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A New Function of GAPDH from *Chlamydomonas reinhardtii*: A Thiol−Disulfide Exchange Reaction with CP12[†]

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ABSTRACT: CP12 is a flexible protein that is well-known to interact with GAPDH, and this association is crucial to the regulation of enzyme activity. This regulation is likely related to structural transitions of both proteins, but the molecular bases of these changes are not yet understood. To answer this issue, we undertook a study based on the use of paramagnetic probes grafted on cysteine residues and followed by EPR spectroscopy. We present a new application of this approach that enables us to probe the functional role of cysteine residues in protein−protein interactions. Algal CP12 contains four cysteine residues involved in two disulfide bridges in its oxidized state and has some α-helical secondary structural elements. In contrast, in its reduced state, CP12 is mainly unstructured and shares some physical properties with intrinsically disordered proteins. Treatment of CP12 with a methane thiosulfonate derivative spin-label (MTSL) led to the labeling of the cysteine residues involved in the C-terminal bridge only as revealed by mass spectrometry. Surprisingly, the partner protein GAPDH induced the cleavage of the disulfide bridge between the cysteine residues of CP12 and the spin-label, resulting in the full release of the label. We showed the existence of a transitory interaction between both proteins and proposed a mechanism based on a thiol−disulfide exchange reaction. The results of this study point out a novel role of the algal GAPDH which is often termed a "moonlighting" protein.

CP12 is an 8.2 kDa protein present in the chloroplasts of most photosynthetic organisms, known to play a role in the formation of a complex with glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] and phosphoribulokinase (PRK), two key enzymes involved in the Calvin cycle (*1*−*5*). In *Chlamydomonas reinhardtii*, CP12 shares some physical properties with intrinsically disordered proteins (IDPs) (*1*, *6*, *7*), being devoid of a stable globular shape in solution (*8*−*10*). It contains four conserved cysteine residues that form two disulfide bridges (C23−C31 and C66−C75) when it is oxidized. The formation of these disulfide bridges upon oxidation plays a key role in the folding of CP12 (*1*). In particular, NMR and CD data showed that oxidized CP12 has some α-helical secondary structure elements and coil segments whereas reduced CP12 is fully disordered. These observations were confirmed by a modeling approach that proposed the existence of two α-helices located in the N-terminal and central parts, whereas the C-terminal part remains flexible (*11*). The kinetic properties of GAPDH were modified upon association with CP12 that acts as a negative regulator of GAPDH activity (*2*, *12*). These kinetic differences were attributed to probable conformational changes in the GAPDH−CP12 complex. More recently, studies of several CP12 mutants (*13*) showed that two regions are involved in the interaction with GAPDH. On the basis of cysteine to serine mutants, it was postulated that the first region essential for the formation of the CP12−GAPDH complex is the C-terminal disulfide bridge (C66−C75), whereas the N-terminal disulfide bridge (C23−C31) is not required. The second one is the negatively charged central region of CP12 which has been predicted to be an α-helix in the modeling of CP12 (*11*) and appeared to be the interaction site. Very recently, it has been shown that reduced CP12 is able to form the GAPDH−CP12 complex (*6*).

At present, no structural data on the possible structural transition undergone by CP12 upon GAPDH binding are available. To answer this issue, we undertook a study based on the use

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[1]Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13); PRK, phosphoribulokinase (EC 2.7.1.19); IDP, intrinsically disordered protein; EPR, electron paramagnetic resonance; MTSL, methanethiosulfonate spin-label; TFE, 2,2,2-trifluoroethanol; TFA, trifluoroacetic acid; CD, circular dichroism; NMR, nuclear magnetic resonance; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MALDI ToF, matrix-assisted laser desorption ionization time of flight.

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of paramagnetic probes grafted on cysteine residues and followed by EPR. Site-directed spin labeling has been extensively used to study conformational changes within structured proteins (*14*−*17*), as well as folding and unfolding processes of structured proteins in the presence of denaturing agents (*18*−*20*). Recently, this approach was used to study IDPs to monitor the disorder-to-order transitions (*21*, *22*). In this work, we present a new application of this approach which enables us to probe the functional role of cysteine residues in protein−protein interactions. Indeed, the GAPDH−CP12 subcomplex is an interesting model as both proteins contain several cysteine residues. Labeling of CP12 was performed, and the sites of the labels were determined by mass spectrometry. In parallel, CD spectroscopy has been used to follow the global folding of CP12. The results obtained with labeled CP12 in the presence of GAPDH revealed an unexpected function involving its cysteine residues.

EXPERIMENTAL PROCEDURES

CP12 Purification. Recombinant CP12 of *C. reinhardtii* with its histidine tag was purified to apparent homogeneity as previously described (*1*). CP12 protein was dialyzed against 50 mM Tris and 100 mM NaCl (pH 8) and stored at −20 °C.

GAPDH Purification. Recombinant *C. reinhardtii* GAPDH was purified to apparent homogeneity using a protocol slightly altered from that of ref (*2*) and kept in 30 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 0.1 mM NAD (pH 7.9) at −80 °C in 10% aqueous glycerol.

Denaturing Gel. SDS−PAGE (12%) performed according to the method described in ref (*23*) was conducted in a Bio-Rad Mini protean system. Proteins were stained with Coomassie Brilliant Blue R250.

In Vitro Reconstitution of the GAPDH−CP12 Complex. CP12 (0.03 nmol), labeled or not, was mixed with GAPDH (0.03 nmol) in 30 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 0.1 mM NAD (pH 7.9) for 1 h at 30 °C. The formation of the GAPDH−CP12 complex with the different CP12s was then checked as previously described (*7*).

GAPDH Activity Measurements. The NADPH-dependent activity of GAPDH was determined as described in ref (*2*). Measurements were performed with 1 nM GAPDH alone or with either CP12 or labeled CP12 at 0.5 μM. All activities were followed by measuring the absorbance at 340 nm using a Pye Unicam UV2 spectrophotometer.

Titration of Thiol Groups on GAPDH. Sulfhydryl groups were quantified using DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] (*24*). GAPDH (1.7 μM) was mixed with 1.5 mM DTNB in a 2 mM EDTA, 0.1 M phosphate buffer at pH 8. Sulfhydryl group titration was then followed by measuring the absorbance at 412 nm using a Pye Unicam UV2 spectrophotometer using an ε^{DTNB} of 13600 M^{-1} cm^{-1}.

Spin Labeling. Before spin labeling was conducted, 20 mM DTT was added to the purified CP12 (730 μg). The mixture was incubated for 30 min at 30 °C to reduce the disulfide bridges. DTT was then removed by gel filtration with a Nick column (2 cm × 1 cm) (GE Healthcare) with 50 mM Tris and 100 mM NaCl (pH 8.0) used as an elution buffer. After gel filtration, the spin-label [either 1-oxyl-2,2,5,5-tetramethyl-δ3-pyrroline-3-methyl methanethiosulfonate (MTSL), 185 Da, Toronto Research Chemicals Inc., or 3-maleimido-proxyl, Aldrich (Figure 3A)] was immediately added to the sample at a molar excess of 10. The reaction was conducted for 1 h in the dark and in an ice bath, under gentle stirring and a continuous flow of argon to avoid oxidation. The excess of unbound spin-label was removed by gel filtration using a PD10 desalting column (GE Healthcare) with the same elution buffer described above. The fractions containing the labeled protein were determined by recording EPR spectra.

EPR Spectroscopy and Data Analysis. EPR spectra were recorded at room temperature (296 ± 1 K) on an ESP 300E Bruker spectrometer equipped with an ELEXSYS Super High Sensitivity resonator operating at X-band (9.9 GHz). Samples were injected into a quartz capillary (20 μL). The microwave power was 10 mW, and the magnetic field modulation frequency and amplitude were 100 kHz and 0.1 mT, respectively.

The concentration of spin-labels was evaluated by double integration of the EPR signal recorded under nonsaturating conditions and comparison with that given by a 3-carboxy-proxyl standard sample. The number of spins per protein was estimated by calculating the ratio of the concentration of spin-labels to the total protein concentration estimated by using the Bio-Rad reagent protein assay (*25*) using BSA as a standard.

In this study, all EPR spectra recorded at room temperature show that the nitroxide probe is in the so-called fast regime of mobility, with an outer line splitting remaining at $2\overline{A} = 3.2mT$, where $\overline{A}$ is the average value of the hyperfine interaction. To describe the mobility of the spin probe, we measured a semi-quantitative parameter: the ratio of the peak-to-peak amplitude of the high- and central-field lines, termed the $h(-1)/h(0)$ ratio, where −1 and 0 correspond to the values of the nuclear magnetic number M_I (*20*).

For the study of spin−spin interaction, the EPR spectrum of a sample of labeled CP12 was recorded at 100 K using an ELEXSYS Bruker spectrometer fitted with an ESR 900 helium-flow Oxford Instrument cryostat. The following experimental conditions were used: microwave power of 1 mW and magnetic field modulation frequency and amplitude of 100 kHz and 0.8 mT, respectively. A monolabeled protein [the C-terminal part (amino acids 401−525) of the nucleoprotein of the measles virus N_{TAIL} labeled at position 496] was recorded as a control.

Effect of GAPDH on Labeled CP12. The effect of *C. reinhardtii* GAPDH on labeled CP12 has been followed by EPR spectroscopy. For the CP12 labeled with MTSL, different tetrameric GAPDH:CP12 molar ratios of 1:1, 1:2, 1:4, and 1:8 were used. For CP12 labeled with 3-maleimido-proxyl, only a tetrameric GAPDH:CP12 molar ratio of 1:1 was used. Moreover, the effect of GAPDH from organisms other than the green algae *C. reinhardtii*, rabbit muscle GAPDH (Roche) and *Saccharomyces cerevisiae* GAPDH (Sigma), was evaluated on labeled CP12. Reciprocally, the effect of the algal *C. reinhardtii* GAPDH was evaluated on two monolabeled proteins: cytochrome c_3 from *Desulfovibrio vulgaris Hildenborough* labeled at position 48 and a totally disordered one, the C-terminal part (amino acids 401−525) of the nucleoprotein of the measles virus N_{TAIL} labeled at position 407. For each sample, the protein concentration was 20 μM.

Circular Dichroism. CD spectra were recorded on a Jasco 815 CD spectrometer using 2 mm thick quartz cells in 10 mM sodium phosphate (pH 6.0) at 20 °C. CD spectra were measured from 260 to 190 nm, at 10 nm/min, and were averaged from four scans. Mean ellipticity values per residue ([θ]) were calculated as described in ref (*26*). Protein concentrations of 0.08 mg/mL were used. Reduced CP12 was obtained by incubation with 30 mM DTT for 1 h at 30 °C, and the final DTT concentration in the cell was 1 mM.

Effect of TFE on CP12 Folding. CD and EPR spectra were recorded in the presence of various concentrations of TFE ranging from 0 to 30% (v/v).

Mass Spectrometry Analyses of Labeled CP12. CP12 and labeled CP12 were diluted in 25 mM ammonium bicarbonate (pH 8) and submitted to digestion by trypsin (Sigma) for 2.5 h at 37 °C. The digested solutions were directly spotted onto a MALDI target plate (0.3 μL) and immediately acidified by addition of a saturated solution of matrix α-cyano-4-hydroxycinnamic acid [0.3 μL of 70% acetonitrile in water with 0.1% TFA (v/v)]. Tryptic peptides were analyzed on the MALDI-ToF Ultraflex mass spectrometer from Bruker Daltonics.

RESULTS

Labeling of CP12 with MTSL and Biochemical Characterization. The EPR spectrum of labeled CP12 with MTSL is shown in Figure 1A. This spectrum has a feature of a nitroxide radical being in a rapid regime of mobility with three narrow lines separated by an outer line splitting of $2\overline{A} = 3.2mT$. The spin quantification gave 1.7 ± 0.1 per CP12, indicating that more than one label was grafted. The spectrum of the same sample was recorded at 100 K and compared to that of a monolabeled protein (Figure 1B) (*21*). The presence of spin−spin interaction was clearly shown by the important broadening of ~1 mT of the labeled CP12 spectrum compared to the reference one. These results show that most of the labeled CP12s have two labels in close vicinity. To determine which cysteine residues were labeled by MTSL, tryptic digestion coupled with mass spectrometry was performed. The attribution of the different fragments showed that only C66 and C75 were labeled (Table 1 of the Supporting Information). This indirectly indicates that the N-terminal disulfide bridge is maintained.

To determine the effect of the label grafting on CP12 on its secondary structure, gel electrophoresis and CD experiments were conducted. Migration properties of the different forms of CP12 were analyzed via SDS−PAGE (Figure 2A). Oxidized CP12 migrated as a protein of 16 kDa, whereas it has an expected molecular mass of 11 kDa, typical of a protein having some IDP features. Some oligomers of 26 and 44 kDa were also observed. Reduced CP12 migrated as a protein of 20 kDa, and no more oligomers were observed. In the absence of DTT, labeled CP12 behaved as nonlabeled oxidized CP12 with an apparent molecular mass of 16 kDa. After reduction with 20 mM DTT, labeled CP12 behaved as nonlabeled reduced CP12 with an apparent molecular mass of 20 kDa. The CD spectrum obtained with oxidized CP12 showed two characteristic minima at 222 and 208 nm, confirming that oxidized CP12 has some α-helical content. The spectrum of reduced CP12 was completely different and characteristic of a totally unstructured protein. The spectrum obtained with labeled CP12 was similar to that of reduced CP12. The addition of TFE, as low as 10% and up to 30%, induced a folding of the labeled CP12 (Figure 2B). To describe the spectral changes, the ratio of the peak to peak amplitude of the high field line $h(-1)$ and the central line $h(0)$ was measured for each TFE concentration. A decrease in the $h(-1)/h(0)$ ratio was observed, indicating that TFE induced a decrease in the mobility of the label (Figure S1 of the Supporting Information).

GAPDH−CP12 Interaction Analyzed by EPR Spectroscopy and Biochemical Experiments. Tetrameric GAPDH

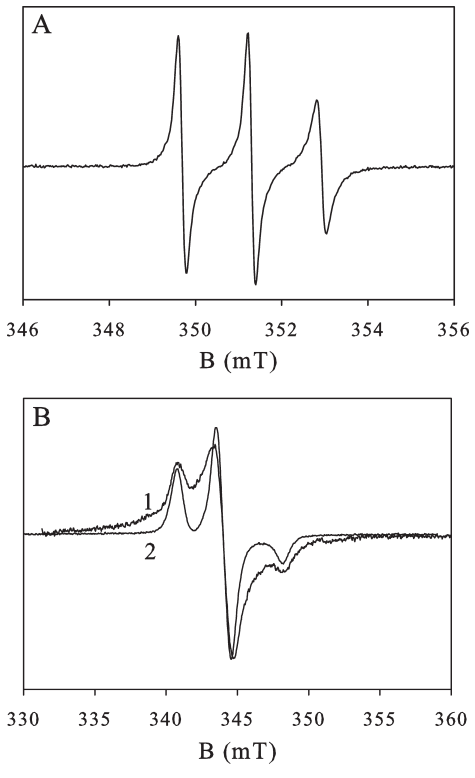

FIGURE 1: EPR spectra of labeled CP12 with MTSL. (A) Spectrum of labeled CP12 recorded at 296 K. (B) Spectra of labeled CP12 recorded at 100 K (spectrum 1) and the monolabeled protein recorded under the same experimental conditions (spectrum 2). The two spectra have been amplitude-normalized. The protein concentration was 20 μM.

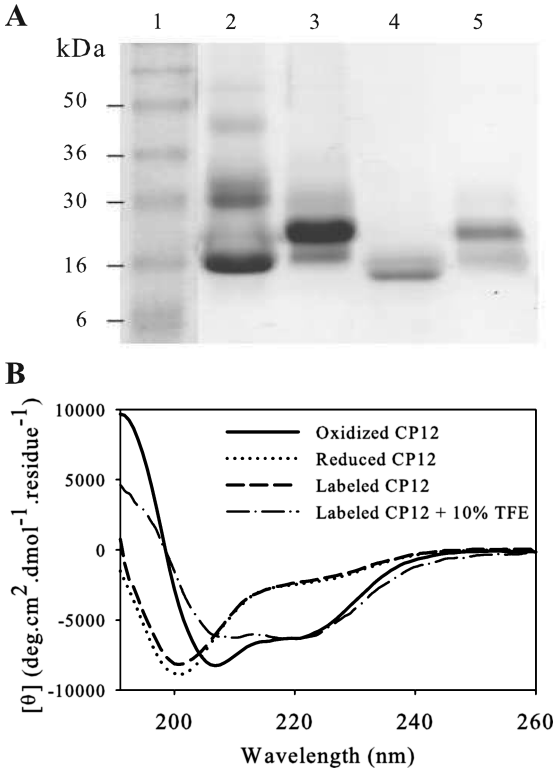

FIGURE 2: Characterization of labeled CP12. (A) Proteins were separated via 12% SDS−PAGE and stained with Coomassie Brilliant Blue R250: protein standard (lane 1), 10 μg of nonlabeled CP12 in the absence of any reducing agent (lane 2) or in the presence of 20 mM DTT (lane 3), and 7 μg of labeled CP12 in the absence (lane 4) or presence (lane 5) of 20 mM DTT. (B) CD spectra were recorded with 8 μM CP12, reduced CP12, or labeled CP12.

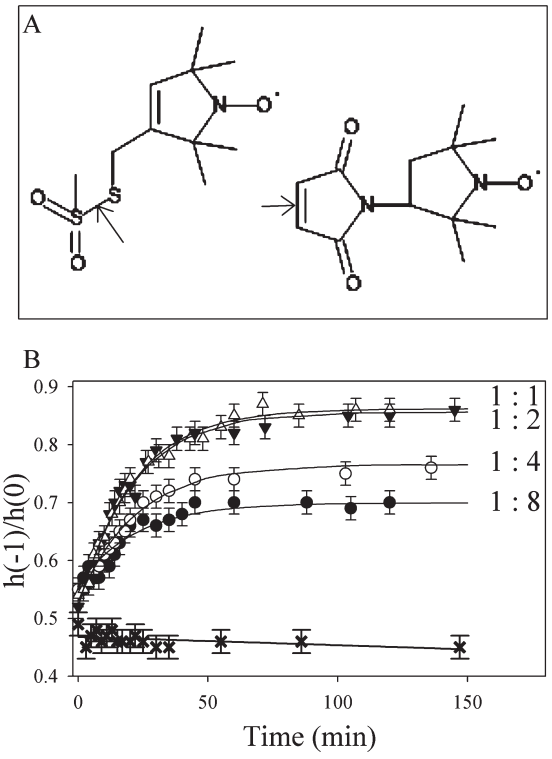

FIGURE 3: Effect of GAPDH on labeled CP12. (A) Chemical formulas of 1-oxyl-2,2,5,5-tetramethyl-δ3-pyrroline-3-methyl methanethiosulfonate (MTSL) (left) and 3-maleimido-proxyl (right). The arrows indicate the position of cleavage on the label that allows the labeling of the protein via a cysteine residue through a disulfide bond with MTSL and a thioether bond with 3-maleimido-proxyl. (B) EPR spectroscopy study. $h(-1)/h(0)$ ratio as a function of time, for different molar ratios: tetrameric GAPDH−(MTSL-CP12) with 1:1 (△), 1:2 (▼), 1:4 (○), and 1:8 stoichiometry (●). Data showing non release of 3-maleimido-proxyl on CP12 by GAPDH are symbolized by crosses. Data were fitted to the single-exponential curve $y = [h(-1)/h(0)]_{max}[1 - \exp(-t/\tau)]$ with the same characteristic time τ of 20 ± 3 min.

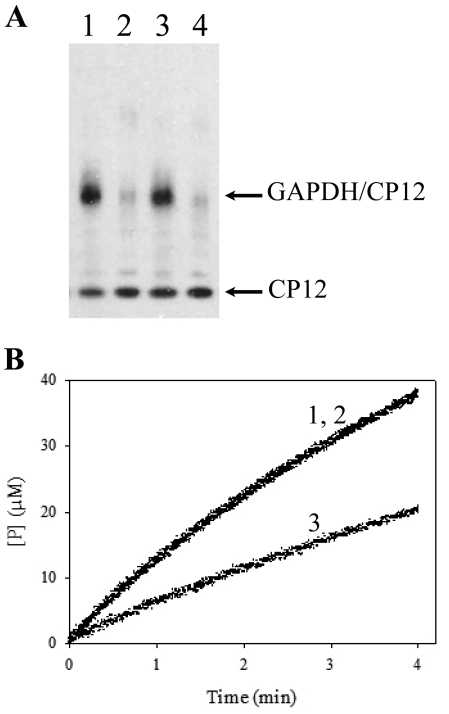

FIGURE 4: Analysis of formation of the GAPDH−CP12 complex. (A) Western blot analysis of the in vitro reconstitution of the GAPDH−CP12 complex. Nonlabeled CP12 (lanes 1 and 3) or labeled CP12 (lanes 2 and 4) was mixed with tetrameric GAPDH in a 1:1 molar ratio (0.03 nmol). Either immediately (lanes 1 and 2) or after 1 h at 30 °C (lanes 3 and 4), proteins were separated onto a native 4 to 15% gradient gel, transferred to a nitrocellulose membrane, and revealed with antibodies raised against CP12. (B) Effect of CP12 on NADPH−GAPDH activity. Progress curves of the NADPH−GAPDH activity for GAPDH alone, $[E]_0 = 1$ nM (curve 1) or with either labeled CP12 (curve 2) or nonlabeled CP12 (curve 3) at 0.5 μM. P stands for the product of the reaction.

and labeled CP12 with MTSL were mixed in different molar ratios and EPR spectra recorded as a function of time (Figure 3B). While in the absence of GAPDH, no change was detected, an evolution of the spectral shape was observed in its presence (Figure S2 of the Supporting Information). After 140 min, the spectrum corresponded to MTSL free in solution, indicating that the label was released from CP12 and the total spin concentration was constant. For tetrameric GAPDH:CP12 molar ratios of 1:4 and 1:8, only partial release of the label has been observed (Figure 3). In contrast, full release of the label was observed with tetrameric GAPDH:CP12 molar ratios of 1:2 and 1:1. As most of the CP12s are bilabeled, this shows that the release of one label corresponds to the action of one monomer of GAPDH. In parallel, the NADPH-dependent activity of GAPDH from the EPR sample was measured at the beginning and after the complete release of the label and did not change.

The labeling of CP12 by MTSL corresponds to the formation of a disulfide bridge between CP12 and the nitroxide radical. CP12 was then labeled with another paramagnetic label, the 3-maleimido-proxyl that introduces a thioether bound between the cysteine residues of CP12 and the label (Figure 3A). No significant change in the EPR signature was observed (Figure 3B).

The mixtures of GAPDH and labeled CP12 were loaded onto a native gel to check the formation of a stable GAPDH−CP12 complex using an in vitro reconstitution test (Figure 4A) (*13*). Using nonlabeled CP12, the presence of the complex was immediately detected, and it was still present after 1 h. For labeled CP12 in the presence of GAPDH, no complex was detected. This result shows that no stable permanent interaction takes place between labeled CP12 and GAPDH.

The effect of labeled CP12 on the NADPH-dependent activity of GAPDH was measured as CP12 can have an inhibitory effect on GAPDH activity (*27*) (Figure 4B). The NADPH−GAPDH activity was reproducibly decreased by a factor of 3 when nonlabeled CP12 was added, but no change was observed with labeled CP12. This observation strengthens the previous results showing the absence of a stable interaction between labeled CP12 and GAPDH.

While all GAPDHs have two conserved cysteine residues present in the active site, some other cysteine residues can also be found, depending on species. GAPDH from *C. reinhardtii* has two additional cysteine residues per monomer. To evaluate the specificity of these cysteine residues, GAPDHs from other species having either no additional cysteine residues (yeast *S. cerevisiae*) or two other cysteine residues located at different positions (rabbit muscle) were used (Figure S3 of the Supporting Information). Four SH groups were quantified with DTNB for the algal and rabbit muscle GAPDH, showing the absence of disulfide. All these GAPDHs were mixed with labeled CP12 in a 1:1 molar ratio, and except for *C. reinhardtii* GAPDH, no effect was observed on the EPR spectrum of labeled CP12 even after 140 min (data not shown).

The ability of *C. reinhardtii* GAPDH to release spin-labels grafted onto proteins other than CP12 has also been tested using two monolabeled proteins. Cytochrome c_3 is a globular protein of 12 kDa, whereas N_{TAIL} is a totally disordered protein of 14.6 kDa. These proteins were thus chosen because of their similar molecular masses and their different structural properties. While *C. reinhardtii* GAPDH cannot remove the label from cytochrome c_3, in the case of the disordered labeled N_{TAIL}, GAPDH is able to induce a slow ($\tau = 50$ min) and partial release of the label (Figure S4 of the Supporting Information).

DISCUSSION

CP12 is a flexible protein well-known to interact with and to regulate GAPDH. This regulation is likely related to structural transitions of both proteins, but the molecular bases of these changes are not yet understood. Site-directed spin labeling combined with EPR spectroscopy is an approach well-suited to addressing this question. It usually consists of grafting onto a cysteine residue a paramagnetic probe that can report changes in its local environment. EPR spectra of nitroxide radicals grafted onto proteins reflect the mobility of the probe linked to movement of the entire protein, local backbone fluctuations, and/or internal dynamics of the spin-labeled lateral chain (*15*, *28*). This technique is highly sensitive in detecting spectral changes related to small changes in the local environment of the label (*21*, *29*, *30*). We show here that this approach can also be used to probe the functional role of cysteine residues.

The labeling reaction with MTSL led to the grafting of two labels at positions C66 and C75 as revealed by mass spectrometry. No labeling was observed on the other two cysteine residues, suggesting that the C23−C31 disulfide bridge is present. This is in agreement with previous results that showed that one pair of cysteine residues was more prone to oxidation than the other (*31*). In particular, after a few hours upon air exposure, reduced CP12 formed one disulfide bond, with two other cysteine residues still reduced. Results reported here identify C23 and C31 as the residues prone to fast oxidation and support the previous hypothesis of a sequential mechanism for reoxidation of the four cysteine residues (*31*).

CD spectroscopy was used to monitor the global folding of CP12. As previously described (*1*), the CD spectrum of the oxidized state of CP12 is characteristic of a protein containing some α-helical contents. In contrast, CD spectra of reduced or labeled CP12 are characteristic of a totally disordered protein. This shows that CP12 underwent a dramatic conformational change after reduction or labeling, leading to an order to disorder transition. However, labeled CP12 does not behave as the reduced form under SDS−PAGE and migrates at the same position as the oxidized protein. The behavior of reduced CP12 under SDS−PAGE is therefore not linked to the absence of some structural elements but rather to the breakage of the N-terminal disulfide bridge. Indeed, labeled CP12 that is unstructured but bears this disulfide bridge migrated as the oxidized protein. This study thus allows us to conclude that the reduction of this disulfide bridge is mainly responsible for the difference in migration via SDS−PAGE between oxidized and reduced CP12.

TFE induces a low-dielectric environment that favors formation of intrapeptide bonds and is well-known to promote folding of unstructured proteins (*21*, *32*, *33*). CD spectra of labeled CP12 in the presence of TFE at a concentration as low as 10% clearly revealed a gain of α-helical content. EPR spectra of labeled CP12 in the presence of TFE also report a gain of rigidity that can be deduced from the decrease of the label's mobility. Although the predicted α-helices are located around the N-terminal disulfide bridge (*11*), the gain of α-helicity in this region can induce slight structural changes in the C-terminal part of the protein. Such an effect was already reported on other proteins undergoing an induced folding (*21*, *22*). These results suggest that although unstructured, labeled CP12 can gain α-helicity if the environment is slightly hydrophobic.

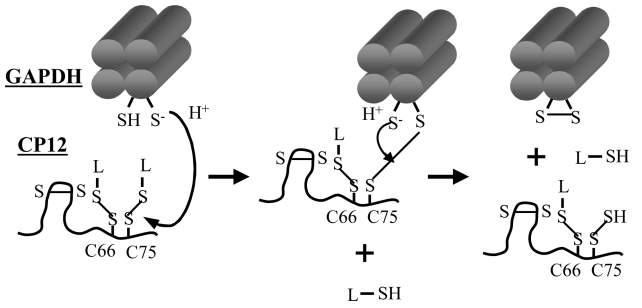

FIGURE 5: Proposed mechanism for the release of the label from CP12 by GAPDH. A thiol−disulfide transfer event occurs between labeled CP12 and the cysteine residues of GAPDH. At the end of the action of one monomer of GAPDH, one label is released and an intramonomeric disulfide bridge is formed within GAPDH. L stands for the nitroxide radical giving the EPR signal.

In vitro reconstitution tests and activity measurements showed the absence of a stable interaction between GAPDH and the labeled protein. On the other hand, the effect of GAPDH on labeled CP12 has been studied by monitoring the evolution of the EPR signal as a function of time. Surprisingly, the presence of tetrameric GAPDH in a 1:1 molar ratio induced a full release of the label in solution. This behavior is quite unusual and to the best of our knowledge only reported for compounds such as DTT (*14*). When 3-maleimido-proxyl is used to label the CP12, GAPDH cannot remove the label. Moreover, the EPR signal did not change in the presence of GAPDH, confirming that no stable interaction between the two partners occurred when CP12 was labeled. These results indicate that, in the case of MTSL labeling, the release involves the breakage of the disulfide bond between CP12 and the label. Thus, GAPDH can catalyze thiol−disulfide exchange reactions with labeled CP12 via a thiol group.

Titration on the algal tetrameric GAPDH gave four cysteine residues per monomer, showing that all SH groups of this protein are rather exposed to the solvent. GAPDH remained active after the release of the label from CP12 and thus rules out the possibility that the cysteine residues involved in the active site (C156 and C160, *C. reinhardtii* numbering) are the ones responsible for the thiol−disulfide exchange. One thus can hypothesize that C21 and C291 (*C. reinhardtii* numbering) are candidates; moreover, modeling of the GAPDH structure (*2*) shows that these two residues are close enough to form a disulfide bridge within a monomer. The active site cysteine residues are far from this disulfide bridge, and the activity of GAPDH is maintained. The effect of GAPDH from yeast, containing no cysteine residues other than the ones in the active site, and from rabbit muscle, containing two other cysteine residues but located at different positions, has been monitored by EPR. These GAPDHs could not release the label from CP12 (data not shown), supporting the role of C21 and C291 of the algal GAPDH in the thiol−disulfide exchange reaction. As release of one label corresponds to the action of one monomer, a model based on the formation of an

intramonomeric disulfide bridge on GAPDH is proposed (Figure 5). The existence of an intermonomeric disulfide bridge on GAPDH can be ruled out because no dimer is observed on a denaturing gel (data not shown). An intermolecular disulfide bridge between GAPDH and CP12 is also ruled out because no covalent complex is observed between these two proteins.

It is well-known that GAPDH has a moonlighting function and has at least 10 distinct, confirmed enzymatic or nonenzymatic activities apart from its function of converting 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate and inorganic phosphate (*34*). Here, for the first time, we showed that the algal GAPDH can be a member of the family of thiol−disulfide catalysts. This mechanism is rather specific as it cannot release the label from globular cytochrome c_3 but can induce a slow and partial release of the label from N_{TAIL}, another IDP. This new function thus seems to be linked to the flexibility of the labeled protein.

The most studied redox system in photosynthetic organisms is the thioredoxin system involved in the regulation of a growing number of target proteins via thiol−disulfide exchange (*35*). In addition, recent studies suggest that glutaredoxins could also play an important role in redox signaling, especially by regulating protein glutathionylation, a post-translational modification whose importance begins to emerge in mammalian cells and, only very recently, in photosynthetic organisms (*36*, *37*). In vivo, for example, cysteine thiols can also be reversibly modified by low-molecular weight compounds such as cysteine, cysteamine, and glutathione (*36*). Through these modifications, oxidants can modulate the activity of a range of proteins in the cells. As important redox regulation takes place in photosynthetic organisms, the thiol disulfide exchange catalyzed by the algal GAPDH might be physiologically relevant.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

EPR spectra with regard to the effect of TFE and GAPDH on labeled CP12 as well as other proteins, alignment of different GAPDHs used in this work, and mass spectrometry data concerning the identification of the labeled site on CP12. This material is available free of charge via the Internet at http://pubs.acs.org.

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