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Molecular Mechanism of NADPH-Glyceraldehyde-3-phosphate Dehydrogenase Regulation through the C-Terminus of CP12 in *Chlamydomonas reinhardtii*

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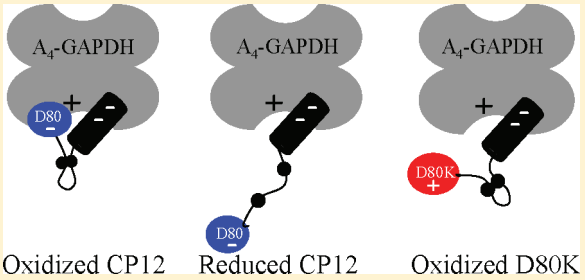
Supporting Information

ABSTRACT: In *Chlamydomonas reinhardtii*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) consists of four GapA subunits. This A_4 GAPDH is not autonomously regulated, as the regulatory cysteine residues present on GapB subunits are missing in GapA subunits. The regulation of A_4 GAPDH is provided by another protein, CP12. To determine the molecular mechanisms of regulation of A_4 GAPDH, we mutated three residues (R82, R190, and S195) of GAPDH of *C. reinhardtii*. Kinetic studies of GAPDH mutants showed the importance of residue R82 in the specificity of GAPDH for NADPH, as previously shown for the spinach enzyme. The cofactor NADPH was not stabilized through the 2′-phosphate by the serine 195 residue of the algal GAPDH, unlike the case in spinach. The mutation of R190 also led to a structural change that was not observed in the spinach enzyme. This mutation led to a loss of activity for NADPH and NADH, indicating the crucial role of this residue in maintaining the algal GAPDH structure. Finally, the interaction between GAPDH mutants and wild-type and mutated CP12 was analyzed by immunoblotting experiments, surface plasmon resonance, and kinetic studies. The results obtained with these approaches highlight the involvement of the last residue of CP12, Asp80, in modulating the activity of GAPDH by preventing access of the cofactor NADPH to the active site. These results help us to bridge the gap between our knowledge of structure and our understanding of functional biology in GAPDH regulation.



Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a ubiquitous enzyme that reversibly catalyzes the dephosphorylation of 1,3-bisphosphoglycerate to form glyceraldehyde 3-phosphate and inorganic phosphate.[1] In chloroplasts, it catalyzes the unique reductive step of the Calvin cycle using either NADH or NADPH as a cofactor, with a marked kinetic preference for NADPH.[2] It can be activated through redox regulation and thereby regulates the Calvin cycle responsible for the assimilation of CO_2. This chloroplast GAPDH exists in several forms. In cyanobacteria, green and red algae, and higher plants, GAPDH is composed of four identical GapA subunits (A_4),[1,3] while in diatoms (heterokont algae), it derives from the GapC glycolytic isoform.[4−8] In higher plants, this enzyme also exists as a heterotetramer composed of two GapA and two GapB subunits (A_2B_2) and as a hexadecamer (A_8B_8).[9,10] GapA and GapB subunits are highly similar, but the GapB subunit contains a regulatory C-terminal extension.[11−13] Chloroplast GAPDHs are activated through redox regulation by the ferredoxin/thioredoxin system,[13−15] and recently, it was shown that chloroplast A_4 GAPDH could be also regulated by glutathionylation, which also protects it from irreversible oxidation under stress.[16]

Most of the information found in the literature concerns the A_2B_2 isoform of GAPDH. This isoform is regulated by the two cysteine residues from the GapB C-terminal extension. In the dark, these residues form a disulfide bridge that induces a conformational change in the C-terminal extension so that the last residue of GapB, glutamate 362 (E362), interacts with the catalytic domain, called the S-loop (D181−A199).[17−19] This conformation seems to prevent the access of NADPH to its binding site and decreases the GAPDH activity with this cofactor. In the light, the disulfide bridge is disrupted by reduced thioredoxin, inducing a conformational change in the enzyme that makes the NADPH binding site accessible. This regulation has not been observed when NADH is the cofactor. The difference lies in the stabilization of the 2′-phosphate group of NADPH by threonine 33 (T33), serine 188 (S188), and arginine 77 (R77) of spinach GAPDH.[20]

The molecular mechanism of the regulation of the A_4 isoform is not well understood. A_4 GAPDH does not possess the C-terminal extension but is regulated during dark−light transitions. The CP12 protein, present in most photosynthetic organisms,[21] is responsible for this regulation by providing the necessary regulatory cysteine residues to A_4 GAPDH.[22−25] CP12 is an 8.5 kDa protein that belongs to the family of intrinsically

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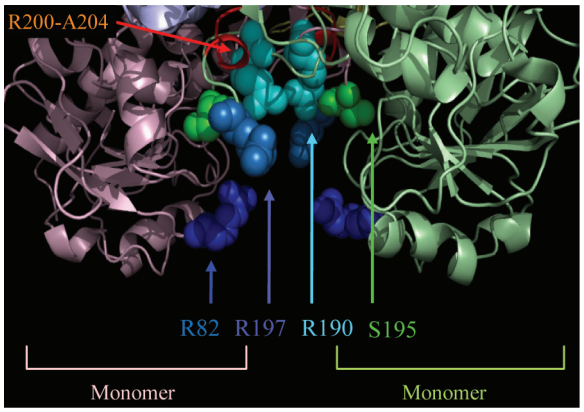

Figure 1. Modeled structure of *C. reinhardtii* GAPDH.[28] Residues R82, R190, R197, and S195 on two monomers of A_4 GAPDH are represented by colored spheres; they are all situated in a groove between the two monomers. The R monomer and the O monomer are colored light pink and light green, respectively. Residues from R200 to A204 are colored red and correspond to GAPDH residues that were previously shown to be protected by CP12 from tryptic digestion.[26]

disordered proteins (IDPs).[22,26] It contains two disulfide bridges, of which the C-terminus appears to be mainly responsible for the redox regulation of A_4 GAPDH.[27] Previously published data have shown that the CP12 binding site on GAPDH involves the S-loop (residues 188−206 according to *Chlamydomonas reinhardtii* nomenclature) and electrostatic interactions.[26−28] However, it has been hypothesized that the C-terminus of CP12 acts like the C-terminus of A_2B_2 GAPDH, in which the last residue, E362, interacts with arginine residues and hinders the entrance of NADPH.[17,18]

Residues arginine 82 (R82), arginine 190 (R190), and serine 195 (S195) that are located in a groove between two monomers on *C. reinhardtii* A_4 GAPDH (Figure 1), corresponding to R77, R183, and S188, respectively, in spinach, were mutated to analyze the physical properties of the mutant proteins, their NADPH specificity, and their interaction with CP12. We also address for the first time whether the C-terminus of CP12 acts like the C-terminus of the GapB subunit of GAPDH.

EXPERIMENTAL PROCEDURES

Production and Purification of Recombinant CP12 and GAPDH. Recombinant wild-type or mutated (D80A or D80K) CP12 protein, with a His tag, was expressed in *Escherichia coli* BL21(DE3)pLysS and purified by Ni-NTA agarose (Qiagen, Courtaboeuf, France) as previously described.[22] E18A, E18K, E40A, and E40K mutants were obtained as described in ref 29. After purification, the CP12 proteins were dialyzed against 50 mM Tris and 100 mM NaCl (pH 8) or 10 mM phosphate (pH 6.5) and stored at −20 °C. Recombinant wild-type or mutated (R82A, R82D, R190A, or S195A) A_4 GAPDHs (EC 1.2.1.13) were expressed in *E. coli* BL21(DE3)pLysS and purified with a DEAE Trisacryl column using a protocol slightly altered from that described in ref 30. The enzymes in 15 mM Tris, 4 mM EDTA, 0.1 mM NAD, and 2 mM dithiothreitol (pH 8.8) were stored with 10% glycerol at −80 °C. Protein concentrations were determined with the Bio-Rad reagent protein assay (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard.[31]

For the D80A and D80K CP12 mutants and for the GAPDH mutants, site-directed mutageneses were performed using the QuickChange kit (Stratagene, La Jolla, CA). The following primers were used: D80A, 5′-GCCGCGTCTACGAGGCTT-AACTCGAGAATCG-3′; D80K, 5′-GCCGCGTCTACGAGA-AGTAACTCGAGAATCG-3′; R82A, 5′-GCAGATCAAGATT-GTGTCCAGCGCCGACCCCCTGCAGCTGCCC-3′; R82D, 5′-GCAGATCAAGATTGTGTCCAGCGACGACCCCCTG-CAGCTGCCC-3′; R190A, 5′-CCTACACCGGTGACCAGC-GCCTGCTGGACGCGTCCC-3′; S195A, 5′-GCGCCTGCT-GGACGCGGCCCACCGCGACCTGCGCC-3′. The mutations at the gene level were confirmed by sequencing (GATC BioTech). The protein sequence for the R190A mutant was checked using tryptic digestion and matrix-assisted laser desorption ionization time of flight to determine if mutation of the residue of interest was correct.

Activity Measurements. To determine the activity of GAPDH, we synthesized 1,3-bisphosphoglyceric acid (BPGA) and determined its concentration as described in ref 30. Kinetic measurements were performed in 50 mM glycyl glycine, 50 mM KCl, 15 mM $MgCl_2$, and 0.5 mM EDTA (pH 7.7) using 0.2 mM NADPH or NADH. For the kinetic characterizations of the GAPDH mutants, the concentration of NADPH or NADH and of BPGA is given in the text. The activity was followed by measuring the change in absorbance at 340 nm using a UV−visible Lambda 25 double-beam Perkin-Elmer spectrophotometer. The inhibition of the NADPH-dependent activity of GAPDH, either wild type or mutant, by the different CP12 proteins was measured by adding various concentrations of CP12 from 0.04 to 19 μM for wild-type CP12, from 0.48 to 19 μM for D80K CP12, and from 0.09 to 18 μM for D80A CP12. At least four measurements were performed for each concentration. The data were fitted either to a hyperbola (eq 1) to determine the catalytic constant (k_{cat}) and the Michaelis constant (K_m) or to a sigmoid equation (eq 2) to determine k_{cat}, n_h (the Hill coefficient), and $K_{0.5}$ (the BPGA concentration for which half of the maximal velocity is obtained).

$$\frac{v}{[E]_0} = k_{cat}\frac{[NAD(P)H]}{K_m + [NAD(P)H]} \quad (1)$$

$$\frac{v}{[E]_0} = k_{cat}\frac{[BPGA]^{n_h}}{{K_{0.5}}^{n_h} + [BPGA]^{n_h}} \quad (2)$$

where v is the rate of the reaction and $[E]_0$ is the total concentration of enzyme in the cuvette.

In Vitro Reconstitution of the GAPDH−CP12 Complexes. Wild-type or mutated CP12 (0.03 nmol) and GAPDH (0.03 nmol) were mixed for 1 h at 30 °C in a buffer that consisted of 30 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.1 mM NAD, and 5 mM cysteine (pH 7.9). The formation of the GAPDH−CP12 complexes was then checked as previously described[26] using native PAGE performed on a 4 to 15% minigel using a GE Healthcare Phastsystem apparatus. The antibodies raised against CP12 were used at a 1:2000 dilution and were revealed using alkaline phosphatase.

Surface Plasmon Resonance and in Vitro Reconstitution of the GAPDH−CP12 Complex. Wild-type or mutated CP12 (50 μg/mL) was coupled through its amine groups to a carboxymethyl dextran-coated biosensor chip (CM5, Biacore, GE Healthcare) following the manufacturer's instructions; 162, 193, and 166 RU (resonance units) of wild-type CP12, D80A,

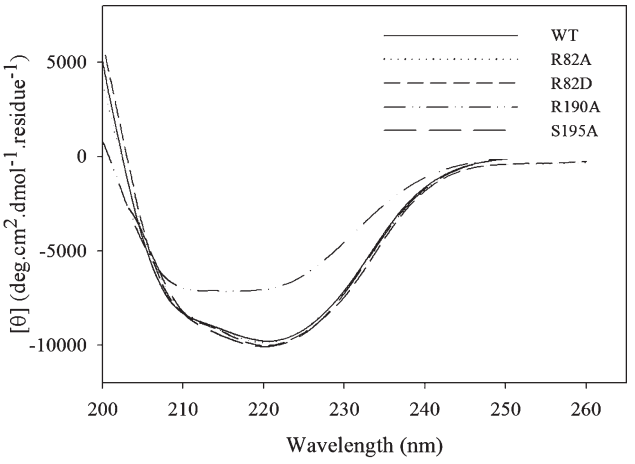

Figure 2. CD spectra of GAPDH and its mutants. Spectra were recorded with 2 μM wild-type or mutated GAPDH. The experiments were conducted in phosphate buffer (pH 7.9). [θ] is mean residue ellipticity.

and D80K, respectively, were immobilized. The interaction with wild-type or mutated GAPDHs was studied using HBS running buffer (BiaCore) with 0.1 mM NAD and 5 mM cysteine (pH 7.9) at a rate of 30 μL/min using a BiaCore T100 instrument. Global fits of the exponential curves (sensorgrams) were realized using Biacore T100 Evaluation (version 2.0), giving values for the dissociation and association rate constants, k_d and k_a, respectively, and the response at equilibrium, R_{eq}. The GAPDH concentrations span 0.1−250 nM. For each concentration, two or three experiments were performed. R_{eq} was then plotted as a function of GAPDH concentration and fitted to a hyperbola (eq 3):

$$R_{eq} = R_{max}[\text{GAPDH}]/(K_D + [\text{GAPDH}]) \quad (3)$$

where K_D is the equilibrium dissociation constant and R_{max} is the maximal analyte binding capacity in response units (RU).

Circular Dichroism. Circular dichroism (CD) spectra were recorded on a Jasco 815 CD spectrometer (JASCO, Bouguenais, France) using 2 mm path length quartz cells in 10 mM sodium phosphate (pH 7.9) at 20 °C. CD spectra were measured from 260 to 190 nm, at a rate of 10 nm/min, and were averaged from four scans. The spectra were corrected for buffer signal. Finally, mean ellipticity values per residue ([θ]) were calculated with the relationship $[\theta]_{mrw,\lambda} = MRW \times \theta_\lambda/(10dc)$, where MRW is the mean residue weight, θ_λ is the observed ellipticity (in degrees) at wavelength λ, d is the path length (0.2 cm), and c is the protein concentration expressed in grams per milliliter.[32] GAPDH at 2 μM was used.

RESULTS

Characterization of the GAPDH Mutants. Far-UV circular dichroism spectroscopy analysis revealed that all GAPDHs (wild type and R82A, R82D, R190A, and S195A mutants) adopt a predominantly α-helical structure, consistent with the characteristic minima at 208 and 222 nm; however, the spectrum of the R190A mutant differs from the spectra of all other GAPDHs (Figure 2). For this mutant, the secondary structure was affected and the percentage of residues in α-helices (26%) was lower than that in the wild type and all other GAPDH mutants (33%). The mutation of the R190 residue also affected the activity of the enzyme, because this mutant had no activity in the presence of NADPH as a cofactor. Thus, this residue is crucial for maintaining a proper enzyme structure.

The kinetic properties of each mutant were studied in the presence of NADPH or NADH as a cofactor (Tables 1 and 2). When the kinetic behavior was determined with both cofactors, the steady-state rate of the mutated GAPDHs, as well as that of the wild-type enzyme, followed Michaelis−Menten kinetics (eq 1). All GAPDH mutants exhibited allosteric behavior with respect to BPGA, irrespective of the cofactor. The rate of reaction (v) was fitted to eq 2. In the presence of NADPH (Table 1), the catalytic constant (k_{cat}) of the R82A, R82D, and S195A mutants decreased by a factor of 2, but the affinity for BPGA was not altered. The mutation of the R82 residue led to a 10-fold increase in the K_m for NADPH but did not affect the kinetics of NADH (Table 2). While the mutation of the S195 residue had no effect on the affinity of GAPDH for NADPH, its affinity for NADH and for BPGA in the presence of NADH was reduced. For the S195A mutant, the values of $K_{0.5}$ for BPGA and K_m for NADH increased 4- and 7-fold, respectively. The catalytic constant, k_{cat}, of the R190A mutant, in the presence of NADH, decreased 10-fold, and the value of the $K_{0.5}$ for BPGA increased. It is interesting to note that the K_m for NADH decreased 12-fold, so the specificity constant (k_{cat}/K_m) of this mutant is the same as that of wild-type GAPDH.

GAPDH−CP12 Interaction. Reconstitution experiments were performed using equimolar concentrations of GAPDH and CP12 to determine whether the GAPDH and CP12 mutants were able to form a complex like the wild-type proteins.[27] The CP12 proteins were recognized by the CP12 antibodies, either alone (Figure 3A, lane 1) or in the GAPDH−CP12 complex (Figure 3A, lane 2). Except for the R190A mutant, wild-type and mutant GAPDHs could form a complex with wild-type CP12 or the D80A mutant (Figure 3A,B). A second form of the complex was detected that might correspond to a higher level of oligomerization of the complex [(CP12−GAPDH)^n]. CP12−GAPDH forms were both recognized by GAPDH antibodies (data not shown). In the presence of the D80K CP12 mutant, the complex could be detected only with wild-type GAPDH (Figure 3C).

The CP12−GAPDH interaction was further characterized by surface plasmon resonance (BiaCore). The sensorgrams, obtained with the S195A GAPDH mutant and wild-type CP12, are shown in Figure 1 of the Supporting Information as an example. The average apparent equilibrium dissociation constants (K_D) were calculated using eq 3. The results are summarized in Table 3.

As previously shown,[22] the affinity between wild-type CP12 and GAPDH was very high (K_D = 0.37 ± 0.06 nM). The mutation of the D80 residue of CP12 affected the affinity for GAPDH as the K_D increased 20- and 50-fold for the D80A and D80K mutants, respectively. When GAPDH was mutated, the K_D obtained with wild-type CP12 increased ~30-fold for R82A and S195A and 86-fold for R82D. These differences in the affinity between GAPDH and CP12 may indicate that these residues are involved in the interaction between these proteins. Other CP12 mutants, E18A, E18K, E40A, and E40K,[29] were also checked for their effects on GAPDH affinity. None of these mutants were significantly affected in their capacity to bind GAPDH, indicating that these residues are not involved in GAPDH−CP12 interaction.

Table 1. Kinetic Parameters of Wild-Type and Mutant GAPDHs Obtained by Steady-State Analysis of the NADPH-Dependent Reaction[a]

	BPGA varied			NADPH varied		
GAPDH	n_h	$K_{0.5}$ (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	specificity constant (s^{-1} μM^{-1})
wild type	1.5 ± 0.1	250 ± 17	419 ± 13	28 ± 3	430 ± 17	15 ± 6
R82A	1.5 ± 0.1	258 ± 32	197 ± 15	300 ± 18	196 ± 5	0.65 ± 0.04
R82D	1.3 ± 0.1	209 ± 23	204 ± 12	202 ± 16	203 ± 7	1.00 ± 0.09
S195A	1.7 ± 0.1	371 ± 37	174 ± 11	28 ± 9	210 ± 17	7.5 ± 1.9
R190A	–	–	0	–	0	–

[a] The concentration of wild-type or mutated GAPDH was 1 nM. The errors represent the standard deviation of three to five experiments.

Table 2. Kinetic Parameters of Wild-Type and Mutant GAPDHs Obtained by Steady-State Analysis of the NADH-Dependent Reaction[a]

	BPGA varied			NADH varied		
GAPDH	n_h	$K_{0.5}$ (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	specificity constant (s^{-1} μM^{-1})
wild type	1.3 ± 0.1	95 ± 10	88 ± 4	120 ± 11	104 ± 3	0.90 ± 0.08
R82A	1.78 ± 0.01	168 ± 8	58 ± 1	157 ± 11	71 ± 2	0.45 ± 0.03
R82D	2.1 ± 0.2	148 ± 8	145 ± 5	102 ± 11	135 ± 30	1.3 ± 0.3
S195A	3.5 ± 0.6	397 ± 27	72 ± 5	872 ± 130	119 ± 10	0.14 ± 0.02
R190A	2.1 ± 0.3	463 ± 75	10 ± 1	10 ± 1	8.4 ± 0.2	0.86 ± 0.15

[a] The GAPDH concentrations were 6.4, 1.6, 13, and 138 nM for the R82A, R82D, S195A, and R190A mutants, respectively. The errors represent the standard deviation of three to five experiments.

The free energy of GAPDH−CP12 binding (ΔG_b) was calculated with eq 4:

$$\Delta G_b = -RT \ln K_a \quad (4)$$

where T stands for temperature and is 293 K, R is the gas constant, and K_a is the association constant ($1/K_D$). The destabilization of the interaction between GAPDH and CP12 (Table 3) that is linked to the point mutations introduced into GAPDH or CP12 was calculated as the difference $\Delta\Delta G_b$ (eq 5).

$$\Delta\Delta G_b = \Delta G_b{}^{WT} - \Delta G_b{}^{mut} \quad (5)$$

A greater effect was observed when the D80 residue of CP12 or the R82 and S195 residues of GAPDH were mutated. The values for the free energy differences were ∼2 kcal/mol in all cases.

Effect of CP12 on GAPDH Activity. The activity of wild-type and mutant GAPDH was measured in the presence of various concentrations of wild-type or mutant CP12 to determine the impact of these mutations on GAPDH regulation by CP12 (Figure 2 of the Supporting Information). When the activity was tested in the presence of NADH as a cofactor, no effect of CP12 could be observed (data not shown). With NADPH as a cofactor, the activity of wild-type, R82A, R82D, and S195A GAPDH was inhibited by wild-type CP12 (Table 4). The inhibition constants (K_I) obtained range from 0.8 ± 0.2 μM for wild-type GAPDH to 2.7 ± 0.7 and 2.1 ± 0.3 μM for the R82A and R82D GAPDH mutants, respectively. In the presence of the D80A CP12 mutant, the activity of all GAPDH mutants was also inhibited and the K_I values were similar to those obtained with wild-type CP12, but K_I increased by a factor of 2 for wild-type GAPDH. When the charge of D80 was reversed (D80K) on CP12, no effect could be observed either on wild-type GAPDH or for any of the GAPDH mutants tested.

DISCUSSION

Molecular modeling of the *C. reinhardtii* GAPDH structure[28] shows that two highly positively charged grooves are present on the enzyme tetramer. Residues R82, S195, R190, and also R197, which was previously studied,[28] are present in the groove between two monomers (Figure 1) and could potentially be involved in the regulation of A_4 GAPDH activity in *C. reinhardtii* and/or in the interaction with CP12. In spinach, residues R77, R183, and S188 of A_2B_2 GAPDH are involved in the NADPH-dependent activity of this isoform.[1] The 2′-phosphate group of NADPH is stabilized by the OH groups of threonine 33 (T33) and, predominantly, of serine 188 (S188) and the NH_2 group of arginine 77 (R77) of this enzyme.[20] This last residue (R77) interacts with the 2′-phosphate group of NADPH, forming a salt bridge.[18,20] The kinetic studies of R82 mutants of algal A_4 GAPDH showed that the K_m for NADPH of GAPDH was markedly increased and became similar to the K_m for NADH. The kinetic preference for NADPH in this mutant was thus lost. These results therefore underscore the major role of residue R82 of GAPDH in the binding and specificity for NADPH and are consistent with those found for residue R77 of spinach GapB. In contrast, substitution of residue S195 with alanine affected the specificity of GAPDH for NADH, but not for NADPH. Contrary to what is observed for spinach GAPDH, the stabilization of the 2′-phosphate of NADPH by the residue serine (S195) on the algal enzyme is thus not a prerequisite for binding this cofactor. Moreover, as shown by circular dichroism, mutagenesis of residue R190 affected the overall secondary structure of GAPDH in *C. reinhardtii* while the equivalent mutation in spinach did not alter the structure of the enzyme.[17] The effect of the mutation of this residue, which is located very deep in the groove (see Figure 1), directly or indirectly leads to a loss of activity with both NADPH and NADH. The structural change induced by

mutation leads to an affinity of this mutant for NADH higher than that of wild-type algal GAPDH. These data indicate that residues R82 and R197 play a similar role in GAPDH from higher plants and algae. However, conserved residues R190 and S195 differ in their effects on the structure and activity of GAPDH in these organisms.

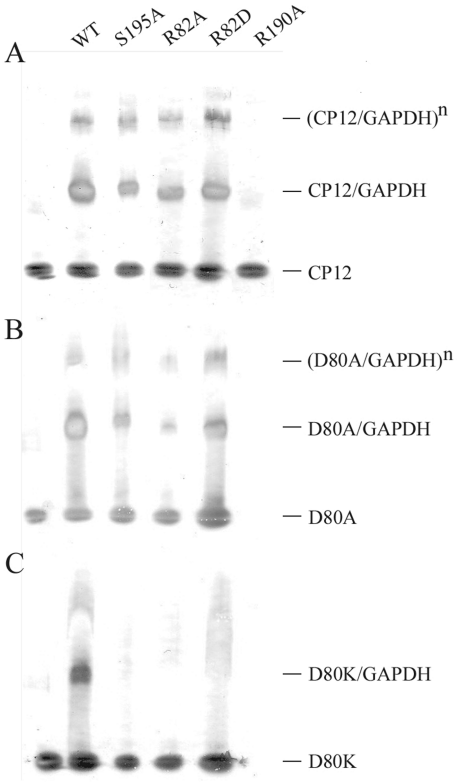

Figure 3. In vitro reconstitution experiments of the GAPDH−CP12 complex. GAPDH and CP12 were mixed in a 1:1 molar ratio. After 1 h at 30 °C, proteins were separated on a 4 to 15% gradient native gel, transferred to a nitrocellulose membrane, and detected with antibodies raised against CP12. Reconstitution mixtures of wild-type and mutated GAPDH with (A) wild-type CP12, (B) D80A CP12, and (C) D80K CP12. We checked that CP12 antibodies did not cross-react with recombinant GAPDH.

In the green alga, CP12 has been shown to interact, through its second α-helix,[27] with S-loop residues of GAPDH[26] (colored red in Figure 1) and is involved in the regulation of GAPDH activity. In the A_2B_2 GAPDH isoform, residue E362 of the GapB extension interacts with the S-loop, preventing the access of NADPH to its binding site. In the A_4 isoform, it is not clear, nor has it ever been shown, how CP12 could play such a role, and which residues are involved in the regulation of GAPDH. Formation of the complex with wild-type CP12 does not occur, but as the overall structure of GAPDH was changed, the involvement of this residue directly or indirectly with binding of CP12 cannot be assessed. The interaction between CP12 and GAPDH mutants R82A, R82D, and S195A is strong because the dissociation constants are in the nanomolar range, and we thus observed formation of the complex in the in vitro reconstitution assay. In addition, the NADPH-dependent activity of GAPDH mutants, like that of wild-type GAPDH, decreases in the presence of wild-type CP12 and thus demonstrates an interaction. However, the affinities of wild-type CP12 for the R82 and S195 GAPDH mutants decreased by factors of 10−30. The difference in the free energies ($\Delta\Delta G_b$) obtained for these mutants is approximately −2 kcal/mol, which probably corresponds to a loss of a hydrogen bond between GAPDH and CP12

Table 4. Inhibition by CP12 of the NADPH-Dependent Activity of GAPDH[a]

GAPDH	WT CP12 (μM)	D80A (μM)
wild type	0.8 ± 0.2	2.0 ± 0.5
S195A	1.6 ± 0.4	1.3 ± 0.2
R82A	2.7 ± 0.7	2.5 ± 0.4
R82D	2.1 ± 0.3	2.4 ± 0.5

[a] The GAPDH activity was measured in the presence of NADPH. Inhibition constants (K_I) were determined by adding various concentrations of wild-type or mutant (D80A) CP12. The values for the D80K mutant are not given as no effect of this mutant was observed on the activity of wild-type and mutant GAPDH. The errors represent the standard deviation of three to five experiments.

Table 3. Dissociation Constants and Quantification of the Destabilizing Effect of the Mutations on the Interaction between CP12 and GAPDH[a]

GAPDH	CP12	K_D (nM)	K_D^{mut}/K_D^{WT}	ΔG_b (kcal/mol)	$\Delta\Delta G_b$ (kcal/mol)
wild type	wild type	0.37 ± 0.06	1	−12.65	0
	E18A	0.50 ± 0.05	1.4 ± 0.3	−12.47	−0.18
	E18K	0.56 ± 0.03	1.5 ± 0.3	−12.41	−0.24
	E40A	0.42 ± 0.05	1.1 ± 0.2	−12.58	−0.07
	E40K	1.03 ± 0.04	2.8 ± 0.5	−12.05	−0.6
	D80A	7.3 ± 0.8	20 ± 4	−10.91	−1.74
	D80K	18 ± 4	49 ± 13	−10.39	−2.26
R82A	wild type	11 ± 3	30 ± 9	−10.67	−1.98
	D80A	12 ± 2	32 ± 8	−10.62	−2.03
R82D	wild type	32 ± 8	86 ± 26	−10.05	−2.60
	D80A	21 ± 3	57 ± 12	−10.30	−2.35
S195A	wild type	9 ± 3	24 ± 9	−10.79	−1.86
	D80A	12 ± 3	32 ± 10	−10.62	−2.03

[a] The dissociation constants were obtained by surface plasmon resonance with GAPDH as the analyte and CP12 as the ligand (immobilized protein). The free energies of the association were calculated according to eqs 4 and 5. The errors represent the standard deviation of three to five experiments.

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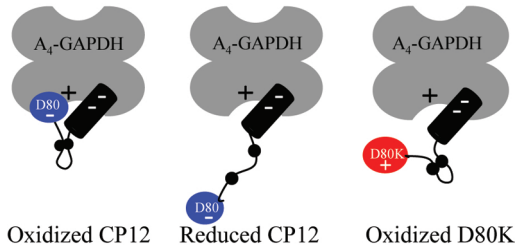

Figure 4. Model of GAPDH regulation by CP12. Cysteine residues of CP12 (shaded in black) are represented as full black circles. The presence of a disulfide bridge, under oxidizing conditions, allows residue D80 on CP12 (colored blue) to interact with GAPDH. Under reducing conditions, the disulfide bridge is disrupted, leading to a conformational change in which residue D80 no longer interacts with GAPDH. The change of a negatively charged residue (D80) to a positively charged one (K, colored red) prevents the C-terminus of CP12 from interacting with GAPDH, even under oxidizing conditions. The minus signs indicate negative charges on CP12, while the plus signs indicate the positively charged residues in the S-loop of GAPDH.

because of the mutations.[33,34] Another form of the CP12−GAPDH complex could also be detected by immunoblotting experiments that might correspond to a higher oligomeric state. In plants, the A_2B_2 form of GAPDH can oligomerize to an A_8B_8 GAPDH that is fully inactive. In this work, a $(CP12-GAPDH)^n$ complex has been detected for the first time in vitro; however, it has not been found within *C. reinhardtii*.

In the oxidized A_2B_2 form of spinach GAPDH, the disulfide bridge of the C-terminal extension of subunit B forces E362 to approach the two arginine residues, R183 and R77. The C-terminal extension of the oxidized enzyme is held against the core structure by this disulfide bond and shields the entrance to the active site of NADPH.[17,18] We checked if the C-terminal residue (D80) of CP12 could play that role for A_4 GAPDH. In the presence of the CP12 mutant, D80A, the NADPH-dependent activity of wild-type GAPDH decreases. The interaction between GAPDH and CP12 (D80A) has also been verified by in vitro reconstitution experiments and quantified by surface plasmon resonance. The interaction is still strong, but there is a loss of free energy (−2 kcal/mol) that may again correspond to the loss of a hydrogen bond.[33,34] Wild-type and D80A CP12 inhibited the NADPH-dependent activity of GAPDH, with K_I values of ∼0.8 and ∼2 μM, respectively. However, when the negative charge of the latter residue on CP12 is replaced with a positive charge (D80K), the NADPH-dependent activity of all mutants and of wild-type GAPDH no longer decreases. Indeed, the interaction with the mutated GAPDHs is no longer observed. In contrast, there is re-formation of the complex with the D80K CP12 mutant and wild-type GAPDH, though no inhibition of the activity was observed. Interestingly, wild-type CP12 in the plant *Arabidopsis* was not able to inhibit GAPDH activity, probably because its affinity for this enzyme was much lower than that obtained for *C. reinhardtii* CP12 for its partner.[35,36]

A conceptual scheme of the GAPDH regulation by CP12 that accounts for these results is presented in Figure 4. Reduced CP12 can interact with GAPDH.[26] However, when CP12 is reduced, it does not inhibit NADPH−GAPDH complex activity (data not shown). The D80K CP12 mutant interacts with wild-type GAPDH but behaves like reduced CP12. It is likely that electrostatic repulsion prevents the interaction of this mutated positively charged residue (K80) with a positively charged region within GAPDH (probably residue R82), and thus, the binding site for NADPH is no longer occluded and NADPH may enter the active site of the enzyme. This interaction between residue D80 of CP12 and a positively charged residue on GAPDH is redox-dependent and depends on the formation of the C-terminal disulfide bridge of CP12[27] (see Figure 3). When this disulfide bridge is present, residue D80 interacts and hinders the entry of NADPH into the active site, but when this disulfide bridge is disrupted, under reducing conditions, this residue moves away, allowing NADPH to enter. This rearrangement is similarly obtained if this residue (D80) is replaced with a positive charge, because of electrostatic repulsion.

In conclusion, basic arginine residues R77, R183, and R191 of spinach GAPDH have been thought to be involved in the interaction with the acidic C-terminal extension of the GapB subunit, and thus, it was hypothesized that CP12 could act in the same way, leading to enzyme regulation.[1] Our results clearly show that the penultimate aspartate on CP12 for A_4 GAPDH, like the penultimate glutamate at the tip of the extension in the GapB subunit, is critical for the inhibitory action observed with A_4 GAPDH. The same type of autoinhibition has been observed with malate dehydrogenase (NADP−MDH) and ribulose bisphosphate carboxylase oxygenase Rubisco activase (α isoform), which possess regulatory cysteine residues[37,38] not present on their unregulated counterparts (NAD−MDH and β isoform of the activase). Thus, mechanisms involving the disulfide bond acting as a barrier to prevent entry into the active site seem to be widespread in photosynthetic organisms. However, our kinetic results underscore the importance of studying dehydrogenases of plants and algae because even though their sequences are similar, they are not identical, and the regulatory mechanisms in higher plants may not be the same as in other photosynthetic organisms, particularly algae.

■ ASSOCIATED CONTENT

Supporting Information. Sensorgrams from surface plasmon resonance experiments with wild-type CP12 and S195A GAPDH as well as the effect of CP12 (wild type, D80A, and D80K) on NADPH−GAPDH activity (S195A, R82A, R82D, and wild type). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BPGA, bisphosphoglyceric acid; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; IDP, intrinsically disordered protein; BSA, bovine serum albumin.

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