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Mass spectrometric analysis of the interactions between CP12, a chloroplast protein, and metal ions: a possible regulatory role within a PRK/GAPDH/CP12 complex

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The small chloroplast protein CP12 plays the role of a protein linker in the assembly process of a PRK/GAPDH/CP12 complex that is involved in CO_2 assimilation in photosynthetic organisms. The redox state of CP12 regulates its role as a protein linker. Only the oxidized protein, with two disulfide bonds, is active in complex formation. Several observations indicating that CP12 might bind a metal ion led us to screen the binding of different metal ions on oxidized or reduced CP12 using non-covalent electrospray ionization mass spectrometry (ESI-MS) experiments. The oxidized protein bound specifically Cu^{2+} and Ni^{2+} (K_d of $26 \pm 1\,\mu M$ and $11 \pm 1\,\mu M$, respectively); other cations such as Fe^{2+} and Zn^{2+} did not bind, while cations such as Cd^{2+} formed non-specific adducts to CP12. Similar results were obtained for metal ions on screening with the reduced CP12. Interestingly, the present results suggest that Cu^{2+} catalyzes the re-formation of the disulfide bonds of the reduced CP12, leading to recovery of the fully oxidized CP12 that is then able to bind a Cu^{2+} ion. Finally the high similarity between CP12 and copper chaperones from *Arabidopsis thaliana*, as judged by hydrophobic cluster analysis, provides additional evidence for the relevance of metal binding for the *in vivo* situation. The findings that CP12 is able to bind a metal ion, and that Cu^{2+} catalyzes the oxidation of the thiol groups of CP12, are new characteristics of this protein that may prove to be important in the regulation of the assembly process of the PRK/GAPDH/CP12 complex. Copyright © 2005 John Wiley & Sons, Ltd.

Many proteins are functional in a combinatorial manner and establish a dynamical network of interactions with other specific proteins. Determination of these interaction patterns is an essential part of functional genomic research. Recently, proteomic approaches, coupled to other methods,[1,2] have been developed to obtain information about the different sets of protein–protein interactions that might exist in the cell depending on its physiological state, the environmental conditions, or genetic background.[3] The analysis of these interactions is probably one of the most important challenges for the years to come.[3]

Among all techniques used to study biological macromolecular complexes, electrospray ionization mass spectrometry (ESI-MS) is well suited to characterize the subunits and ligand binding stoichiometry, as well as the regulatory properties of non-covalently bound species; the subject has been well reviewed by Heck and Van Den Heuvel.[4] Advantages of this method lie in its speed, sensitivity and accuracy. The technique possesses several advantages versus other spectroscopic methods: (i) it requires moderate amounts of protein; (ii) all metal ions are theoretically observable regardless of their electronic properties; and (iii) it is well suited for competition experiments.

Determination of equilibrium constants by ESI-MS has usually involved titration experiments; a frequent assumption made for this approach is that the ionization efficiencies for both complexed and non-complexed species are the same. Thus, it is still debatable whether or not the relative ion abundances reflect the relative concentrations of species in solution. However, it is not possible to determine the signal-response factor of a non-covalent complex in solution unless its dissociation constant is already known. To overcome these problems, different groups have introduced an internal standard that allows relative quantitation of complexed and free species,[5] or a so-called R-factor that gives a relative measurement of the signal response for free and complexed species.[6] However, most of the examples in which ESI was used for determination of binding constants did not consider

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these aspects. Nonetheless, the results obtained were in reasonable agreement with the values determined by solution-phase methods.

Numerous applications of ESI-MS to the study of non-covalently bound complexes have been reported; such studies determine complex stoichiometry or describe biological properties of protein–ligand, protein–protein, protein–metal ions, DNA–ligand complexes, etc. In addition, this approach also allows the determination of dissociation constants of weakly bound non-covalent complexes, with K_d values ranging from milli- to micro-molar.[7–10]

A protein complex involved in the Benson-Calvin cycle, responsible for CO_2 assimilation in photosynthetic organisms, has been isolated from plants.[11,12] It is composed of phosphoribulokinase (PRK)/glyceraldehyde-3-phosphate dehydrogenase (GAPDH)/CP12, and has also been found in green algae such as *Chlamydomonas reinhardtii*[13,14] and *Scenedesmus obliquus*,[15,16] and in the cyanobacterium *Synechocystis* PCC6803.[14] The small nuclear-encoded 8.5-kDa protein, named CP12, has been recently identified in most of these complexes whether they originated from higher plants, green algae or cyanobacteria.[14,17] This protein consists of about 80 residues with four conserved cysteine residues. It also shares sequence similarities with part of the C-terminal extension of the GAPDH B subunit that contains the regulatory cysteine residues,[16] suggesting similarity to GAPDH, a redox regulator of the CP12 protein activity.[18]

Secondary structure predictions suggest that CP12 might possess two peptide loops formed via two disulfide bonds between the two N-terminal cysteine residues and the two C-terminal ones.[17] It was also proposed that these putative loops play a role in the formation of the complex, and particularly that the interaction between GAPDH and CP12 occurs at the C-terminal peptide loop of CP12, while the interaction of CP12 with PRK occurs at the N-terminal peptide loop of CP12.[14] Recently, the role of CP12 in the assembly process of a PRK/GAPDH/CP12 complex from *C. reinhardtii* was characterized by surface plasmon resonance and *in vitro* reconstitution experiments.[19] It was shown that the first step in the assembly process of the complex is the association of CP12 with GAPDH that results in a change of conformation and kinetic properties of GAPDH. This GAPDH/CP12 sub-complex then becomes associated with PRK.[19] These findings indicated that CP12 may act as a linker in the assembly of the PRK/GAPDH/CP12 complex. Only the oxidized CP12, with its two disulfide bonds, was able to act as a linker, signifying that the role of CP12 in complex assembly is dependent on its redox state. A change in redox state of CP12 is also accompanied by a structural change; circular dichroism and nuclear magnetic resonance spectra showed that oxidized CP12 is mainly composed of α-helices plus random coil, while upon reduction it becomes disorganized and very flexible. CP12 has physicochemical properties similar to those of intrinsically unstructured proteins (IUP) which are involved in protein–protein interactions, and play major roles in cellular functions.[20–25]

Another intriguing observation regarding CP12 is that the recombinant protein purified by affinity chromatography on a nickel column has a yellow color, even after extensive dialysis with buffer depleted in metal and imidazole. The absorption spectra from 280 to 600 nm were similar to those of ferredoxin, with a broad peak around 410 nm. Functional domain prediction using a NCBI conserved domain search suggested that a zinc-binding domain might exist in CP12. All these observations led to the hypothesis that CP12 could bind a metal ion. Therefore, non-denaturing MS experiments were designed in order to (i) identify which metal ions CP12 would be able to bind, and (ii) to measure the dissociation constants of complexes between CP12 and those metal ions that could specifically bind to CP12, and hence characterize the specificity of this binding.

EXPERIMENTAL

Materials

Most metal ions, as $CaCl_2$, $CdSO_4$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $ZnSO_4$, $NiSO_4$, $Fe(NO_3)_3$, $FeSO_4$, $(NH_4)_2Fe(SO_4)_2$ and $CuCl_2$, were supplied by Merck. EDTA and dithiothreitol (DTT) were from Sigma. EndoLysC was obtained from Pierce Chemicals (Rockford, IL, USA).

CP12 purification

Recombinant wild-type *Chlamydomonas* gene and mutant H47L CP12 were purified to apparent homogeneity, as previously described.[19] Standard and reverse primers used for mutant H47L recombinant CP12 were 5′-CTC AGC GCT GCT GTC TCG CTT AAG AAG GAT GCC GTC-3′ and 5′-GAC GGC ATC CTT CTT AAG CGA GAC AGC AGC GCT GAG-3′, respectively. The histidine tag was removed using Factor Xa, according to the manufacturer's instructions (Novagen). Site-directed mutagenesis was performed using a QuickChange™ site-directed mutagenesis kit (Stratagene). Introduction of the point mutation H47L and absence of additional mutations were confirmed by DNA sequencing.

Mass spectrometric sample preparation

Prior to MS experiments, CP12 was desalted using an ultra-free membrane filter with a cut-off of 3000 Da (Millipore). CP12 concentration was then determined with the Bradford assay using bovine serum albumin (BSA) as standard.[26] For MS analyses in denaturing conditions, the CP12 was diluted in a water/MeOH/acetic acid solution (50:50:1, v/v/v). For measurements under non-denaturing conditions, the protein was diluted in 20 mM ammonium acetate (pH 6.8) at a protein final concentration of 5 or 2 μM. Cation-binding studies were performed by adding increasing metal-ion concentrations to a fixed protein concentration of 5 μM.[27] Reduced CP12 was obtained after incubation of the oxidized form of the protein with 1 mM DTT (pH 7.8) at 37°C for 1 h. When necessary the cysteine residues were alkylated with 5 mM iodoacetamide (IAA) solution. Then the reduced, or the reduced and alkylated, protein was desalted as described above and was directly analyzed by MS under denaturing or non-denaturing conditions, as well as under strongly acidic conditions ($HCOOH/H_2O$, 70:30, v/v).

Electrospray mass spectrometry (ESI-MS)

Metal ion/protein complexes were analyzed using a hybrid Qq-TOF mass spectrometer (Q-Star Pulsar, Applied Biosystems, Les Ulis, France) fitted with an ionspray source. Instrumental parameters (values of ion spray voltage (ISV),

nebuliser and curtain gas (GS1 and GS2), declustering potential (DP1 and DP2) and focusing potential (FP)) were optimized in order to obtain the best sensitivity without disrupting non-covalent interactions. The parameters used were the following: GS1 = 55 psi, GS2 = 45 psi, DP1 = 150 V, FP = 125 V, DP2 = 15 V. The mass spectrometer was calibrated with a 3 mM cesium iodide solution in 50:50 isopropanol/water (v/v) for an m/z range 580–3000. Samples were introduced into the mass spectrometer at a flow rate of 5 µL/min.

During MS experiments designed to characterize non-covalent complexes, the acquisition time was shortened to 30 s to get rid of memory effects due to metal-ion retention in the PEEK tube during the course of a MS run from one experiment to another, while still attaining a good signal-to-noise ratio. To avoid the non-specific accumulation of these metal ions, the tubing was washed with 200 µM EDTA in a 200 mM ammonium acetate solution prior to any measurements.

MALDI-MS

To assess whether the two disulfide bonds of oxidized CP12 occurred between the pairs of cysteine residues C23 and C31, and C66 and C75 (*C. reinhardtii* numbering), the protein was digested with EndoLysC at 37°C for 15 h in 20 mM ammonium bicarbonate (pH 7.8), with a protease-to-substrate ratio of 1:100 (w/w). The digest mixture was then divided into two parts. The first aliquot was acidified with acetic acid in order to prevent disulfide bond recombination, and the second was treated with 1 mM DTT (pH 7.8). Peptide mapping of both these digest fractions was recorded using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) Voyager DE STR mass spectrometer (Applied Biosystems, Les Ulis, France). MALDI mass spectra were acquired in the reflectron mode, with an m/z range 500–3000, a grid percentage of 60%, a delayed extraction time of 300 ns, and a laser intensity setting of 1869. Calibration was performed with a standard peptide mixture solution (Pepmix 4, LaserBioLabs, Sophia-Antipolis, France).

Determination of the dissociation constants

Data were fitted to theoretical curves defined by Eqns. (1), (2) and (3), given below, using ORIGIN Pro 7 software (Copyright, 1991–2003, OriginLab Corp., Northampton, MA, USA[28]).

Alkylation experiments

The free SH groups in CP12 were quantified and mapped by alkylation with IAA as previously described.[29] After desalting, samples were analyzed by MALDI-TOFMS.

Hydrophobic cluster analyses

We used the bidimensional hydrophobic cluster analysis (HCA),[30,31] which offers the possibility to add information about secondary structures to the lexical analysis of the considered sequences. The sequence is handled on a duplicated α-helical net in which hydrophobic amino acids (V, I, L, F, M, Y, W) are contoured. The defined hydrophobic clusters (i.e. hydrophobic amino acids that are separated from each other by at least four non-hydrophobic residues, a connectivity distance linked to the use of an α-helical support) were shown to mainly correspond to the internal faces of regular secondary structures (α-helices or β-strands).[32] Conservation of hydrophobic cluster features which participate in the protein core, together with a similar texture of sequence similarities, are associated with the maintenance of a similar structure and often help and/or allow the alignment procedure for highly divergent sequences (typically in the 10–20% sequence identity range, below the so-called twilight zone of 25–30% identity).

RESULTS

Metal-ion screening on oxidized CP12

Mass spectrometric analysis in denaturing conditions gave a molecular mass in good agreement with the theoretical molecular mass of oxidized CP12 (8734.3 ± 0.2 Da, data not shown). Under non-denaturing conditions, the oxidized CP12 (CP12ox) was characterized by a charge-state distribution (CSD) of ions spanning from +4 to +8 charges (Fig. 1(A)), with major charge states +5 and +6. To avoid any discrimination effects all charge states were considered for metal-ion screening, as well as for calculation of the dissociation constants. According to the measured masses, under both denaturing and non-denaturing conditions, the CP12ox possesses two disulfide bridges that are formed between cysteine residues C23 and C31, and C66 and C75, as confirmed by the peptide mapping of the EndoLysC-digested CP12ox (data not shown).

We first screened the complexation properties of different metal ions by recording the ESI mass spectra of 5 µM CP12 in the presence of a 20-fold molar excess of metal ions. Different behaviors were observed, depending on the nature of the metal ion bound to CP12. For example, in the presence of Fe^{2+} and Fe^{3+}, no protein/metal complex was observed (Fig. 1(B)). For other metal ions such as Cd^{2+}, several species were observed, the major one corresponding to the free CP12ox and others to CP12ox complexed with one to three Cd^{2+} ions (Fig. 1(D)). This result was obtained for a protein/metal-ion ratio 1:20 or lower. The presence of a high amount of cadmium-ion adducts with the CP12ox suggests a non-specific binding of this cation to the protein. In contrast, for Cu^{2+} (Fig. 1(C)) and Ni^{2+} (not shown), the major species observed in the mass spectra was the CP12ox complexed with a single Cu^{2+} or Ni^{2+} cation.

To discriminate between non-specific and specific binding, we considered the distribution of species; if the distribution displayed a statistical distribution of a large number of ion adducts on the protein, the binding was considered non-specific. In contrast, if only one protein/metal species was observed, independently of the protein/metal-ion ratio and with a well-defined stoichiometry, this interaction was considered as specific. According to this criterion, only the binding of Cu^{2+} and Ni^{2+} to CP12ox could be considered as specific, which led us to determine the dissociation constants of the CP12ox/Cu^{2+} and CP12 ox/Ni^{2+} complexes.

Calculation of dissociation constants of CP12ox/Cu^{2+} and CP12ox/Ni^{2+} complexes

As the CSDs of the free and complexed forms of CP12ox were identical, it was concluded that it could be approximated that the binding of the ligand did not promote any drastic
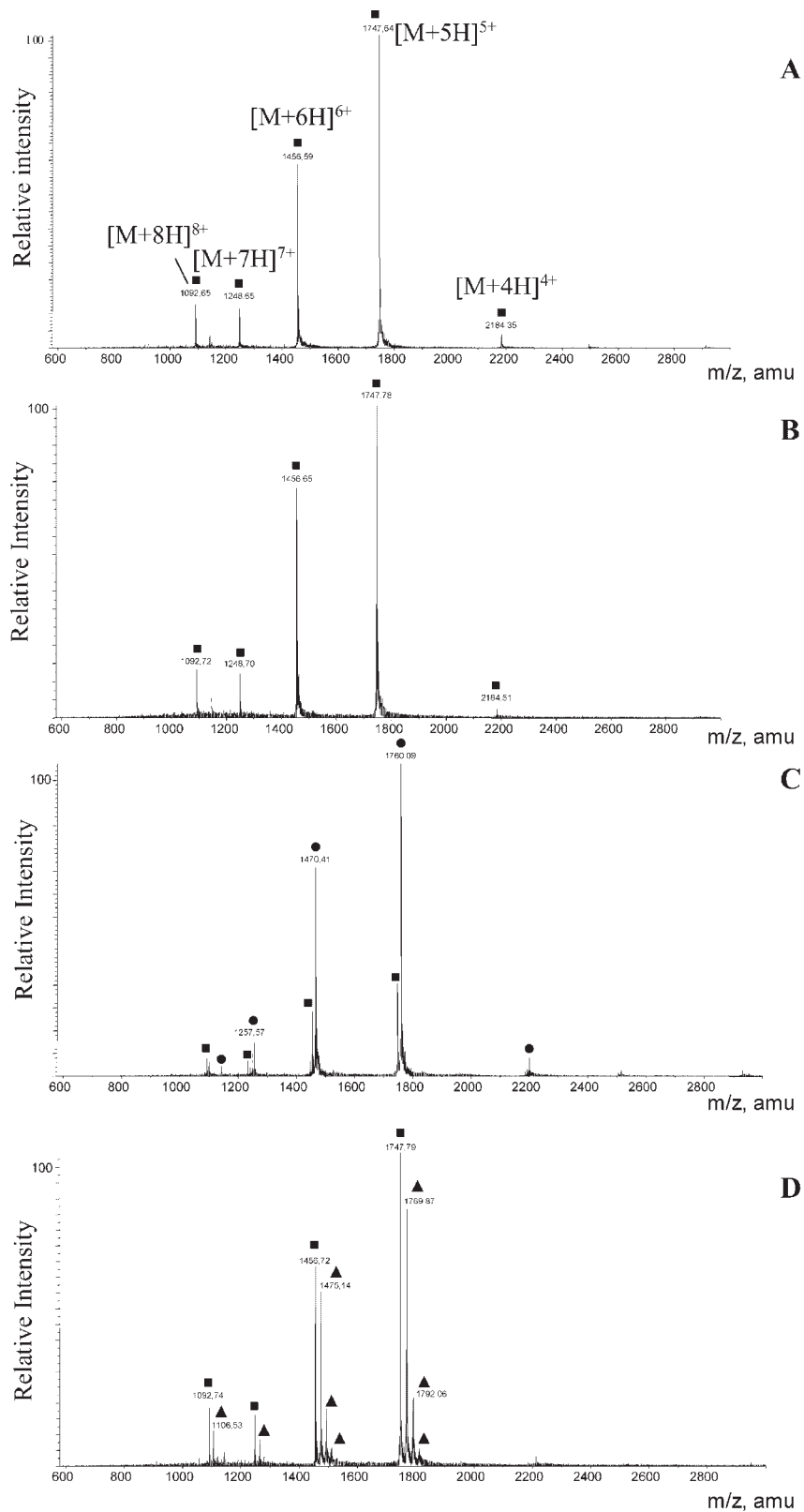
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Figure 1. ESI mass spectra of the oxidized CP12 protein without or with metal ions. (A) ESI mass spectrum of the free oxidized CP12 protein. (B, C, D) ESI mass spectra of the oxidized CP12 protein in the presence of Fe^{2+} (B), Cu^{2+} (C) and Cd^{2+} (D), at a molar ratio protein/metal ion of 1:20 with a fixed protein concentration of $5\,\mu M$. Labels: ■: free CP12ox, ●: CP12/Cu^{2+}complex, ▲: CP12/Cd^{2+}complexes.
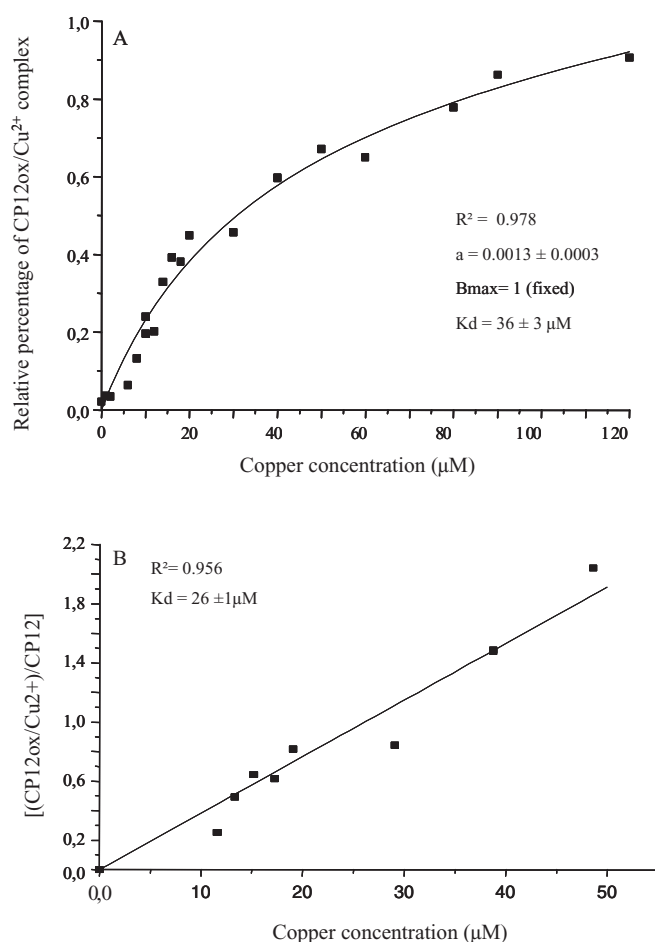
Figure 2. Titration of the CP12ox by Cu^{2+}. Saturation (A) and linear correlation (B) curves for the formation of the CP12ox/Cu^{2+}complex as a function of increasing concentrations of metal ion (from 0 to 120 μM), with a fixed protein concentration of 2 μM. Linear regression calculations (Eqn. (3)) allowed determination of a dissociation constant of 26 ± 1 μM for the CP12ox/Cu^{2+}complex.

conformational changes of CP12ox and that, most importantly, both forms had similar desorption and ionization yields. Thus, the two CP12ox species (CP12ox alone and complexed to a metal) differed only by a mass increase due to the binding of the metal ions. On this basis the relative ion abundances recorded for the free and complexed CP12ox can be directly linked to their relative concentrations in solution. To determine the dissociation constants, we recorded the mass spectra of CP12ox with increasing concentrations of metal ions (Cu^{2+} or Ni^{2+}), ranging from 0 to 120 μM, at a fixed protein concentration of 2 μM. Dissociation constants were then deduced from the relative concentrations of bound and free CP12ox. The experimental data were first fitted to a hyperbola:

$$[PM]/[P]_{tot} = [M]/(K_d + [M]) \tag{1}$$

where $[P]_{tot}$ is the total protein concentration, [M] the free metal-ion concentration, [PM] the CP12/metal-ion complex concentration, and K_d the dissociation constant.

The experimental data followed a sigmoidal curve (Figs. 2(A) and 3(A)) and did not fit the theoretical curve

deduced from Eqn. (1). The sigmoid may be explained by an underestimation of the relative intensity of CP12ox bound to the metal ion at low CP12/metal-ion ratios because of a competition between the specific binding of the metal ion to CP12ox and adsorption onto the PEEK tube. In addition, at high metal-ion/CP12 ratios the concentration of complexed CP12ox is over-estimated, even with a short acquisition time, because of the formation of additional CP12ox/metal-ion complexes with residual cations released by the PEEK tube prior to the transfer of the molecules in the gas phase. To take this non-specific binding into account, the data were fitted to the following equation:

$$[PM]/[P]_{tot} = [M]/(K_d + [M]) + a[M] \tag{2}$$

where the term a[M] corresponds to the proportion of the metal ion responsible for the over-estimation of the CP12/metal-ion complex.

The resulting correlation coefficients were 0.978 and 0.927 for Cu^{2+} and Ni^{2+}, respectively. The worst fitted parts corresponded to the beginning and the end of the curves. To avoid these problems, only the central parts of the curves were fitted to Eqn. (3). In this case, the proportion of the complexed species is linearly related to the ligand concentration:

$$[PM]/[P] = 1/(K_d)[M] \tag{3}$$

Using a linear regression with Eqn. (3), the dissociation constants of CP12ox/Cu^{2+} and CP12ox/Ni^{2+} were determined (Figs. 2(B) and 3(B)). K_d values were 26 ± 1 μM for the CP12ox/Cu^{2+} complex and 11 ± 1 μM for the CP12ox/Ni^{2+} complex.

Effect of the His 47 Leu mutation on metal-ion binding

Amino acids that are involved in the binding of cations in proteins are well characterized. Among them, histidine plays an important role.[33] The comparison of amino acid sequences of various CP12s from different organisms revealed the presence of a highly conserved histidine residue in position 47,[19] which could potentially play a role in the binding of a metal ion. Screening of the binding properties of the different metal ions for the H47L CP12 mutant showed that the mutation did not affect the behavior of CP12 towards the metal ions tested (data not shown). In addition, the dissociation constant calculated in the presence of Cu^{2+} was similar to that obtained with the wild-type CP12ox (27 ± 1 μM, data not shown). Hence, His 47 is not essential for metal-ion binding.

Metal screening on reduced CP12: catalysis of disulfide bond formation by Cu^{2+}

Since it has been shown previously that, upon reduction of its two disulfide bridges, CP12 becomes more flexible and unstructured, a study of the reduced CP12 (CP12red) was conducted to determine whether the greater flexibility and the availability of four free SH groups would change the metal-ion-binding properties of CP12 (Fig. 4).

Surprisingly, MS control analysis on CP12red under both non-denaturing and denaturing conditions gave a molecular mass of 8737.9 ± 0.2 Da, which is 1 Da less than expected (Fig. 4(A)). This extremely reproducible result was obtained with several independent sample preparations. Moreover,
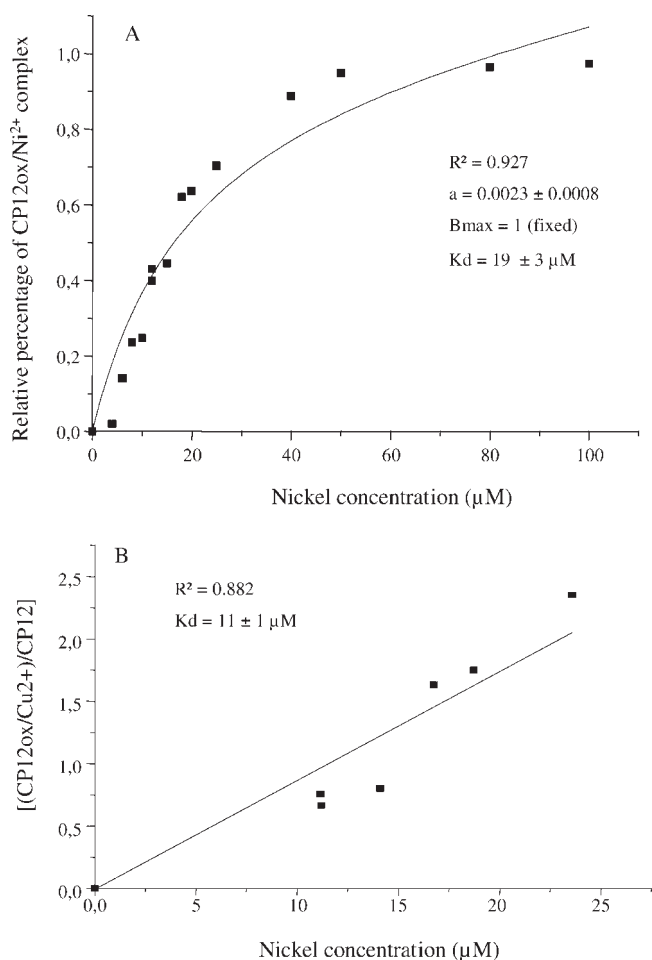
Figure 3. Titration of the CP12ox by Ni^{2+}. Saturation (A) and linear correlation (B) curves for the CP12ox/Ni^{2+} complex as a function of increasing concentrations of Ni^{2+} (from 0 to 110 μM), with a fixed protein concentration of 2 μM. Linear regression calculations (Eqn. (3)) allowed determination of the dissociation constant of 11 $\pm$ 1 μM for the CP12ox/Ni^{2+} complex.

the CP12red was not stable during the course of the experiments (1 day). Its average mass varied from 8737.9 $\pm$ 0.2 Da at the beginning of the experiment to 8734.4 $\pm$ 0.2 Da, corresponding to the fully oxidized CP12, strongly suggesting that the disulfide bonds spontaneously formed in solution during the course of the experiment. The next measurements were performed on freshly reduced CP12 protein.

In order to determine whether or not the presence of a thiolate among the four cysteine residues could be responsible for the mass difference of 1 Da, the CP12red was alkylated with IAA and digested by EndoLysC. The MALDI mass spectrum obtained for the resulting peptide mixture showed that the protein bore four alkylated cysteine residues (data not shown), indicating that the 1 Da mass difference was not linked to the presence of a mixed population of reduced and oxidized CP12 in the sample. In contrast, the homogeneity of the CP12 population in its reduced form is in agreement with the presence of three SH groups and a more reactive thiolate. To confirm this hypothesis, the mass spectrum of CP12red was recorded under very acidic

conditions (70% formic acid, data not shown). In this case no mass difference was observed, with a measured mass of 8738.8 $\pm$ 0.2 Da, indicating the presence of four SH groups. This set of data demonstrated that the CP12red contains three SH groups and a thiolate (S^-) group under non-acidic conditions.

Under non-denaturing conditions, the ESI mass spectrum of reduced CP12 was characterized by a CSD of ions spanning from +4 to +8 charges (Fig. 4(A)); the major charge states of CP12red are +5 and +6. For most of the metal ions studied, screening of the binding of metal ions on CP12red gave the same results as those described for CP12ox, in terms of specificity and CP12/cation peak intensities. The non-specific adduct formation with Cd^{2+} (Fig.4(A)) was, however, more pronounced, as the peak corresponding to free CP12red was of very low intensity. Intense peaks corresponding to up to eight Cd^{2+} adducts were observed (Fig. 4(D)). In contrast to the CP12ox, the major peaks of the CP12red/Cd^{2+} complex corresponded to CP12red with four and five Cd^{2+} adducts. This result can be attributed to a larger accessibility of the reduced CP12 to the metal ions, due to its unstructured and flexible properties after reduction by DTT.[19]

The greatest difference was observed in the presence of Cu^{2+} (Fig. 4(B)). At a molar protein/metal-ion ratio of 1:20, the major species observed with CP12ox was the protein complexed with one Cu^{2+}, whereas, with the reduced CP12, the major species was the free protein (60% intensity) with the complexed form representing 40% of the total CP12 protein intensity. It was very striking that the measured mass of the free CP12 corresponded to that of the CP12ox (8734.6 $\pm$ 0.2 Da), and that the measured mass of the CP12red/Cu^{2+} complex was identical to that of the mass of the CP12ox/Cu^{2+} complex. This result strongly suggests that Cu^{2+} regenerates the oxidized form of CP12, which would then be able to bind one Cu^{2+} cation.

To test this hypothesis we attempted to observe re-oxidation intermediates, by recording re-oxidation kinetics of the CP12red in the presence of smaller CP12red/copper ratios (1:1 and 1:2; data not shown). Mass spectra were acquired for 10 min and masses of the free CP12 were determined every 30 s. This experiment confirms that the first species observed corresponds to the CP12red and that the m/z values of the CP12 charge states shifted towards those of the oxidized form of CP12 in the presence of copper. Given the redox properties of copper, we hypothesized that the oxidation of the thiol groups of cysteine residues could occur through the formation of an intermediate complex that would include one or two atoms of Cu^{2+} cross-linked by cysteine residues. The presence of a majority of free CP12 (60%), although Cu^{2+} was added in huge excess, was intriguing (Fig. 4(B)). Even if part of the copper was used to regenerate disulfide bonds, which led it to be present in a redox state not suitable for complexation with CP12, one would still expect the CP12ox/Cu^{2+} complex to be the main species in the mass spectrum. In the absence of any structural information on the free CP12 in its different redox states and intermediates, it would be hazardous to make any assumptions as to the putative structural changes that may occur upon re-oxidation of CP12 by copper. Nonetheless, it cannot be excluded that the
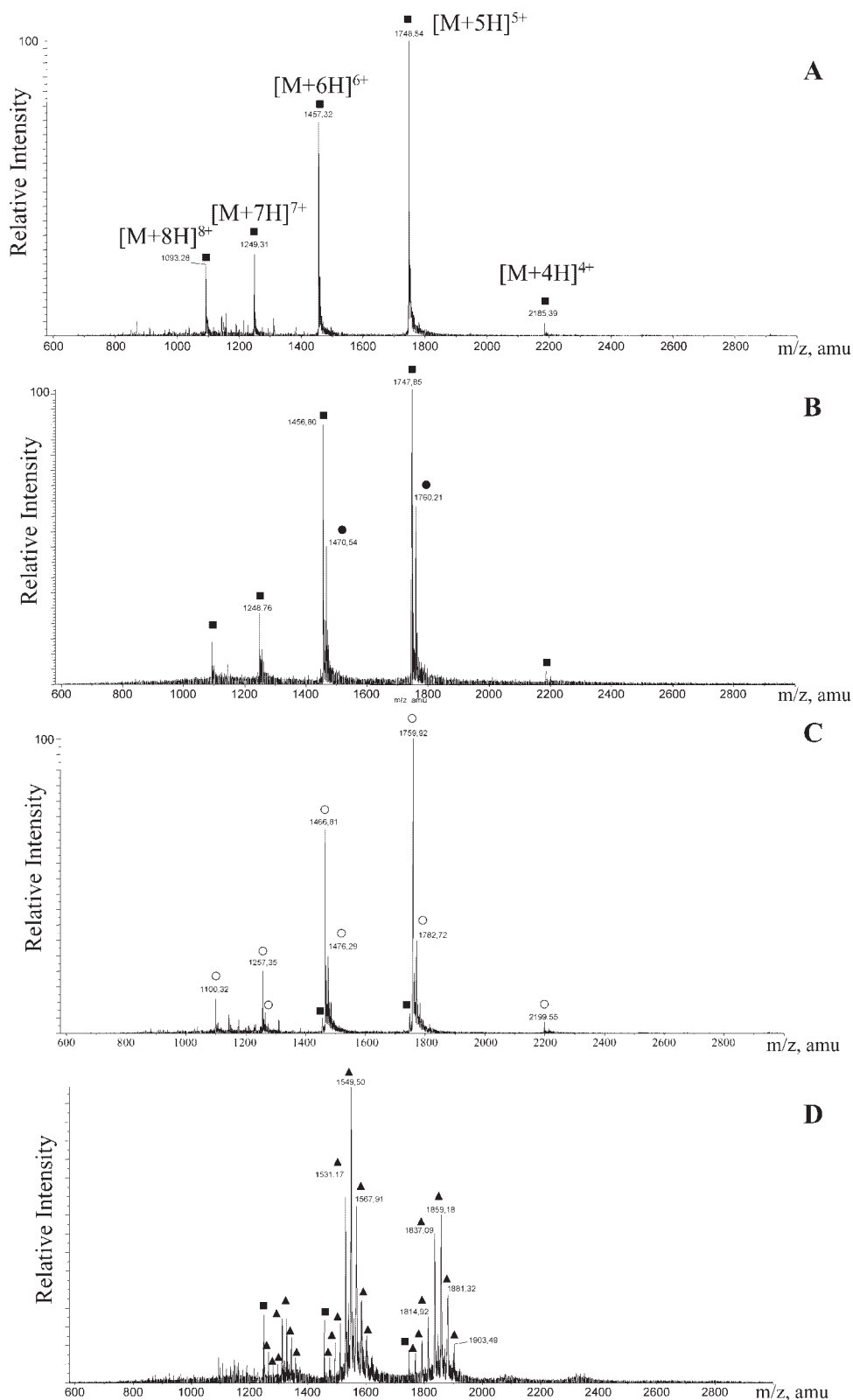
Figure 4. ESI mass spectra of the reduced CP12 protein without or with metal ions. (A) ESI mass spectrum of the free reduced CP12 protein. (B, C, D) ESI mass spectra of the reduced CP12 protein in the presence of Cu^{2+} (B), Ni^{2+} (C) and Cd^{2+} (D), at a molar ratio concentration of 1:20 with a fixed protein concentration of 5 μM. Labels ■: free CP12red, ●: CP12/Cu^{2+} complex, ▲: CP12/Cd^{2+} complexes, ○: CP12/Ni^{2+} complex.

free newly re-oxidized protein could correspond to a modified structure of CP12. Study of the Ni^{2+} cation binding to CP12red gave results very similar to those for CP12ox, with the CP12red/Ni^{2+} complex representing the major form

of the species and a low abundance of free CP12red (Fig. 4(C)). This indicates that CP12red binds Ni^{2+} in the same manner as CP12ox, and that thiols are not involved in Ni^{2+} complexation.
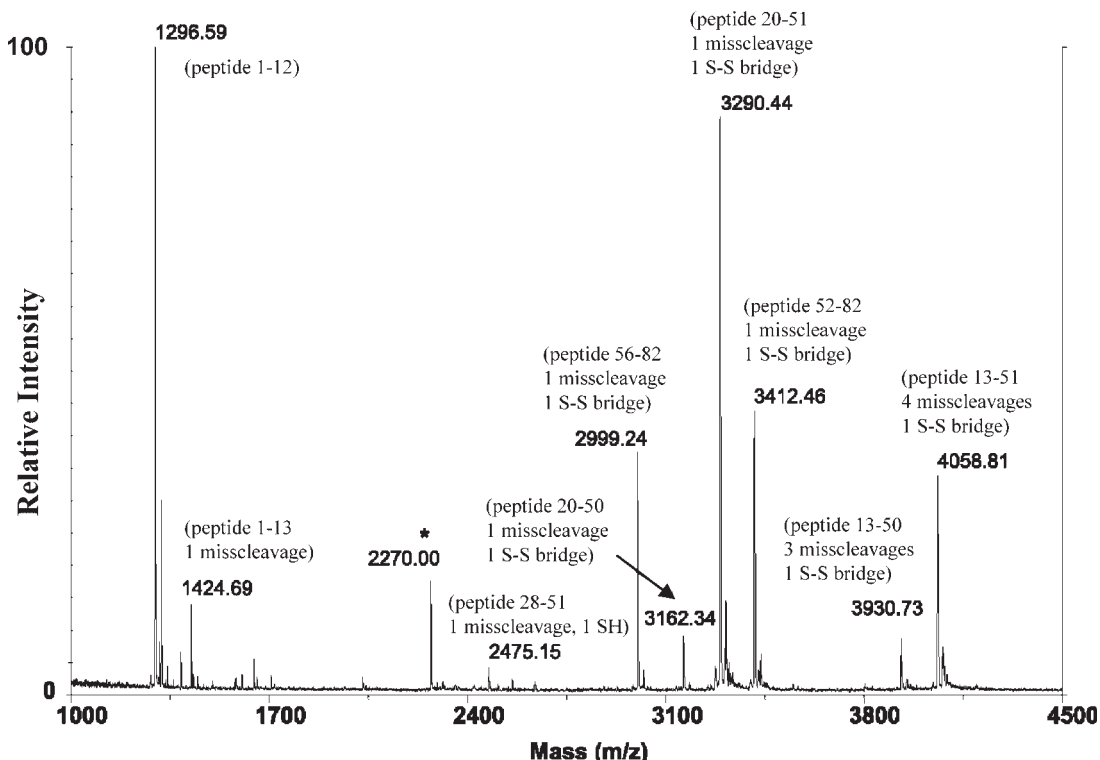
Figure 5. MALDI mass spectrum of the EndolysC peptides of CP12*ox protein obtained after addition of copper, showing the correct disulfide bond rearrangement upon copper binding. The stars correspond to unidentified peptides (e.g. non-specific cleavages).

DISCUSSION

Contrary to our expectations, our results unambiguously showed that CP12 does not bind Zn^{2+}, ruling out our first hypothesis suggesting the presence of a zinc-binding domain. In contrast, we showed that, in its oxidized form, the small chloroplast protein CP12 specifically binds Cu^{2+} and Ni^{2+} with a low affinity (dissociation constants of 26 ± 1 and $11 \pm 1 \, \mu M$, respectively). In particular, the K_d value for Cu^{2+} is within the range of those obtained with the prion protein (K_d of about $14 \, \mu M$)[34] or copper chaperone proteins.[5,35]

We then studied the behavior of the reduced CP12 to test whether the metal-ion-binding properties of the reduced form could be altered because of the presence of four free cysteine residues, or because of the structural disorganization that occurs upon reduction of CP12. We showed that the nickel ion binds to CP12red, with the same stoichiometry and peak-ratio intensity for both the CP12red/Ni^{2+} and CP12ox forms. In contrast, Cu^{2+} binding exhibits a striking difference. Under non-denaturing conditions, when Cu^{2+} is added to the CP12red solution, restoration of the disulfide bonds between the C23-C31 and C66-C75 cysteine residues was observed, leading to a newly oxidized CP12 (denoted CP12*ox) which has two disulfide bonds involving the same pair of cysteine residues as the original CP12ox (Fig. 5). This newly formed oxidized CP12 (CP12*ox) is then able to bind another Cu^{2+} cation. A similar disulfide bond formation catalyzed by Cu^{2+} and followed by the binding of the same metal was reported by McEwan *et al.*[36] They suggest that Cu^{2+} may catalyze the formation of two disulfide bonds on

the Sco protein, a protein required for copper delivery to cytochrome c oxidase. Moreover, this finding is also supported by the results obtained by Jensen *et al.*,[37] who showed that Cu^{2+}, but not Cu^+, is responsible for cysteine residue oxidation.

Surprisingly, the main species observed after re-oxidation of the cysteine residues by addition of a large excess of Cu^{2+} (protein/metal-ion ratio of 1:20) is the free CP12*ox. In the presence of such a high amount of Cu^{2+} in solution one would expect the main species to be the CP12*ox/Cu^{2+} complex, as was the case for the original CP12ox. Even if part of the Cu^{2+} is used for disulfide rearrangement, the remaining Cu^{2+} in solution should suffice to promote the formation of the CP12*ox/Cu^{2+} complex. This discrepancy could be explained by either (i) the production of a CP12*ox protein with a structure less capable of binding Cu^{2+}, or (ii) the requirement of a non-stoichiometric higher amount of Cu^{2+} to re-oxidize the disulfide bonds. Regarding the first possibility (i), a different conformation for CP12*ox cannot be deduced from the CSD, as it was similar for both CP12*ox (Fig. 4(B)) and CP12ox (Fig. 1(A)). Protein structure recovery upon binding of a metal ion has already been observed for other proteins, such as prion proteins upon Cu^{2+} binding,[38] human α-prothymosin in the presence of Zn^{2+},[21] or calbindin in the presence of Ca^{2+},[39,40] but such structural modifications are only subtle and cannot be observed by CSD changes in ESI mass spectra. Consequently, at this point, one cannot rule out the possibility that disulfide bond formation of CP12 by Cu^{2+} could result in a CP12*ox having a slightly different conformation compared with the original CP12ox. As proposition (ii), the disulfide bond re-oxidation of CP12red could require a higher amount
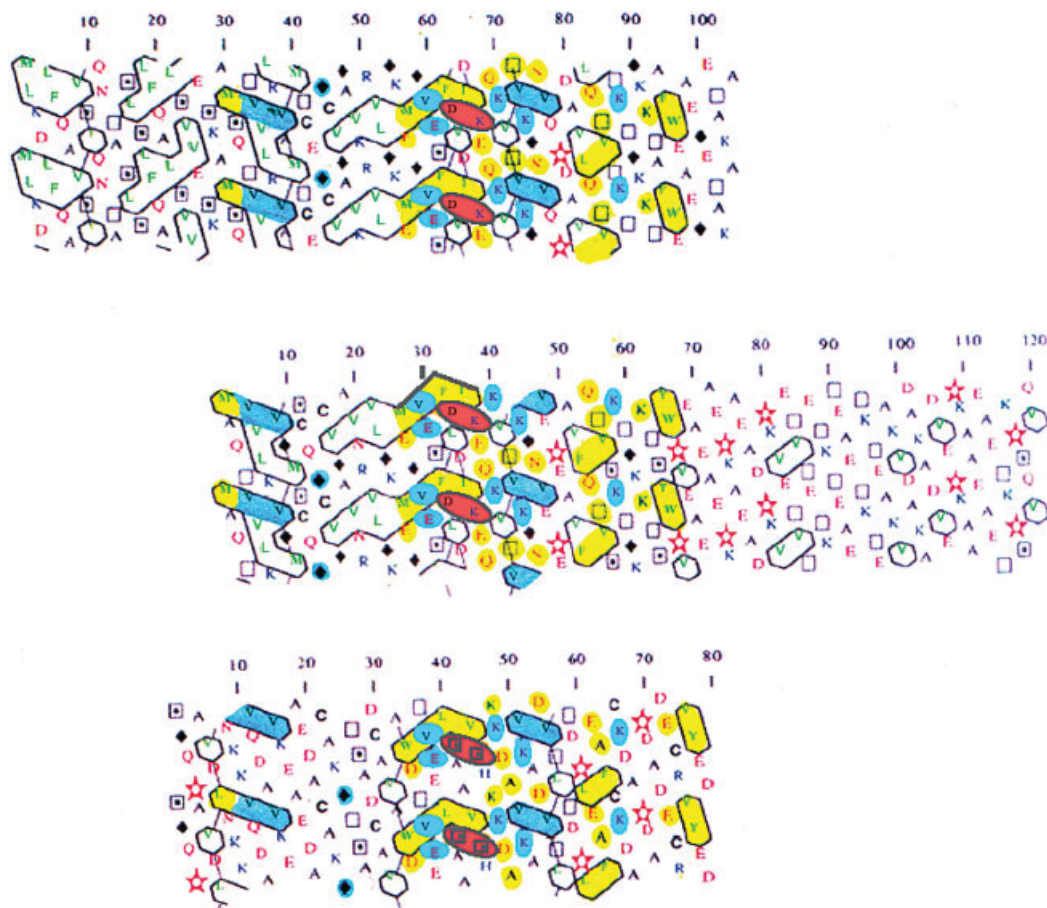
Figure 6. HCA plot comparison of copper chaperones from *Arabidopsis thaliana* and CP12 from *Chlamydomonas reinhardtii*. Two copper chaperones from *Arabidopsis* (accession number: AAK 64002, AAC33510) and CP12 from *Chlamydomonas* are represented. Special symbols, a diamond, a star, a square and a square with a point, are used for proline, glycine, threonine and serine. Hydrophobic residues are boxed and formed clusters that mainly correspond to the internal sides of regular secondary structures. Sequence homologies are associated with blue (identical residues), yellow (conserved substitutions) and red color (semi-conservative substitutions).

of Cu^{2+} if the catalysis occurred in a non-stoichiometric manner. This last proposition is supported by the results of Jensen *et al.*, who showed that thiol re-oxidation is induced by Cu^{2+} in a non-stoichiometric manner.[37]

At this point, the mechanism responsible for the re-formation of the two disulfide bonds remains unclear. The disulfide bond rearrangement could occur via the binding of one Cu^{2+} tetra-coordinated to the four thiols of the CP12red, catalyzing their re-oxidation, or via the binding of one Cu^{2+} to two cysteine residues, catalyzing the formation of a first disulfide bond, with the second pair of cysteine residues being re-oxidized afterwards. The oxidation kinetics experiment that we performed does not enable us to rule out one of the two hypotheses. It is possible that, in the case of a sequential mechanism, the formation of the first disulfide bond could be too fast to be observed. This mechanism could be supported by the existence of a more reactive pair of cysteine residues, favored by the presence of a thiolate on the CP12red. It is also noteworthy that the reduced CP12, when left in the presence of air for a few hours, showed the formation of one disulfide bond with the two remaining cysteine residues still reduced. Consequently, a sequential re-oxidation to form disulfide bonds is the most likely mechanism for CP12.

All the present MS experiments clearly showed that only the natural oxidized form of CP12 and the form that originates from the reduced CP12 upon Cu^{2+} binding are able to bind Cu^{2+}, and that cysteine residues are not involved in the metal-ion binding. However, disulfide bridges are essential for CP12 re-folding prior to metal-ion binding. Consequently, copper binding may involve carboxylic groups of glutamic or aspartic residues or the imidazole of histidine residues.[21] Since sequence analysis showed that histidine 47 was strongly conserved among different CP12 sequences from many organisms, a CP12 H47L mutant was tested for its capacity to bind Cu^{2+}. No effect was observed, thus rejecting the involvement of this residue in metal-ion binding.

The finding that CP12 is able to bind a metal ion is another new characteristic of this protein that may prove to be important in the redox regulation of the assembly process of the PRK/GAPDH/CP12 complex. This result is similar to that of Hsp33[41,42] and Toc 64,[43] two proteins that are regulated by reversible disulfide bond formation induced by Cu^{2+}. Plants are faced with the challenge to maintain metal homeostasis, and copper chaperones like those in mammals or yeast have been described. Recent work on the *Arabidopsis* genome suggests that these chaperones have acquired

additional functions and played different roles in copper homeostasis.[44] All these points led us to perform hydrophobic cluster analysis (HCA) to compare CP12 and copper chaperones. Indeed, the sensitivity of HCA has often been successfully used to identify new domains[45,46] and/or to link orphan sequences to particular structural and functional families.[47–49] In the case of CP12, the HCA result shown in Fig. 6 demonstrated a high structural similarity with copper chaperones from *A. thaliana*.[43,50,51] Knowing that these copper chaperones have been shown to be involved in copper homeostasis and to facilitate copper transfer to specific partners, we can hypothesize that CP12 is a metallochaperone that can play a role in the PRK/GAPDH activity regulation during the light and dark activation of the Calvin-Benson cycle.

CONCLUSIONS AND FUTURE DIRECTIONS

The experiments reported here show that oxidized CP12 specifically binds Cu^{2+} and Ni^{2+} with K_d values calculated from the MS data of $26 \pm 1\,\mu M$ and $11 \pm 1\,\mu M$, respectively. Similar results were obtained with the reduced CP12. Interestingly, our results showed that Cu^{2+} catalyzes the re-formation of the disulfide bonds of the reduced CP12, leading to the recovery of the fully oxidized CP12 that is then able to bind a Cu^{2+} ion. Finally, the high similarity between CP12 and copper chaperones from *Arabidopsis thaliana*, as judged by HCA, is additional evidence for the relevance of metal binding *in vivo*.

To go further in our understanding of CP12 copper regulation, the confirmation of the binding constant values for copper and nickel could be assessed by alternative solution-based techniques such as differential scanning calorimetry. In addition, the design of limited proteolysis experiments or electron capture dissociation experiments, associated with site-directed mutageneses, would also be very helpful to identify the site of metal-ion binding.

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