Spontaneous Assembly of Photosynthetic Supramolecular Complexes as Mediated by the Intrinsically Unstructured Protein CP12*

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CP12 is a protein of 8.7 kDa that contributes to Calvin cycle regulation by acting as a scaffold element in the formation of a supramolecular complex with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK) in photosynthetic organisms. NMR studies of recombinant CP12 (isoform 2) of Arabidopsis thaliana show that CP12-2 is poorly structured. CP12-2 is monomeric in solution and contains four cysteines, which can form two intramolecular disulfides with midpoint redox potentials of −326 and −352 mV, respectively, at pH 7.9. Site-specific mutants indicate that the C-terminal disulfide is involved in the interaction between CP12-2 and GAPDH (isoform A4), whereas the N-terminal disulfide is involved in the interaction between this binary complex and PRK. In the presence of NAD, oxidized CP12-2 interacts with A4-GAPDH (KD = 0.18 μM) to form a binary complex of 170 kDa with (A4-GAPDH)-(CP12-2) stoichiometry, as determined by isothermal titration calorimetry and multivariate light scattering analysis. PRK is a dimer and by interacting with this binary complex (KD = 0.17 μM) leads to a 498-kDa ternary complex constituted by two binary complexes and two PRK dimers, i.e., (A4-GAPDH)-(CP12-2)2-(PRK)2. Thermodynamic parameters indicate that assembly of both binary and ternary complexes is exoeogenic although penalized by a decrease in entropy that suggests an induced folding of CP12-2 upon binding to partner proteins. The redox dependence of events leading to supramolecular complexes is consistent with a role of CP12 in coordinating the reversible inactivation of chloroplast enzymes A4-GAPDH and PRK during darkness in photosynthetic tissues.

The photosynthetic reduction cycle for carbon organization (Calvin cycle) is a finely regulated metabolism that plants keep tuned with light reactions of photosynthesis under variable environmental conditions. Thioredoxins and metabolic intermediates play essential signaling roles within this complex regulatory system (1). Two nonconsecutive enzymes of the Calvin cycle, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK), are regulated in this way, both individually and through the reversible formation of supramolecular complexes (2–8).

Chloroplast GAPDH is mainly heteromeric in land plants, with homologous A and B subunits occurring in stoichiometric ratio (9, 10). The B-subunits confer regulatory properties, and the enzyme oscillates between a fully active A2B4 tetramer at one extreme and a partially inhibited A8B8 hexadecamer at the other (11). Partially polymerized intermediates like A4B4 have also been reported (12–14). Thioredoxins and metabolites directly regulate AB-GAPDH activity and strongly affect the equilibrium between active tetramers and aggregated forms (8, 10).

A second isoform of chloroplast GAPDH in land plants is a stable homotetramer of A-subunits (A4-GAPDH) (9, 15), similar to Calvin cycle GAPDH of lower photosynthetic organisms (5, 6, 16). A4-GAPDH only accounts for a minor portion of total chloroplast GAPDH activity in land plants (14, 15). Due to the absence of B-subunits, A4-GAPDH is not directly regulated by thioredoxins and metabolites (8, 17, 18), although the reversible glutathionylation of the active site cysteine 149 provides a mechanism of A4-GAPDH regulation that may be relevant under stress (19). Alternatively, reversible down-regulation of A4-GAPDH activity can be achieved through formation of a supramolecular complex with PRK and the regulatory peptide CP12 (2–8, 20). In land plants, PRK itself undergoes a light/dark modulation mediated by thioredoxins (21), but once incorporated into the complex, both A4-GAPDH and PRK activities are inhibited in a coordinated manner (7). Similar to A8B8-GAPDH, the stability of the supramolecular complex involving A4-GAPDH, CP12, and PRK is controlled by thioredoxins and several cofactors, including NAD(H) and NADP(H), ATP, and 1,3-bisphosphoglycerate (3–7). It is generally agreed that aggregated forms of GAPDH (A8B8) and GAPDH-CP12-PRK complexes are prevalent in chloroplasts in the dark, whereas illumination favors the accumu-

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2 The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PRK, phosphoribulokinase; ITC, isothermal titration calorimetry; MAL5, multivariate light scattering; QELS, quasirelaxing light scattering; CTE, C-terminal extension of GAPDH subunit B; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).
luation of fully active GAPDH tetramers ($A_2B_2$, $A_4$) and PRK dimers (4–6, 8, 12, 13, 22, 23).

The acronym CP12 refers to small proteins of nearly 80 amino acids, widespread in oxygenic photosynthetic organisms (24, 25), apparently lacking an ordered structure in solution (20). Interestingly, the C-terminal half of CP12 is closely related to the C-terminal extension (CTE) of GAPDH B-subunits (24). Both the CTE and the C-terminal part of CP12 contain a couple of conserved cysteines, which can form an intramolecular disulfide and are potential targets of thioredoxin regulation (3, 8, 10, 17, 26–28). In addition, most CP12 proteins contain a second pair of conserved cysteines in the N-terminal half of the molecule also able to form a disulfide bond (2, 25). However, the redox properties of CP12 disulfide bonds are unknown, and it is difficult to predict whether CP12 could exist in different redox states in vivo. Studies on the green algae *Chlamydomonas reinhardtii* (20) and the flowering plant *Arabidopsis thaliana* (7) help to trace the sequence of events during the formation of CP12-mediated supramolecular complexes. First, $A_4$-GAPDH (in complex with NAD) interacts with oxidized CP12, and then oxidized PRK can participate in the assembly of the ternary complex, resulting in strongly inhibited enzyme activities.

In this paper, we shall investigate some biochemical-molecular features of *A. thaliana* CP12-2 and properties of the supramolecular complexes it forms with $A_4$-GAPDH and PRK. The CP12-2 isoform, produced by one of three CP12 genes known for this species (8), was chosen as a model, since the expression pattern of the CP12-2 gene in different *Arabidopsis* organs and conditions strictly followed that of GAPDH, PRK, and other Calvin cycle genes (8, 29). The resulting model supports the view that these supramolecular complexes represent an instrument for photosynthetic organisms to finely modulate Calvin cycle turnover in response, for example, to changes in light intensity as commonly occur in natural environments and to safely and reversibly store photosynthetic enzymes in an inactive conformation during the night.

**EXPERIMENTAL PROCEDURES**

*Protein Expression and Purification*—Heterologous expression and purification of recombinant $A_4$-GAPDH (At3g26650), PRK (At1g32060), CP12-2 (At3g62410), and CP12-2 site-specific mutants of *A. thaliana* were performed as described (7). NMR analyses were performed on uniformly $^{15}$N-labeled His-tagged CP12-2 samples obtained by transformed *Escherichia coli* BL21 (DE3) cells grown in M9 minimal medium containing 1 g/liter $^{15}$NH$_4$Cl (Euriso-top, France) as the sole nitrogen source. An overnight culture of 25 ml in M9 medium was transferred to 500 ml of fresh M9 medium, both supplied with kanamycin (50 $\mu$g/ml) and grown at 37 °C under shaking. When optical density at 600 nm reached 0.6–1.0 units, expression was induced by the addition of 0.4 mM isopropyl-β-D-thiogalacto-pyranoside. Since M9 minimal medium prevented rapid cell growth, the induction phase was prolonged for 15 h before cells were collected by centrifugation (10,000 rpm; 15 min). CP12-2 was purified from the resulting pellet as previously described (7). Purified proteins were quantified by absorbance at 280 nm (7), desalted in appropriate buffers, and stored at $-20$ °C. Concentrations of purified proteins are all referred to native con-

formsations (CP12-2 monomers, $A_4$-GAPDH tetramers, and PRK dimers).

**CP12-2 Site-specific Mutants**—Site-specific mutants of recombinant CP12-2 were obtained as previously described (26). PCR primers were the following (mutation sites are underlined): C22S(up), 5'-AACGCTAGGAGACCTTGCAGCCGATCC-3'; C22S(down), 5'-ATCGCCCAGAAGCTCTGAGCTTCC-3'; C73S(up), 5'-ACAATCTGTGACCAACAGGAGTTCCGTACTTACG-3'; C73S(down), 5'-TGTTGTAAGTACGGGACTCCTTTGCTACAGG-3'. The presence of mutations was confirmed by DNA sequence analysis.

**NMR Spectra**—Uniformly $^{15}$N-labeled CP12-2, provided with His tag, was oxidized by the addition of 20 mM oxidized DTT (Sigma). After 16–18 h of incubation at 4 °C, the sample was desalted in 25 mM potassium phosphate buffer, pH 7.0, and concentrated. NMR samples were typically 300 or 600 µl of 1 mM CP12-2 solution in 25 mM potassium phosphate buffer, pH 7.0, 5% (v/v) 2H$_2$O, 0.05% (w/v) sodium azide.

Two-dimensional $^1$H-$^1$N HSQC (30) spectra were recorded with 256 and 2048 complex points in F1 and F2 dimensions, respectively, at 20 °C on a Bruker Avance 800-MHz spectrometer equipped with a triple-resonance ($^1$H, $^{13}$C, $^{15}$N) probe, including field $xyz$ gradients.

Spectra were processed using Topspin version 1.3 (Bruker). Chemical shifts were referenced to internal $d_5$-3-(trimethylsilyl) propionic acid, according to Ref. 31.

**Analysis of Thiol Groups and Redox Titration of CP12-2**—Protein thiol analyses and redox titrations were performed with pure CP12-2 in 100 mM Tricine-NaOH, pH 7.9. Redox titration experiments were performed with 70 µM CP12-2 incubated for 3 h at 25 °C with variable ratios of reduced and oxidized DTT (20 mM total concentration) in a final volume of 500 µl. After incubation, samples were desalted with PD10 columns (GE Healthcare) equilibrated with 100 mM Tricine-NaOH, pH 7.9. In order to avoid any possible DTT contamination, only the first 2 ml of eluted samples were collected. Control experiments were performed under the same conditions but in the absence of CP12-2. Absorbance at 280 and 412 nm was recorded immediately before and after the addition of 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The number of solvent-accessible thiol groups under different redox conditions was calculated from the ratio between the absorbance at 412 nm (molar extinction coefficient of 14,150 M$^{-1}$cm$^{-1}$ for 2-nitro-5-thiobenzoate (thiolate) dianion) (32) and the absorbance at 280 nm (molar extinction coefficient of 8,370 M$^{-1}$cm$^{-1}$ for CP12-2) (7).

Redox titration results were fit by nonlinear regression (CoHort Software) to the Nernst equation for two redox components (26). Midpoint redox potentials are reported as average values ± S.D. of triplicate experiments.

**Isothermal Titration Calorimetry (ITC)**—Calorimetric measurements were carried out using a VP-ITC MicroCalorimeter (MicroCal Inc., Northampton, MA). Each experiment was performed at a constant temperature of 30 °C and consisted of 25 injections of 10-µl aliquots, repeated every 200 s. All samples were degassed by stirring under vacuum before use. Heat of dilution, measured by control experiments in which samples were injected into a buffer-filled cell, was subtracted. Signals recorded in each experiment were integrated using OriginPro.
CP12-mediated Assembly of Supramolecular Complexes

The presence of ligand (CP12-2) in the cell and macromolecule (A4-GAPDH) in the syringe was taken into account in the elaboration of primary data.

Calorimetric titrations of ternary complex formation were performed with 5 μM preformed binary complex in 25 mM potassium phosphate, 0.2 mM NAD, pH 7.5, in sample and reference cells, whereas the syringe was filled with 70 μM PRK dissolved in the same buffer. Thermodynamic parameters of binary and ternary complex formation are reported as average values ± S.D. of triplicate experiments.

**Dynamic Light Scattering (MALS-QELS)**—Purified single proteins and preformed binary and ternary complexes were analyzed by size exclusion chromatography connected to a multiangle light scattering (MALS) equipped with QELS module (quasielastic light scattering) for R_h measurements. 100-μl samples were loaded on a Superdex 200HR column (GE Healthcare) equilibrated in 25 mM potassium phosphate, pH 7.5, 1 mM EDTA, 150 mM KCl, with 0.2 mM NAD (for A4-GAPDH, binary, and ternary complexes) or without NAD (for PRK and CP12-2). A constant flow rate of 0.6 ml min⁻¹ was applied. Elution profiles were detected by an Optilab rEX interferometric refractometer and a Dawn EOS multiangle laser light-scattering system at 690 nm (Wyatt Technology Corp.). Data acquisition and processing were carried out using ASTRA 5.1.9.1 software (Wyatt Technology). Determination of molecular masses and hydrodynamic radii are reported as mean values ± S.D. of duplicate experiments.

**RESULTS**

CP12-2 of A. thaliana Is Intrinsically Unstructured and Monomeric—NMR analysis of CP12-2 in the oxidized state revealed that most of the amide proton resonances are localized between 8.5 and 8.0 ppm, the so-called random coil region, strongly suggesting that CP12-2 is mainly unstructured (Fig. 1). Only a few residues located at the C terminus exhibit amide chemical shifts outside the random coil range, indicating structuration (33). Consistent with the importance of disulfide bridges in this respect, the reduction of oxidized CP12-2 by DTT led to typical random coil signals (not shown), as previously described for *Chlamydomonas* CP12 (20).

The calculated molecular mass of recombinant CP12-2 (after proteolytic cleavage of the His tag) was 8.7 kDa. Although oxidized CP12-2 behaves as a protein of 29 kDa in size exclusion chromatography (7), MALS-QELS analysis of the protein eluted from the size exclusion column yielded a molecular mass of 9 ± 1 kDa, conclusively demonstrating that under native conditions, CP12-2 is a monomer (Table 1).

**TABLE 1**

| Sample                     | Calculated | Measured |
|---------------------------|------------|----------|
|                           | m          | R_h m    |
|                           | Da         | nm       |
|                           |            |          |
| CP12-2                    | 8.7        | 1.63     |
| A4-GAPDH                  | 148.8      | 4.21     |
| PRK                       | 77.6       | 3.39     |
| (A4-GAPDH) + (CP12-2)     | 166.2      | 4.36     |
| (A4-GAPDH) + (CP12-2) + (PRK) | 1833 | 6.25     |

* The theoretical hydrodynamic radius R_h for a spherical protein of a given M_w was calculated on the basis of the empirical equation M_w = 4/3πR_h³/V_p, where N_A is Avogadro’s number, f/b is the ratio of frictional coefficients (set to 1.2 for spherical proteins), and V_p is the partial volume set to 0.73 for a spherical protein (45).

* Since chromatographic runs were performed in the presence of 0.2 mM NAD, molecular masses were calculated on the assumption that each A4-GAPDH tetramer bound four NAD molecules.

* Calculated molecular mass for a binary complex with stoichiometry (A4-GAPDH)-(CP12-2)₂.

* Calculated molecular mass for a ternary complex with stoichiometry (A4-GAPDH)-(CP12-2)₂-(PRK)₂.

7.5 software supplied with the instrument. The thermodynamic binding parameters (dissociation constant (K_D), variations of enthalpy (ΔH), Gibbs free energy (ΔG), entropy (ΔS), and the number of binding sites (n)) were obtained by nonlinear regression of the integrated heat plots, according to the “one set of sites” model of the software.

Calorimetric titrations of binary complex formation were carried out with 15 μM oxidized CP12-2 in 25 mM potassium phosphate, 0.2 mM NAD, pH 7.5, in both sample and reference cells, whereas the syringe was filled with 52 μM А4-GAPDH in the same buffer. Although CP12-2 behaved as a ligand, it was not filled in the syringe because of its very high heat of dilution.

FIGURE 1. NMR spectra of CP12-2. Two-dimensional 1H-15N HSQC spectra of the His-tagged oxidized CP12-2 from *A. thaliana* recorded at 1H = 800 MHz and 20 °C with 16 transients by t1 increment.
**CP12-mediated Assembly of Supramolecular Complexes**

**CP12 Redox Properties**—To gain insight into the redox properties of CP12-2 cysteines, redox titrations were performed in the presence of DTNB as a probe to reveal free protein thiols under varying redox conditions. Fully reduced/oxidized samples were obtained following equilibration with 20 mM reduced or oxidized DTT, which was then removed by desalting. Although reduced CP12-2, whose amino acid sequence includes four Cys residues, was found by DTNB titration to contain four reactive thiols (4.5 ± 0.5), oxidized CP12-2 had none (−0.3 ± 0.1), indicating that both CP12-2 disulfides could be redox-titrated by DTT plus DTNB. Data from redox titrations of purified CP12-2 were therefore fitted to a Nernst equation for two different thiol/disulfide equilibria, equally contributing to the total redox response. At pH 7.9, the midpoint redox potentials ($E_{m,7.9}$) of CP12-2 disulfides were estimated as −326 ± 2 and −352 ± 6 mV, respectively (Fig. 2).

**Interaction between A$_4$-GAPDH, CP12-2, and PRK**—Size exclusion chromatography carried out in the presence of NAD, combined with MALS-QELS analysis, demonstrated that a stable binary complex of 170 ± 14 kDa was reconstituted by incubating oxidized CP12-2 (9 ± 1 kDa; MALS) with A$_4$-GAPDH complexed with NAD (146 ± 2 kDa; MALS; Table 1). Since MALS data are independent of protein conformational effects, they do suggest that two CP12-2 molecules can bind to one A$_4$-GAPDH tetramer, giving rise to an (A$_4$-GAPDH)-(CP12-2)$_2$ binary complex (calculated molecular mass = 166 kDa; Table 1).

The affinity between A$_4$-GAPDH and CP12-2 was analyzed by isothermal titration calorimetry (ITC) (Fig. 3). Such interaction was characterized by a $K_a$ of 0.18 ± 0.02 μM. In agreement with MALS analysis, two binding sites for CP12-2 were detected per A$_4$-GAPDH tetramer ($n = 1.9 ± 0.2$), with no evidence for different affinities between binding sites. Binding of two CP12-2 molecules to each A$_4$-GAPDH tetramer was exothermic ($\Delta H = −15$ kcal mol$^{-1}$), and although leading to a simultaneous decrease in entropy ($T\Delta S = −5$ kcal mol$^{-1}$), the process was exoergic overall ($\Delta G = −9.4$ kcal mol$^{-1}$; Table 2).

The interaction between oxidized PRK and preconstituted (A$_4$-GAPDH)-(CP12-2)$_2$ binary complexes was also investigated by multinic light scattering. Binding of PRK (85 ± 7 kDa; MALS) to the binary complex gave rise to a ternary complex of 498 ± 6 kDa (MALS), likely to include two dimers of PRK plus two (A$_4$-GAPDH)-(CP12-2)$_2$ binary complexes (calculated molecular mass = 488 kDa; Table 1). The stoichiometric ratio of two A$_4$-GAPDH subunits per PRK subunit in the 498-kDa ternary complex was well compatible with the relative intensities of Coomassie-stained bands in denaturing gel electrophoresis (not shown).

Thermodynamic parameters of the interaction between PRK and (A$_4$-GAPDH)-(CP12-2)$_2$ were also determined by ITC (Fig. 3).
TABLE 2
Thermodynamic parameters of binary and ternary complex formation at 30 °C, as determined by ITC

| Complex | $n$ | $K_D$ | $\Delta H^\circ$ | $T\Delta S^\circ$ | $\Delta G^\circ$ |
|---------|-----|-------|------------------|------------------|-----------------|
| (A$_x$-GAPDH) + CP12-2 | 1.9 ± 0.2 | $0.18 \pm 0.02$ | $-15 \pm 2$ | $-5 \pm 2$ | $-9.4 \pm 0.1$ |
| (A$_x$-GAPDH)-(CP12-2)$_2$ + PRK | 1.3 ± 0.1 | $0.17 \pm 0.09$ | $-20 \pm 4$ | $-11 \pm 3$ | $-9.3 \pm 0.2$ |

* Thermodynamic parameters of association refer to mol of A$_x$-GAPDH (first line) or PRK (second line).
* CP12-2 molecules per A$_x$-GAPDH.
* PRK dimers per (A$_x$-GAPDH)-(CP12-2)$_2$ binary complexes.

DISCUSSION

CP12 is a widespread regulatory protein of oxygenic photosynthetic organisms that contributes to the regulation of carbon metabolism by producing supramolecular complexes with two enzymes, GAPDH and PRK, accounting for most of the energetic needs of the Calvin cycle (2, 3). Why land plants, which possess distinct systems to regulate both GAPDH (mainly AB isoform) and PRK under variable light conditions (1, 8), need to maintain this additional and more ancient regulatory system based on CP12 is a matter of debate. It has been argued that CP12 provides plasticity and coordination to the regulatory network modulating the Calvin cycle (7); the minor isoform A$_x$-GAPDH is unregulated in the absence of CP12, and PRK too becomes more sensitive to modulators (e.g. 1,3-bisphosphoglycerate and pyridine nucleotides) when embedded in the complex. Moreover, CP12 could link PRK also to the more abundant AB isoform of GAPDH (3, 4), thereby further contributing to the coordinated control of the Calvin cycle. However, since antisense tobacco plants with reduced levels of CP12 show a severely altered leaf morphology (36), we should
assume that the function of CP12 might well extend beyond the Calvin cycle. Interestingly, a recent analysis of protein-protein interaction maps from different eukaryotes revealed that poorly structured proteins (i.e., they interact with many partners and often have regulatory functions) (37). The interaction of CP12 with partners other than just GAPDH and PRK, although speculative, may be a possible explanation for the aberrant leaf morphology of CP12 antisense tobacco plants (36).

Although the fully oxidized form of chloroplast CP12-2 of *A. thaliana* contains two intrachain disulfide bridges (Fig. 1), solution NMR studies show that it is a poorly structured protein even under oxidizing conditions, attaining almost complete unstructuration when the cysteines are reduced. This lack of structure was supported by several bioinformatic predictors based on different algorithms (38) (not shown). Like CP12 of *C. reinhardtii* (21), also *A. thaliana* CP12-2 can thus be considered an intrinsically unstructured protein (35, 39), and this property related to its regulatory function (20, 34, 37, 40). In contrast with other reports (3, 41) but consistent with its disordered nature, the CP12-2 failed to interact with itself, as shown by dynamic light scattering analysis (MALS-QELS). Previous and significantly higher estimates of CP12 molecular mass based on size exclusion chromatography (29–70 kDa) (3, 6, 7) were apparently biased by the intrinsic disorder of CP12 (39).

Oxidized CP12-2 and A4-GAPDH from *Arabidopsis* interact together with a dissociation constant in the submicromolar range (*K_D* = 0.18 μM measured by ITC) (i.e., a 450-fold lower affinity with respect to *Chlamydomonas* (*K_D* = 0.4 nM, surface plasmon resonance)) (20). The exceptionally high stability of the complex in *Chlamydomonas* may be related to the inhibitory effect of CP12 on GAPDH activity in this species (28, 42), whereas little inhibition, if any, was observed in the plant protein unless PRK was also recruited in a ternary complex (7). A4-GAPDH is the only chloroplast GAPDH isoform in *C. reinhardtii* (25), and CP12 may uniquely provide a way to regulate GAPDH activity in these green algae, whereas *Arabidopsis* and land plants in general also contain an AB-GAPDH isoform, which is itself autonomously regulated (8, 10). Thus, the relevant role of CP12 in land plants may be to create a direct connection between A4-GAPDH and PRK, leading to coordinate down-regulation of these key enzymes, both quite active in the free state (7).

The stoichiometry of the binary complex (A4-GAPDH)-(CP12-2)2 was assessed by isothermal titration calorimetry (CP12-2/A4-GAPDH = 1.9 ± 0.2) (Table 2) and confirmed by light scattering determination of the molecular mass (MALD-TOF-MS, 170 ± 14 kDa; calculated, 166 kDa; Table 1). That one A4-GAPDH tetramer is able to bind two CP12 monomers is also consistent with the homology between CP12 and the CTE...
of subunits B of GAPDH, whose primary sequence is in the main highly related to A subunits. The crystal structure of spinach A₂B₂-GAPDH in the oxidized state shows that each CTE, partially structured by a disulfide bridge, is located within a wide cleft delimited by each pair of A/B-subunits (10). GAPDH tetramers (A₂B₂ or A₄) (10, 15) contain two symmetrical clefts of this type, and it is likely that CP12-2 may bind to A₂-GAPDH in the same way that CTE is located in A₂B₂-GAPDH. This conclusion is also supported by site-specific mutant C73S of CP12-2 (Fig. 5), and analogous site-specific mutants of CP12 from other species (3, 6, 28), which also fail to form the C-terminal disulfide and are consequently unable to build up a stable complex with GAPDH.

A chloroplast complex of proteins, including GAPDH, CP12, and PRK, with a molecular mass of 460–640 kDa has been known for years (2–7) and has been explained by a model with (GAPDH-CP12-PRK), stoichiometry, based on the purported capability of CP12 to dimerize (3, 5). However, the observed A₄-GAPDH binding to two CP12-2 monomers suggests a different interpretation, also compatible with the molecular mass of the Arabidopsis ternary complex (498 ± 6 kDa; MALS), (i.e. two (A₂-GAPDH)-(CP12-2), binary complexes interacting with two PRK dimers (for a calculated molecular mass of 488 kDa (Table 1). The hydrodynamic radius of this ternary complex (7.0 ± 0.1 nm) was slightly higher than expected (6.2 nm, Table 1), indicating deviation from a theoretical spherical shape. Based on the apparent symmetry of both (A₄-GAPDH)-(CP12-2), binary complexes and PRK homodimers, we propose that the architecture of the ternary complex includes two binding sites for PRK on each binary complex and two binding sites for binary complexes on each PRK dimer. As a result, a toric supramolecular complex would finally be assembled as depicted in the model of Fig. 6. This model requires a cautious interpretation of microcalorimetric data, because ternary complex formation actually involves two steps: the first interaction between PRK and the binary complex and the following dimerization of the transient complex (Fig. 6). Since thermodynamic parameters of ternary complex formation (Table 2) are related to the whole process, the meaning of the stoichiometric parameter n is uncertain in this case.

Arabidopsis CP12-2 primary sequence contains four cysteines, and two internal disulfides are formed under oxidizing conditions (3, 43). The C-terminal disulfide (Cys64-Cys73) is homologous to the regulatory disulfide of A₂B₂-GAPDH (3, 10, 24). Redox titration analysis of CP12-2 revealed that the most negative midpoint redox potential (Eₘ,-7.9 = −352 mV) was identical to that of spinach A₂B₂-GAPDH (−353 mV (26) and was therefore attributable to the C-terminal disulfide. Surprisingly, the Eₘ,-7.9 of the second disulfide of CP12-2 (−326 mV, N-terminal) was identical to that of spinach PRK (−330 mV) (7), although no sequence similarities exist between the two proteins. The N-terminal disulfide was not required for binary complex formation with GAPDH, but the ternary complex of 498 kDa did not form in its absence (see also Refs. 3 and 28). Since PRK does not bind to CP12-2 alone (7, 20), it is likely that the CP12-2 domain, including the N-terminal disulfide, forms a binding site for PRK together with A₂-GAPDH. However, the cyanobacterium Synechococcus PC7942 presents a

GAPDH-CP12-PRK ternary complex of about 500 kDa, although CP12 of this species has no N-terminal cysteines (6).

The different redox properties of the two disulfides of Arabidopsis CP12-2 and the sequential formation of the supramolecular complex (7, 20) fit within a plausible physiological scenario. During transition from saturating light to suboptimal light availability, the incipient oxidation of chloroplast thioredoxins (Eₘ,-7.9 = −351 mV) for thioredoxin f) (44) would cause the formation of the C-terminal disulfide of CP12-2 (Eₘ,-7.9 = −351 mV) and then favor the formation of the binary complex with A₂-GAPDH bound to NAD ((A₂-GAPDH)-(CP12-2)),. By approaching darkness, further oxidation of thioredoxins would lead to N-terminal disulfide formation in CP12-2 (Eₘ,-7.9 = −327 mV) and formation of the regulatory disulfide in PRK (Eₘ,-7.9 = −329 mV). These conditions would promote the formation of a transient complex ([(A₄-GAPDH)-(CP12-2),](PRK)), with a suggested toric structure.

![FIGURE 6. A schematic diagram of supramolecular complex formation of A₄-GAPDH, CP12-2, and PRK, compatible with current evidence.](Image)

The sequence of interactions is depicted as a function of the redox state of thioredoxins in chloroplasts, known to be largely reduced in the light and oxidized in the dark. In the light, reduced thioredoxins keep reduced both CP12-2 and PRK. During light to dark transition, partially oxidizing conditions would cause the formation of the C-terminal disulfide of CP12-2 (Eₘ,-7.9 = −351 mV) and then favor the formation of the binary complex with A₂-GAPDH bound to NAD ((A₂-GAPDH)-(CP12-2)),. By approaching darkness, further oxidation of thioredoxins would lead to N-terminal disulfide formation in CP12-2 (Eₘ,-7.9 = −327 mV) and formation of the regulatory disulfide in PRK (Eₘ,-7.9 = −329 mV). These conditions would promote the formation of a transient complex ([(A₄-GAPDH)-(CP12-2),](PRK)), which rapidly dimerizes to a ternary complex ([(A₄-GAPDH)-(CP12-2),](PRK)), with a suggested toric structure.
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