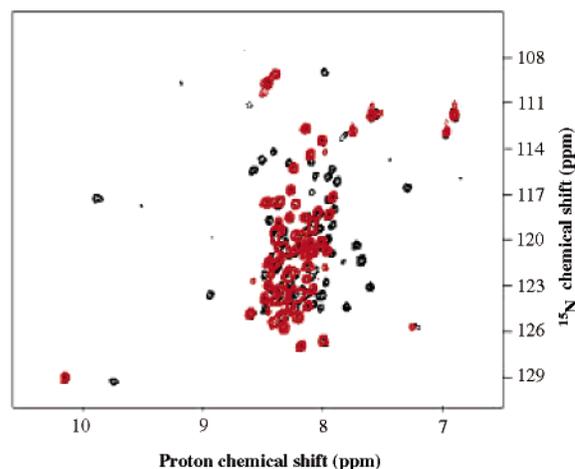


FIGURE 5: ^{15}N -HSQC spectra of the oxidized and reduced form of *C. reinhardtii* His-CP12. In black: HSQC spectrum at 400 MHz of oxidized His-CP12 (0.9 mM) at 27 °C in 50 mM NaCl, 50 mM phosphate buffer, pH 6.0; four transients by t1 increment. In red: HSQC spectrum at 400 MHz of reduced CP12 (0.3 mM) at 27 °C in 50 mM NaCl, 50 mM phosphate buffer pH 6.0, 10 mM DTT; 16 transients by t1 increment.

third of the residues exhibit chemical shifts which are far from the random coil values (Figure 5), suggesting that CP12 is only partially structured. These features, together with the absence of nOe transfer strongly suggest that CP12 exhibits highly dynamic properties. This is further corroborated by ^1H - ^{15}N nOe experiments which show only very weak positive heteronuclear nOe effects indicative of high mobility (data not shown).

The reduction of oxidized CP12 caused a very large change in the NMR spectrum. All the correlations in the HSQC having chemical shift values different from those of a random coil disappeared, and all the chemical shift values of the correlations were those for a typical unstructured peptide (Figure 5). In addition, all the heteronuclear nOe effects became very negative, indicating that reduced CP12 was much more mobile than the oxidized form. Thus, CP12 became completely unstructured upon reduction, in agreement with the CD experiments.

DISCUSSION

CP12 Is a Conserved Protein Involved in Complex Assembly. We have cloned the full sequence of CP12 cDNA

from the unicellular green alga *C. reinhardtii*. It is highly homologous to previously sequenced CP12s from higher plants and the cyanobacterium *Synechocystis PCC6803* (4–6) (Figure 1). All CP12s cloned to date have four cysteine residues which form two intramolecular disulfide bridges, one between cysteines 1 and 2 and the other between cysteines 3 and 4 (6). We confirmed the presence of these residues in *C. reinhardtii* CP12, in particular cysteines 1 and 4, which had not been observed in the partial sequence previously obtained (5). Sequence alignment shows major differences between higher plant CP12s, cyanobacterial hypothetical and *C. reinhardtii* sequences. First, the presence of a proline residue between each pair of cysteines in higher plant CP12s is probably responsible for the formation of two loops in oxidized CP12 (4, 6). The prokaryotic and algal CP12s lack the proline residue between cysteines 1 and 2, although the proline residue between Cys 3 and 4 is present in the CP12 of all organisms. Second, the CP12s of higher plants have a set of compensating charged residues at their N-terminus that may enable the protein to form dimers by electrostatic interactions (6). The hypothetical sequences of cyanobacteria lack the charged residues responsible for the dimerization of CP12. This “motif” may be present in *C. reinhardtii* CP12, but the amino acid residues putatively involved are different from those of higher plants. The most conserved part is a region of 10 residues between cysteines 2 and 3 (Figure 1), suggesting that this region may play another, yet undefined, functional role. This study is currently under investigation.

We investigated the role of CP12 in the assembly pathway of the PRK/CP12/GAPDH complex. Our *in vitro* reconstitution assays show that oxidized CP12, in addition to modifying GAPDH kinetic properties and probably conformation (13), also acts as a linker in the assembly of the PRK/CP12/GAPDH complex. The formation of this complex may be a prerequisite for light-activation of the enzymes and subsequent docking of the other enzymes of the Benson-Calvin cycle as higher supramolecular complexes have been described (23–26). Complex reconstitutions with reduced CP12 were possible in the presence of 5 mM Cys, but not 1 mM DTT (data not shown). In the presence of the weak reducing agent (5 mM Cys), the disulfide bridges form again prior to complex reconstitution. These results corroborate those obtained by Wedel et al, who showed that CP12 mutants in which Cys 1 and/or 4 were mutated into Ser failed to reconstitute the complex (6). They confirm that the presence of disulfide bridges is essential for complex reconstitution. Furthermore, alkylation of the native complex shows that CP12 within this complex is present under an oxidized form (data not shown).

We have performed SPR binding experiments to gain further insight into the action of CP12 and into the assembly pathway of the PRK/CP12/GAPDH complex. We have tested different combinations using two partners, one ligand and one analyte, to determine which couple of partners had the lowest dissociation constant or the highest affinity. When CP12 was used, these studies also enabled us to determine the role of CP12 in the assembly of the complex. On the basis of the following data from SPR binding experiments, *in vitro* reconstitution assays and the result of purifying native enzymes, we propose a model for the assembly of the PRK/CP12/GAPDH complex and the role of CP12:

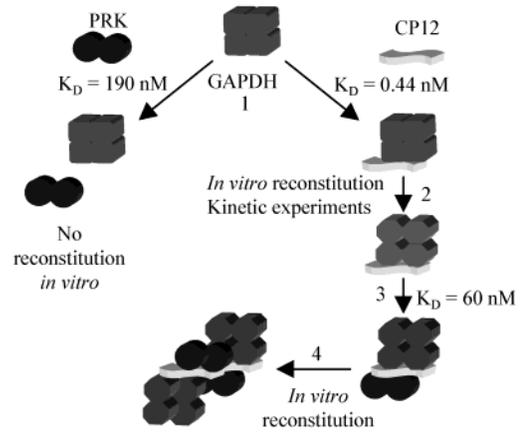


FIGURE 6: Model describing the assembly of PRK/CP12/GAPDH complex of *C. reinhardtii*. (1) GAPDH has a much higher affinity for CP12 than for PRK and thus associates with the small protein to form a very stable complex. (2) Binding of CP12 to GAPDH causes the conformation of GAPDH to change (13). According to Wedel et al (6), CP12 binds to GAPDH through its C-terminus. (3) GAPDH acquires a higher affinity for PRK and forms a subcomplex composed of one dimer of PRK, one tetramer of GAPDH and one monomer of CP12. As described by Wedel et al (6), we assume that PRK interacts with the GAPDH/CP12 complex via the N-terminus of CP12. (4) The final complex may finally be obtained by dimerization. The interactions involve CP12/CP12 contacts and additional enzyme/enzyme interactions.

(i) CP12 does not co-purify with PRK when the native enzyme is isolated. The notion of a weak interaction between PRK and CP12 is also supported by the high value of the K_d for the interaction of PRK with oxidized CP12 (1.3 μM). Thus, PRK does not seem to interact with CP12 to form a stable PRK/CP12 complex.

(ii) On the contrary, native isolated GAPDH co-purifies with CP12 and forms a very stable GAPDH/CP12 complex. We have previously shown that the association of CP12 with recombinant GAPDH changes the kinetic properties of the enzyme (13). The K_d for the interaction of oxidized CP12 and GAPDH is extremely low (0.44 nM). Furthermore, *in vitro* reconstitution assays using recombinant GAPDH and PRK, but no CP12, show that these two enzymes do not form a stable complex. The K_d for the interaction of recombinant GAPDH (devoid of CP12) with PRK is very high (0.19 μM) compared to the one obtained for the interaction of the GAPDH/CP12 (native GAPDH) complex with PRK (62 nM). These results show that the GAPDH that is bound to CP12 and has undergone a conformation change has more affinity for PRK than does GAPDH alone.

Thus, the first step in the assembly of the complex is the association of CP12 with GAPDH and the subsequent change of the conformation of the enzyme. This GAPDH/CP12 complex then becomes associated with PRK to form a half-complex (Figure 6), defined as one unit. This unit must then dimerize to give the native complex composed of two dimers of PRK, two tetramers of GAPDH and, probably, two monomers of CP12. The stoichiometry was determined by immunoblots (data not shown). Wedel et al proposed that dimerization involves electrostatic interactions between negatively and positively charged residues of the N-terminal of CP12 (6). The K_d for the dimerization of oxidized CP12 by SPR is high (51 μM). Yet, CP12 probably undergoes an poorly structured to structured transition upon association with GAPDH and PRK and the K_d for the dimerization of

CP12 could change. However, prokaryotic CP12s do not have such oppositely charged amino acid residues, and given the small size of CP12 we propose that the dimerization of the complex involves both contacts between the two enzymes and the two CP12s of each unit (Figure 6).

Redox States and Structural Transitions of CP12. We have shown that reduced CP12 was not able to reconstitute the complex. To link these results with a possible structural transition of CP12 upon reduction, we have analyzed the conformations of CP12 in solution by CD and NMR. The CD spectra show that oxidized CP12 has a secondary structure, mainly composed of α helices plus some random coils. The NMR experiments, however, indicate that CP12 does not adopt a rigid 3D structure in solution. The heteronuclear nOe experiments on His-CP12 indicate that the secondary structures are mobile one respect to another in solution. Thus, even if CP12 has secondary structures, their relative mobility makes the resolution of its 3D structure most unlikely and the NMR datasets obtained preclude the construction of a model. Since recombinant CP12 had two disulfide bridges, we reduced the protein with DTT and studied the effect of reduction on the structure of CP12. The structure of His-CP12 changes dramatically upon reduction; it loses almost all its α -helices and becomes highly disorganized, almost unfolded and much more flexible and mobile than oxidized CP12. These results show that flexible oxidized CP12 may act as a linker in complex formation, whereas reduced and unstructured CP12 loses its capacity to form the complex. Such structural transitions with loss of function are also observed with small proteins, such as prion proteins. Upon transition from the normal form to the scrapie (infectious) form, the prion protein undergoes a structural transition from α -helical to β -sheet structures, as shown by CD and FTIR experiments (27).

The lack of overall organization and the great flexibility of oxidized CP12 observed by NMR and CD are in good agreement with the role of this protein as linker. The flexibility and the net negative charges of CP12 may increase its reactive area and its "stickiness", compared to rigid partners. It thus facilitates specific interactions and increases the rates of specific macromolecular interactions (28, 29). Such behavior resembles that of "intrinsically unstructured proteins" or "IUPs" (30–32). These proteins are often involved in protein–protein interactions and play major roles in cellular functions, such as DNA and RNA binding, transcriptional regulation, signal transduction, and cell cycle regulation (30). They are found in a wide variety of macromolecular complexes: Nup2p and the nuclear pore complex (33), Securin and cyclin B (34), complexins and SNARE complexes (35–37), IF1 and the regulation of ATPases (38–40), p21 or p27kip1 and regulation of CDK2, cyclin A or PCNA (41, 42). Like CP12, their flexibility favors the binding of several partners and the possibility of presenting larger interaction surfaces, compared to rigid partners. A recent survey of several genomes indicated that 34% of all proteins have at least one disordered segment (32). CP12 proteins thus appear as one of the few known IUPs specific to plants, in addition to Dsp16, a dehydrin-related protein, involved in the response to abiotic stresses such as drought, low temperature, and salinity (43), and a manganese-stabilizing protein (MSP) of 33 kDa which is part of photosystem II (44).

Interestingly, IUPs display residual secondary structures and hence are not true random-coil proteins (29). It has been speculated that these residual structures would be the same as those observed in the complex (31). CP12 probably has the same behavior and should become structured upon association with the other proteins of the complex. NMR techniques are now accurate enough to study the structure of a small [^{15}N] labeled protein that forms part of a macromolecular complex (45). This matter is currently under investigation.

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