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Incorporation of Sulfide Ions into the Cadmium(II) Thiolate Cluster of *Cicer arietinum* Metallothionein2

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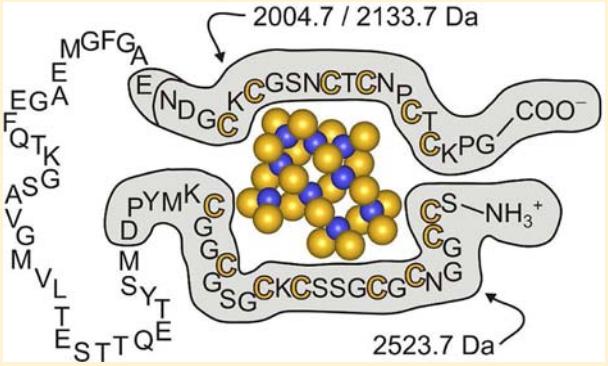
Supporting Information

ABSTRACT: The plant metallothionein2 from *Cicer arietinum* (chickpea), cic-MT2, is known to coordinate five divalent metal ions such as Zn^{II} or Cd^{II}, which are arranged in a single metal thiolate cluster. When the Zn^{II} form of the protein is titrated with Cd^{II} ions in the presence of sulfide ions, an increased Cd^{II} binding capacity and concomitant incorporation of sulfide ions into the cluster are observed. The exact stoichiometry of this novel cluster, its spectroscopic properties, and the significantly increased pH stability are analyzed with different techniques, including UV and circular dichroism spectroscopy and colorimetric assays. Limited proteolytic digestion provides information about the spacial arrangement of the cluster within the protein. Increasing the Cd^{II} scavenging properties of a metallothionein by additionally recruiting sulfide ions might be an economic and very efficient detoxification strategy for plants.



INTRODUCTION

Exposure to heavy-metal ions such as Cd^{2+} induces the enzymatic synthesis of a class of small peptides known as phytochelatins (PCs), oligomers of glutathione with typically 2−6 repeating units and the general formula $(\gamma\text{-EC})_nG$. PCs have been identified particularly in higher plants, marine and freshwater algae, fission yeast, and filamentous fungi.[1] More recently, it was found that the genome of the nematode *Caenorhabditis elegans* encodes a protein with PC synthase activity, conferring an increased Cd^{II} tolerance to the animal as well as to yeast cells in complementation assays.[2,3]

The cadmium γ-glutamyl peptide complexes of fission yeast, *Schizosaccharomyces pombe*, were the first in which an additional incorporation of sulfide ions was observed.[4] Two different complexes can be isolated from *S. pombe*, a high-molecular-weight complex containing sulfide ions, $Cd_{5.4}S[(\gamma\text{-EC})_3G]_4$, next to a smaller amount of a sulfide-free low-molecular-mass complex, $Cd_{1.8}[(\gamma\text{-EC})_3G]_2$.[4,5] The high-molecular-mass complex can be transformed into the low-molecular-mass complex by acidification and reneutralization, which removes the labile sulfide ions in the form of hydrogen sulfide. In vitro treatment of the low-molecular-mass complex with Na_2S and Cd^{II} results in a complex with even higher Cd^{II} and sulfide binding abilities and a $Cd^{II}/S^{2-}/(\gamma\text{-EC})_3G$ ratio of 1.8:1:1.[4] The incorporation of sulfide ions into cadmium(II) thiolate clusters causes a red shift of the cluster-specific ligand-to-metal charge-transfer (LMCT) bands observed in the UV spectra from 250 nm in the sulfide-free complexes to ∼265 nm or even ∼310 nm for the sulfide-containing complexes found in vivo and prepared in vitro as described above, respectively. It was also shown that increasing $CdCl_2$ concentrations in the growth media (up to 2 mM) lead to a linear increase of the cellular sulfide production and a shift in the ratio of the two in vivo produced PC complexes toward the high-molecular-mass complex.[6] Hence, the incorporation of sulfide ions can be directly linked to an increased Cd^{II} binding capacity of PCs in yeast, probably enhancing the effectiveness of the detoxification system. In addition, sulfide ions were shown to increase the thermodynamic as well as pH stability of the cadmium γ-glutamyl peptide complexes.[7] A more detailed analysis of the in vitro complex with a Cd^{II}/S^{2-} ratio of 1.8:1 revealed the formation of nearly monodisperse particles with a diameter of ∼18 Å.[8] They contain a crystallite CdS core and show an X-ray diffraction pattern closely similar to the six-coordinate rock-salt structure. This is remarkable considering that naturally occurring CdS adopts a four-coordinate zinc-blende structure and only converts to the rock-salt structure at high pressure.

Similar to *S. pombe*, plants also produce low- and high-molecular-weight Cd-PC complexes depending on the respective growth conditions. Sulfide ions were analogously identified mainly in the higher-molecular-weight complexes, i.e., in *Lycopersicon esculentum* (tomato), *Silene cucubalus* (bladder campion), *Brassica juncea* (indian mustard), and *Rauvolfia serpentina* (snakeroot).[9−13] In the latter, even three different kinds of complexes varying in the cysteine (Cys) thiolate/S^{2-}/Cd ratios were identified: a low-molecular-weight complex with a ratio of 3:0:1,

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a medium-molecular-weight complex with 2.5:0:1, and a higher-molecular-weight complex with a ratio of 1.5:0.5:1. Hence, with increasing molecular weight and, most importantly, upon incorporation of sulfide ions, the total S/Cd^{II} ratio is lowered. This led to the hypothesis that recruiting sulfide ions as additional ligands makes Cd^{II} detoxification far more economic for the organism than deploying the amino acid Cys as the sole ligand.[13] Additionally, because sulfide ions are formed prior to Cys in the biosynthetic assimilatory sulfate reduction pathway, substituting at least part of the Cys ligands by sulfide ions decreases the energy consumption for the synthesis of metal chelators even further.

More recently, sulfide ions were also detected in metallothioneins (MTs) from different species, when the proteins were expressed recombinantly in *Escherichia coli*.[14] In contrast to PCs, MTs are a superfamily of ubiquitous genetically encoded Cys-rich proteins with a molar mass of usually less than 10 kDa. The range of proposed functions includes among others the homeostasis of essential metal ions such as Zn^{2+} and Cu^{+}, detoxification of heavy-metal ions such as, for example, Cd^{2+}, Hg^{2+}, and Pb^{2+}, and scavenging of reactive oxygen species under stress conditions.[15,16] Metal ions are coordinated in the form of metal thiolate clusters; more recently, also histidine (His) coordination of divalent metal ions was observed in certain MT forms.[17,18] Plant MTs are characterized by a high sequence diversity and accordingly are further divided into four subfamilies, designated as p1, p2, p3, and pec or p4.[16,19,20] Usually, representatives of all four subfamilies can be detected in a single plant, showing differential expression depending on, among others, the tissue localization, growth stage, and growth conditions. Peculiar is the occurrence of long Cys-free amino acid stretches or linker between Cys-rich regions.[21]

As observed so far and in contrast to the PC complexes, the effect of sulfide ions on the metal-ion binding capacities of MTs is distinctively smaller. For example, mammalian MTs are able to coordinate seven Zn^{2+} or Cd^{2+} ions. In the presence of sulfide ions, this binding capacity is retained; i.e., a range of 6.4−7.3 bound divalent metal ions was observed, while between 1.5 and 3.5 equiv of sulfide ions are associated with the proteins.[22] When analyzed separately, however, the two domains of mouse MT4 can bind slightly more metal ions, i.e., coordination of 3.9 Cd^{2+} ions and 5 sulfide ions in the β-domain and 4.6 Cd^{2+} ions and 1.7 sulfide ions in the α-domain compared to 3 and 4 divalent metal ions in the absence of sulfide ions, respectively.[22] The results for the yeast MT CRS5 paint a similar picture. Conveying the divalent metal ion-to-Cys stoichiometry from the metal thiolate clusters of mammalian MTs to the 19 Cys residues of CRS5, a range of 6.3−6.9 metal ions is calculated; hence, this protein should in theory be able to coordinate 6−7 Zn^{2+} or Cd^{2+} ions. Recombinant expression in non-Zn^{II}-supplemented media yields lower amounts, i.e., observation of a Zn_4CRS5 species,[23] while Zn^{2+} and Cd^{2+} supplementation produces species with the stoichiometric composition $Zn_{5.7}S_{0.4}$ and $Cd_{7.4}S_{1.8}$, respectively.[24] The signals in the electrospray ionization mass spectrometry (ESI-MS) spectra with the highest intensity can be assigned to Zn_6- and Zn_7CRS5 for the Zn^{II} form and Cd_7CRS5 for the Cd^{II} form, respectively, while additionally lower-intensity signals of Zn_6S_2CRS5 or Cd_7S_2- and Cd_8S_2CRS5 can be observed. More heterogeneous are the results obtained for the recombinant expression of the plant MT2 from *Quercus suber* (cork oak). This MT subfamily features 14 Cys residues and should be able to coordinate around 5 divalent metal ions according to the range of 4.7−5.1 metal ions calculated as for CRS5 above. However, significantly lower amounts of metal ions were detected in the Zn^{II} forms, while among others, a Cd_5MT2 species was observed in the ESI-MS spectra (Table 1).[25−28]

Table 1. Stoichiometric Composition of Zn^{II} and Cd^{II} Species Obtained by Recombinant Protein Expression of Cork Oak MT2 in *E. coli* as Well as Different Species Observed in ESI-MS Spectra of the Proteins

	ESI-MS	
stoichiometric composition[a]	species with higher-intensity signals	species with lower-intensity signals
$Zn_{3.5}$[28]	Zn_4	Zn_3, Zn_5
$Zn_{4.2}$[25]	Zn_4	
$Zn_{3.5}S_1$[26,27]	Zn_4	Zn_3, Zn_4S_2
$Cd_{6.3}$[28]	Cd_6	Cd_7
$Cd_{5.6}S_3$[27]	Cd_6S_4	Cd_5
$Cd_{5.3}S_{2.2}$[26]	Cd_5	Cd_6S_4
$Cd_{6.3}S_{2.4}$[26]	Cd_6S_4	Cd_7S_4
$Cd_{6.7}S_{2.9}$[26]	Cd_6S_4	Cd_7S_4

[a]Metal-ion concentrations were determined with inductively coupled plasma atomic emission spectroscopy (ICP-AES) as well as the total sulfur content, i.e., Cys and Met residues, of the acidified samples, which was used to calculate the protein concentrations. Sulfide ions were quantified as H_2S using gas chromatography coupled to a flame photometric detector.

In the presence of sulfide ions, binding of 2−3 sulfide ions enables the additional coordination of 1−2 Cd^{II} ions by the protein and, hence, increased the Cd^{II} binding capacity of this plant MT2 form by up to 40%. It should be noted that all sulfide-containing MT forms described above were overexpressed in the form of glutathione *S*-transferase (GST) tagged proteins in *E. coli*.

On basis of the property of cork oak MT2 to coordinate higher amounts of Cd^{II} in the presence of sulfide ions, we investigated this property further with the aim of characterizing the formed cadmium(II) sulfide thiolate complex in more detail. We chose the MT2 protein from *Cicer arietinum* (chickpea), cicMT2, which was previously characterized in the absence of sulfide ions.[29] cicMT2 is able to coordinate five Zn^{II} or Cd^{II} ions in a cluster arrangement connecting the N- and C-terminal Cys-rich regions with each other.

EXPERIMENTAL SECTION

Chemicals and Enzymes. LB broth (Miller), 1,4-dithio-DL-threitol, and isopropyl-β-D-thiogalactopyranosid were purchased from Chemie Brunschwig AG (Basel, Switzerland), tris(hydroxymethyl)-aminomethyl hydrochloride (Tris-HCl) from Calbiochem (VWR International AG, Lucerne, Switzerland) and Chelex 100 resin from Bio-Rad (Reinach, Switzerland). All other chemicals were ACS grade from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). *Tritirachium album* proteinase K was obtained from Qbiogene (Lucerna Chem AG, Lucerne, Switzerland). The deionized water used for the preparation of all solutions was vacuum-degassed for approximately 30 min and nitrogen-saturated for at least 1 h. Where strictly anaerobic conditions were required, solutions were rendered oxygen-free by three freeze−thaw cycles under vacuum.

Overexpression and Purification of Zn_5-cicMT2. Construction of the plasmid containing the *cicmt2* gene was described previously along with the growth condition for the overexpression of Zn_5-cicMT2 in *E. coli* Bl21 (DE3) cells, the purification, the determination of protein concentrations via thiol group quantification, and analysis of the metal-ion content with flame atomic absorption spectroscopy (F-AAS) using an AA240FS spectrometer (Varian AG, Zug, Switzerland).[29]

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Cd^{II} Titration of Zn_5-cicMT2 in the Presence of S^{2-} Ions. To a 10 μM solution of Zn_5-cicMT2 containing 1 mM Tris-HCl (pH 7.5) was added 10 equiv of S^{2-} in the form of a 40.7 mM Na_2S solution, which was standardized by iodine titration using $NaIO_3$.[30] After 10 min of incubation at room temperature, up to 15 equiv of $CdCl_2$ were added. After the addition of each equiv, the solution was incubated for 30 min at room temperature before a UV spectrum was recorded.

Preparation of the Cd/S-cicMT2 Complex. The Cd/S-cicMT2 complex was prepared as described above starting from 2 mL of a 30 μM Zn_5-cicMT2 solution. Subsequently, the sample was purified with size-exclusion chromatography (SEC) as described previously for Zn_5-cicMT2.[29] The protein purity and molecular weight of Cd/S-cicMT2 were examined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Tris–tricine buffer system.[31] Modification of the samples to break up the cluster structure was carried out with 3-(bromomethyl)-2,5,6-trimethyl-1*H*,7*H*-pyrazolo(1,2-α)-pyrazole-1,7-dione (monobromobimane, mBBr) without the addition of ethylenediaminetetraacetic acid or a reducing agent.[32] The stoichiometry of Cd/S-cicMT2 was determined as described below.

UV–Vis and Circular Dichroism (CD) Spectroscopic Measurements. UV–vis absorption spectra were recorded on a Cary 500 scan spectrophotometer (Agilent Technologies AG, Basel, Switzerland) over the range of 200–400 nm at room temperature at a scanning speed of 600 nm min^{-1} and expressed as molar absorptivity (M^{-1} cm^{-1}). CD spectra were measured on a J-810 spectropolarimeter (Jasco Inc., Japan) over the range of 200–400 nm at room temperature at a scanning speed of 10 nm min^{-1} with four spectral accumulations and expressed as molar ellipticity (deg cm^2 $dmol^{-1}$).

Quantification of S^{2-} Ions in the Cd/S-cicMT2 Complex. (a) *2,2′-Dithiopyridine (2-PDS) assay*: A total of 500 μL of a SEC-purified Cd/S-cicMT2 sample was acidified with HCl to pH 1 in order to release the coordinated S^{2-} ions in the form of H_2S. The acidified solution was then centrifuged under vacuum at room temperature for 20 min using the Eppendorf Concentrator 5301 to remove released H_2S. To estimate the volume decrease due to the additional evaporation of water, the sample weight as well as the Cd^{II} concentration (F-AAS) was determined before and after the centrifugation step and used to correct the values obtained below. Thiol groups and S^{2-} ions were quantified with 2-PDS.[29,33] The 2-PDS assay was performed before and after acidification and centrifugation under vacuum to determine the combined content of Cys thiol groups and sulfide ions or solely the amount of Cys thiol groups, respectively. The difference of both measurements was used to calculate the amount of S^{2-} ions per protein in the Cd/S complex. Amino acid analysis (AAA) was used to verify the protein concentration in the Cd/S complex before S^{2-} ion release. (b) *Methylene blue (MTB) assay*: S^{2-} ions were quantified as described with small modifications.[34] All reagents required for the assay as well as the samples were prepared inside a N_2-purged glovebox. A total of 0.1 mL of a 20 mM *N,N*-dimethyl-*p*-phenylenediamine sulfate solution in 7.2 M HCl was mixed with 1 mL of the Cd/S-cicMT2 sample in a 1.5-mL Eppendorf tube, rapidly followed by the addition of 0.1 mL of a 30 mM $FeCl_3$ solution in 1.2 M HCl. The tube was closed tightly immediately and vortexed vigorously in the dark for 20 min. Subsequently, absorption at 650 nm originating from produced MTB was measured with UV–vis spectroscopy. A calibration curve was determined with a series of S^{2-} standards with concentrations of 0, 2, 5, 10, 20, 50, and 80 μM prepared from the standardized stock solution of 40.7 mM Na_2S. The presence of Cd^{II} ions did not influence the assay at the concentrations used. (c) *Inductively coupled plasma mass spectrometry (ICP-MS)*: ICP-MS was performed with a Varian 820-MS instrument to determine the total sulfur and cadmium concentrations in both, a purified sample of Cd/S-cicMT2, and a sample after acidification and degassing. Also, Cd_5-cicMT2 was analyzed for comparison.

Spectrophotometric pH Titration of Cd/S-cicMT2. A total of 800 μL of a 10 μM sample in 1 mM Tris-HCl (pH 7.5) was titrated with N_2-saturated HCl in volume increments of 0.5–1 μL. After each acid addition, a UV absorption spectrum as well as the pH value of the solution was recorded. The entire titration was carried out under a flow of argon. *Origin 7.0* (Origin Lab Corp., Northhampton, MA) was applied for curve fitting of the experimental data with pK_a equations as described.[35]

Limited Proteolytic Digestion of Cd/S-cicMT2 with *T. album* Proteinase K. Limited proteolytic digestion was performed as described previously.[29,36] The major fraction obtained during SEC purification of the digestion mixture was further analyzed by AAA and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry as reported.[29]

RESULTS AND DISCUSSION

Cd^{II} Titration of Zn_5-cicMT2 in the Presence of S^{2-} Ions. Zn_5-cicMT2 was overexpressed in the form of a fusion protein with a self-cleavable Intein-tag in *E. coli*. In contrast to the results reported with the GST–fusion system, no sulfide ions were detected in the purified protein.[14,26,27] Nevertheless, a broadening or shift of the LMCT bands of the Cd^{II} form at 250 nm toward higher wavelength, i.e., 275 nm, was observed after accidental dialysis of the protein in an untreated dialysis membrane containing residual sulfide ions. The apparent incorporation of sulfide ions into the cadmium(II) thiolate cluster could be reproduced under defined conditions when Zn_5-cicMT2 was titrated with increasing amounts of Cd^{II} ions in the presence of 10 equiv of sodium sulfide. An absorption increase of the LMCT bands around 275 nm is observed up to the addition of approximately 9 equiv of Cd^{II} ions (Figure 1a). In comparison, in the absence of sulfide ions, only 5 equiv of Cd^{II} are incorporated.[29] It has to be noted that the shoulder at 275 nm is not observed, when either Zn_5-cicMT2 are mixed with 10 equiv of Na_2S (dash-dotted line in Figure 1a) or 10 equiv of Na_2S is titrated with up to 15 equiv of Cd^{II}. Interestingly, the apparent incorporation of Cd^{II} ions into cicMT2 takes place, although the ionic product $[Cd^{2+}][S^{2-}]$ of the titration solution already by far exceeds the value of the solubility product of CdS, i.e., 1.40×10^{-29} M^2 at 25 °C,[37] after the addition of the first 1 equiv of Cd^{II}, i.e., 10^{-9} M^2. To evaluate, if indeed bound sulfide ions are responsible for the increase and shift of the LMCT bands relative to experiments in the absence of sulfide, a sample of Cd/S-cicMT2 was acidified to pH 2 with HCl, placed in the vacuum concentrator to remove released H_2S, and reneutralized to pH 7.5. A UV spectrum was taken, which resembles closely the spectrum of Cd_5-cicMT2, indicating that the shoulder at 275 nm was indeed caused by sulfide, and additional Cd^{II}, ions (Figure 1b).

Bathochromic shifts of the LMCT bands are characteristic for the formation of cadmium thiolate clusters in MTs and have been associated with the transition of initially terminal thiolate ligands to bridging ones, i.e., coordination of one thiolate group to two Cd^{II} ions, in the cluster structure.[38] Analogous band shifts to higher wavelengths were also observed for thiol-capped CdS particles when the sizes of the clusters were increased from approximately 1.8 to 2.5 nm, which is also roughly the size range of the MT structures known so far.[39] Broadening of the absorption envelope, observed, e.g., in sulfide ions containing the cluster structure $[Cd_{10}S_4(SPh)_{16}]^{4-}$, to approximately 310 nm was attributed to LMCT transitions involving the μ_3-S^{2-} ions.[40,41] Ab initio calculations have shown that the differences between the highest occupied and the lowest unoccupied molecular orbitals, i.e., the HOMO-LUMO gaps, in cadmium thiolate clusters decrease with increasing cluster size but that also the transition to structures with highly coordinated sulfur atoms, e.g., μ_3- or even μ_4-S^{2-} ions, has a major influence.[42,43]

To further characterize the Cd/S-cicMT2 form, a CD spectrum was taken and compared to the sulfide-free Cd^{II} form (Figure 1c). The CD spectrum of Cd/S-cicMT2 features intense dichroic bands at (+) 244 nm ($[\theta] = 2.5 \times 10^4$ deg cm^2 $dmol^{-1}$),

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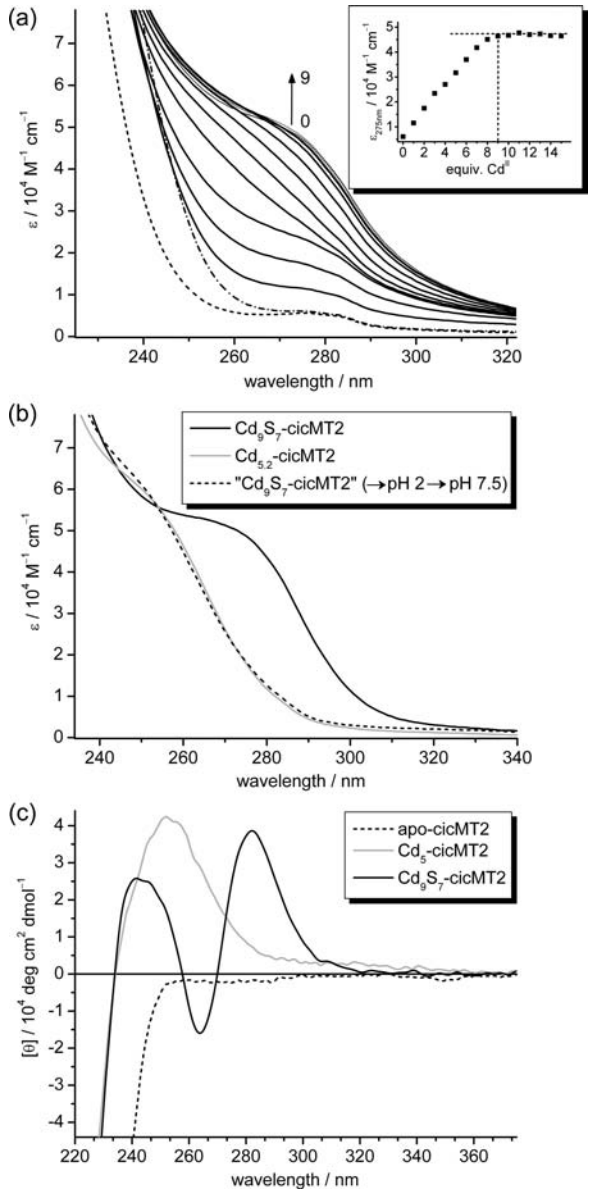

Figure 1. (a) UV spectra of the titration of Zn_5-cicMT2 (dashed line) with increasing equivalents of Cd^{II} in the presence of 10 equiv of S^{2-}. The dash-dotted spectrum is obtained after the addition of 10 equiv of S^{2-} in the form of Na_2S to Zn_5-cicMT2. The inset shows the plot of the molar absorptivity at 275 nm against the number of Cd^{II} equivalents added. (b) UV spectra of $Cd_{5.2}$-cicMT2 (dashed line), Cd_9S_7-cicMT2 (black), and the Cd_9S_7-cicMT2 species after acidification, vacuum centrifugation, and reneutralization to pH 7.5 (gray). (c) CD spectra of apo-cicMT2 (dashed line), $Cd_{5.2}$-cicMT2 (gray), and Cd_9S_7-cicMT2 (black).

(−) 264 nm (1.6×10^4 deg cm^2 dmol^{-1}), and (+) 282 nm (3.9×10^4 deg cm^2 dmol^{-1}) with two inflection points at 255 and 272 nm, respectively. The CD profile is thus significantly different from that of Cd_5-cicMT2, which is characterized by a single intense dichroic band at (+) 253 nm ($[\theta] = 4.2 \times 10^4$ deg cm^2 dmol^{-1}), corroborating an alteration of the cluster structure in Cd/S-cicMT2 relative to Cd_5-cicMT2. Transitions in the range of 250−300 nm have also been observed in the CD spectra of the recombinantly expressed mammalian MT1 metallothionein upon sulfide incorporation.[22]

For a more detailed characterization, a larger amount of Zn_5-cicMT2 was titrated with Cd^{II} ions in the presence of 10 equiv of sulfide ions, and the resulting mixture was subjected to SEC. The elution profile (Figure 2b) shows clearly that a certain

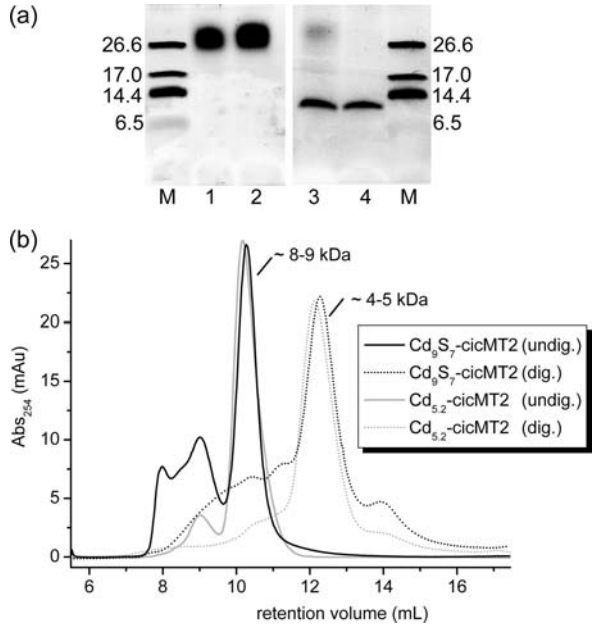

Figure 2. (a) Silver-stained SDS-PAGE (12%) of unmodified and mBBr-modified $Cd_{5.2}$-cicMT2 (lanes 1 and 4) and Cd_9S_7-cicMT2 (lanes 2 and 3) relative to a peptide marker (lanes marked with M). (b) Size-exclusion chromatogram of undigested and proteinase K digested Cd_9S_7-cicMT2 (black solid and dotted lines) and $Cd_{5.2}$-cicMT2 (gray solid and dotted lines).

amount of aggregation and dimerization of the protein took place (7.5−9.7 mL), but also a monomeric species is obtained (~10.3 mL). The elution times of Cd_5-cicMT2 and Cd/S-cicMT2 are very similar, suggesting that the two species have similar hydrodynamic radii. To analyze the mass and size of the two protein forms in more detail, after purification with SEC, both were subjected to SDS-PAGE (Figure 2a). The silver-stained gel shows a single band each for Cd_5- and Cd/S-cicMT2 with an apparent molar mass slightly above 27 kDa (lanes 1 and 2). The difference between the apparent molar masses and expected masses, i.e., 8.5 kDa for Cd_5-cicMT2, can be explained by only partial denaturation of the protein structure with SDS, while the metal thiolate clusters are left untouched. This results in lower charge-to-protein mass ratios with respect to the proteins used in the molar mass marker (lanes M).[29,35] However, the metal clusters can be disrupted by thiol group modification with mBBr.[29,32,35] The completely denatured proteins migrate in SDS-PAGE at an apparent molecular weight of ~10 kDa (lanes 3 and 4), which is in accordance with the theoretically expected molecular mass of apo-cicMT2 after modification of all 14 cysteine residues (7′969.9 Da + 14*190.2 Da = 10′632.7 Da).[29] Hence, the results from SDS-PAGE are in agreement with the results from the chromatogram that both species have the same hydrodynamic radius. In addition, the equal apparent molar masses of the partially SDS denatured proteins (lanes 1 and 2) indicate that the preserved clusters keep the partially denaturated polypeptide chains in similar structural arrangements. That is, it is unlikely that, for example, the metal ions in Cd_5-cicMT2 are arranged only in a single cluster, while Cd/S-cicMT2 contains two.

Stoichiometry of the Cd/S-cicMT2 Complex. To quantify the number of sulfide ions incorporated into Cd/S-cicMT2,

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Table 2. Original Concentration Data for Cd_5-cicMT2 and Cd/S-cicMT2 Measured with Various Methods[a]

	sample	$[Cd^{2+}]$ (F-AAS)	$[Zn^{2+}]$ (F-AAS)	$[-S^-]$ (+ $[S^{2-}]$) (2-PDS)	[cicMT2] (AAA)	$[S^{2-}]$ (MTB)	[total S] (ICP-MS)	$[Cd^{2+}]$ (ICP-MS)
Cd_5-cicMT2	**1**	304(3)	1(1)	822(7)	60.6			
	2						142(1)	44(1)
Cd/S-cicMT2	**1**	164(1)	1(1)	392(1)	18.3			
	1[b]	164(1)	1(1)	267(1)				
	2	79(1)	1(1)	185(1)		62(1)		
	3						111(1)	39(1)
	3[b]						[33(1)]	[34(1)]

[a]All concentrations are given in micromolars. [b]Acidified and vacuum-degassed sample. Errors are given for the 3σ level.

Table 3. Summary of Calculated and Measured Data for Analysis of the Cd^{2+} and S^{2-} Contents in Cd_5-cicMT2 and Cd/S-cicMT2[a]

	sample	$[Cd^{2+}]$	[cicMT2]	$[Cd^{2+}]$/[cicMT2]	$[S^{2-}]$	$[S^{2-}]$/[cicMT2]	[total S]/$[Cd^{2+}]$
Cd_5-cicMT2	**1**	304(3)	58.7[b], 60.6[c]	5.2, 5.0			3.5[h], 3.6[h]
	2	44(1)	7.9[d]	5.6			3.2[h]
Cd/S-cicMT2	**1**	164(1)	18.3[c], 19.1[e]	9.0, 8.6	136[g], 125[g]	7.4, 6.5	2.8[I], 2.9[I]
	2	79(1)	8.8[f]	9.0	62(1)	7.0	2.8[I]
	3	39(1)					2.8[j]

[a]All concentrations are given in micromolars. [b]Calculated from $[-S^-]$ (+$[S^{2-}]$) (Table 2, column 5) considering 14 Cys residues per protein. [c]Value from AAA (Table 2, column 6). [d]Calculated from [total S] (Table 2, column 8) considering 14 Cys and 4 Met residues per protein. [e]Calculated from $[-S^-]$ (+$[S^{2-}]$) (Table 2, column 5) of the acidified and vacuum-degassed sample considering 14 Cys residues per protein. [f]Calculated from the difference between ($[-S^-]$ + $[S^{2-}]$) (Table 2, column 5) and $[S^{2-}]$ (Table 2, column 7) considering 14 Cys residues per protein. [g]Calculated from the difference between ($[-S^-]$ + $[S^{2-}]$) (Table 2, column 5) and ($[cicMT2]^{b,d}$·14) (Table 3, column 4). [h][total S] calculated from [cicMT2] (Table 3, column 4) considering 14 Cys and 4 Met residues per protein. [I][total S] calculated from the sum of [cicMT2] (Table 3, column 4) considering 14 Cys and 4 Met residues per protein and $[S^{2-}]$ (Table 3, column 6). [j]Data taken from Table 2, columns 8 and 9.

the S^{2-} concentration was determined using a combination of three different methods (Table 2). The 2-PDS assay is commonly used in MT research to determine protein concentrations via the quantification of thiolate groups. Because 2-PDS undergoes the same reaction with sulfide ions, the assay can also be applied to determine the combined content of the Cys thiolate groups and sulfide ions in a protein sample. Cd^{II} ions do not interfere with the assay under the conditions and at the concentrations used in the experiments. A comparison of the combined content of the Cys thiolate groups and S^{2-} ions with the protein concentration obtained from AAA yields a sulfide content of approximately 7.4 ions per protein molecule (Tables 2 and 3). To assess the protein concentration directly with the 2-PDS assay, the sulfide ions in Cd/S-cicMT2 were protonated with HCl and, subsequently, the formed H_2S was removed by centrifugation under vacuum. A comparison of the 2-PDS assay results with the values obtained from the same sample before acidification yields a S^{2-} content of approximately 6.5 ions per protein molecule. As a third method, the S^{2-} content was determined directly by spectrophotometric measurement of the amount of MTB produced upon reaction of the sulfide ions in a strongly acidic solution with *N*,*N*-dimethyl-*p*-phenylenediamine in the presence of ferric chloride. In a similar experiment, this method has been also used to determine the sulfide content in the Cd-γ-Glu peptides of *S. pombe*.[44] The colorimetric MTB assay yields a S^{2-} content of approximately 7.0 ions per cicMT2 molecule.

The content of Cd^{II} ions was determined with F-AAS and varies between 8.6 and 9.0 per Cd/S-cicMT2 molecule depending on the method used for determination of the protein concentration (Table 3). The Zn^{II} content was lower than 0.1 equiv in all samples (Table 2). Coordination of nine Cd^{II} ions per cicMT2 molecule is in agreement with the results from the titration of Zn_5-cicMT2 with Cd^{II} ions in the presence of S^{2-} described above (Figure 1a).

Methods that allow the parallel determination of the total sulfur and cadmium concentrations in a sample are ICP-MS and ICP-AES. For the Cd_5-cicMT2 sample, the ICP-MS measurement gave a sulfur-to-Cd^{II} ratio of 3.2 (Table 3). This allows one to directly calculate a Cd^{II} content of 5.6 ions per protein molecule because only Cys and Met residues are contributing to the total sulfur content. Hence, the Cd^{II} content is higher than values determined with combinations of the 2-PDS assay, AAA, and F-AAS, although only slightly. For the Cd/S-cicMT2 complex, a ratio of 2.8 was determined in agreement with the values obtained with the other methods (Table 3); however, the initially unknown number of S^{2-} ions renders the calculation of the complex stoichiometry impossible without additional methods. A large number of stoichiometries, e.g., $Cd_7S_{1.6}$-, $Cd_8S_{4.4}$-, $Cd_9S_{7.2}$-, and $Cd_{10}S_{10}$-cicMT2, or species with even higher Cd^{II} contents are equally consistent with the experimental value. In an effort to determine the sulfide content, also ICP-MS with the same Cd/S-cicMT2 sample after acidification and vacuum degassing was performed, but the resulting total S content is far too low and would indicate an initial sulfide content far above 30 ions per cicMT2 molecule (Table 2, values given in square brackets). However, the underestimation of sulfur in acidified protein samples with ICP-MS is a known problem reported previously in the literature.[26]

Taking together all results with the different analytical methods (Tables 2 and 3), the composition of the Cd/S-cicMT2 species can best be given as $Cd_{8.9(2)}S_{7.0(5)}$-cicMT2 or approximately Cd_9S_7-cicMT2.

Effect of the Sulfide Ions on the Cluster Stability. To compare the pH stability of the metal-ion binding of Cd_9S_7-cicMT2 with the one of Cd_5-cicMT2, a pH titration was performed and the metal ion release followed with UV spectroscopy

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by means of a decrease of the LMCT band at 275 nm (Figure 3). Down to a pH value of ~5.5, the absorption at 275 nm shows

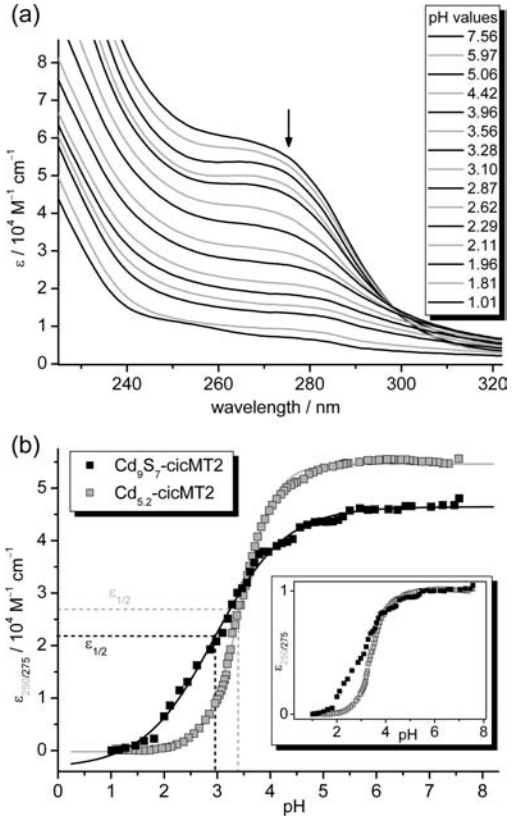

Figure 3. (a) UV spectra of titration of Cd_9S_7-cicMT2 with increasing amounts of HCl. The arrow indicates a decrease in the absorptivity at 275 nm. (b) Plots of molar absorptivity at 275 or 250 nm against the respective pH values for the pH titration of Cd_9S_7-cicMT2 (black squares) and $Cd_{5.2}$-cicMT2 (gray squares),[29] respectively. Data points are fitted with eq 1 (Table 4) to determine the apparent pK_a values of the sulfur ligands (solid lines). Dotted lines indicate the molar absorptivity value at half-maximum absorptivity. The inset shows the plots normalized onto each other to allow for easier comparison.

only little changes but decreases rapidly below 5.5. At around pH 1.0, the point of complete Cd^{II} ion release from the protein is reached. The overall apparent pK_a value of the metal-binding ligands, i.e., the Cys thiolate groups together with the S^{2-} ions, was determined by curve fitting of the pH titration data as described (Table 4).[35]

With 2.95(3), this value is by nearly 0.5 units lower than the pK_a value obtained for the Cys residues in $Cd_{5.2}$-cicMT2, i.e., 3.439(6).[29] Hence, Cd_9S_7-cicMT2 features a higher overall stability against pH-dependent metal loss, obviously brought about by the bound sulfide ions. Similarly, the apparent pK_a values of the cadmium γ-glutamyl peptide complexes from *S. pombe* decreased from ~5.4 to ~3.9 upon the incorporation of sulfide ions.[7] Accordingly, the pH stabilities of both the sulfide-free and sulfide-containing cadmium(II) complexes of cicMT2 are significantly higher than those for the corresponding complexes of fission yeast. Taking a closer look at the plots of molar absorptivity against the respective pH value (Figure 3b), it is apparent that for both species the pH-dependent metal loss starts already below a pH value of 5.5. Differences become apparent below approximately pH 3.0−3.5, especially when the normalized plots were examined (Figure 3b, inset). While the

Table 4. Apparent pK_a Values[a] of Cys Residues (and Sulfide Ions) in Cd_9S_7- and Cd_5-cicMT2

	Cd_9S_7-cicMT2	Cd_5-cicMT2[29]
Equation 1		
A_{MT}	46299 ± 265	54607 ± 162
A_{MTHn}	−3213 ± 749	−225 ± 246
pK	2.95 ± 0.03	3.439 ± 0.006
n	0.66 ± 0.02	1.38 ± 0.03
Equation 2		
A_{MT}	46662 ± 1675	
A_{MTHo}	36817 ± 3294	
A_{MTHm+o}	15119 ± 2191	
$A_{MTHn+m+o}$	0 ± 1450	
pK_3	4.6 ± 0.5	
pK_2	3.27 ± 0.04	
pK_1	2.09 ± 0.05	
o	0.8 ± 0.3	
m	1.7 ± 0.3	
n	2.1 ± 0.4	

[a]Curve fitting of the pH titration data was performed with the following two equations:

$$A_{total} = \frac{A_{MT} + A_{MTH_n} 10^{n(pK-pH)}}{1+10^{n(pK-pH)}} \quad (1)$$

$$A_{total} = \frac{A_{MTH_{m+o}} + A_{MTH_{n+m+o}} 10^{n(pK_1-pH)}}{1+10^{n(pK_1-pH)}} + \frac{A_{MTH_o} + A_{MTH_{m+o}} 10^{m(pK_2-pH)}}{1+10^{m(pK_2-pH)}} \frac{A_{MT} + A_{MTH_o} 10^{o(pK_3-pH)}}{1+10^{o(pK_3-pH)}} \quad (2)$$

A_{MT} is the absorptivity of the fully metal-ion-loaded protein (=A_{max}), A_{MTHn} (eq 1) or $A_{MTHn+m+o}$ (eq 2) denote the value obtained for apo-MT after acidification (=A_{min}), and n, m, and o are measures for the slopes of the curves.

curve for the pH titration of $Cd_{5.2}$-cicMT2 shows a steep decrease of the absorptivity and a complete metal-ion release at pH ~2, the curve for Cd_9S_7-cicMT2 is more shallow and the lowest absorptivity value, i.e., complete loss of Cd^{II} ions, is reached at pH ~1. Accordingly, the metal-ion release range is broader for the species containing sulfide ions, and the corresponding decreased steepness of the plot is reflected by the lower value for n in eq 1 used for the curve fit (Table 4), i.e., 0.66(2) in Cd_9S_7-cicMT2 compared to 1.38(3) in $Cd_{5.2}$-cicMT2.[35] Having in mind that the free ligands contributing to the LMCT bands at 275 nm have three different pK_a values, i.e., hydrogen sulfide with 7.02 and 14.9 and the Cys thiol groups with 8.57, we also attempted a curve fit of the pH titration data with an equation considering three different pK_a values (eq 2, Table 4). Disregarding the highest pK_a value of 4.6(5), which is, on the one hand, relatively poorly defined and, on the other hand, also observed in experiments with MTs devoid of sulfide ions, we can deduce two well-defined pK_a values. The pK_a value of 3.27(4) is similar to the apparent pK_a value of the Cys residues in $Cd_{5.2}$-cicMT2 [3.439(6)] and hence can be assigned to the Cys thiolate groups in Cd_9S_7-cicMT2. The pK_a value of 2.09(5) should then arise from the incorporated sulfide ions. Assuming that the pK_a values of both ligands, Cys thiolate groups and sulfide ions, show a decrease in the presence of Cd^{II} ions of similar magnitude, i.e., by roughly five pK_a units, the value of 2.09(5) could indeed originate from the second pK_a value of hydrogen sulfide (7.02). This view is corroborated

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by the respective absorptivity decrease accompanying the titration steps. The absorption decrease caused by protonation of the 14 Cys thiolate groups amounts to ~31′500 M^{-1} cm^{-1} (=46′662 − 15′119), while the absorption decreases during the final protonation of the seven coordinated sulfide ions by ~15′100 M^{-1} cm^{-1} and thus roughly by 50% of the value observed for the Cys residues in agreement with the number of protonated ligands in both cases.

Cluster Arrangement in Cd_9S_7-cicMT2. The metal thiolate clusters of MTs have been shown to protect the peptide backbone to a certain extent against proteolytic cleavage.[29,36,45,46] In addition, the metal thiolate clusters usually stay intact at physiological pH even if backbone cleavage occurs between amino acid residues belonging to the same cluster. Digestion of the sulfide-free $Cd_{5.2}$-cicMT2 form with the subtilisin-related serine protease *T. album* proteinase K already showed that the N- and C-terminal Cys-rich regions of the protein are coeluting from the SEC column, corroborating the view that the Cd^{II} ions are coordinated in a single metal thiolate cluster arrangement.[29] In order to evaluate the number and size of the metal clusters formed in Cd_9S_7-cicMT2, an analogous digestion experiment was performed. The subsequent separation of the digestion mixture with gel filtration under nondenaturing conditions results in a broad elution profile with a major sharp peak at 12.5 mL (Figure 2b). The elution volume of this peak is significantly larger than that for the undigested protein (10.3 mL) but is nearly identical with the one observed for the digested $Cd_{5.2}$-cicMT2 species. The ratio between sulfur from Cys thiolate groups and S^{2-} and Cd^{II} ions was determined in the peak fraction with the 2-PDS assay and F-AAS, respectively, and yields a value of 2.3, which is the same as that for the undigested protein. Hence, it can be assumed that neither sulfide ions, Cys residues, nor Cd^{II} ions were lost from the cluster during the digestion procedure. The MALDI-TOF spectrum of the major peak fractions collected exhibits three major signals: one at 2004.7 Da matching the mass for residues 62−81 (calcd 2005.2 Da), one at 2133.7 Da corresponding to residues 61−81 (calcd 2133.8 Da), and the third one at 2523.7 Da corresponding to residues 2−28 (calcd 2523.8 Da; Figure 4). The assignment was further confirmed by AAA (Supporting Information). Quantification of the Cys residues was not possible. Hence, all available data strongly suggest the presence of a single metal cluster in Cd_9S_7-cicMT2 in analogy to $Cd_{5.2}$-cicMT2. A metal cluster with comparable stoichiometry is realized in the model compound $[S_4Cd_{10}(SPh)_{16}]^{4-}$ (Figure 4c).[47] Structure analysis of $[S_4Cd_{10}(SPh)_{16}]^{4-}$ shows that the 10 Cd^{II} ions and 20 sulfur ligands constitute four fused adamantanoid cages with six inner Cd^{II} ions capped by the four sulfide ligands and the other four Cd^{II} ions by 16 thiolate ligands. Considering the similarity between Cd_9S_7-cicMT2 and $[S_4Cd_{10}(SPh)_{16}]^{4-}$ with respect to the total amount of coordinated metal ions and sulfur ligands, i.e., 21 in Cd_9S_7-cicMT2 compared to 20 in $[S_4Cd_{10}(SPh)_{16}]^{4-}$, the formation of just a single metal cluster in Cd_9S_7-cicMT2 is theoretically feasible. In addition, the Cys-free linker region in cicMT2 connecting the N- and C-terminal Cys-rich regions is long enough to easily enable the accommodation of such a larger cluster without a major change in the overall protein size. It should be emphasized, however, that the detailed structures of both clusters in the model compound and in cicMT2 will significantly differ from each other especially considering the different amounts of sulfide ions and thiolate ligands in both compounds. A second aspect to consider is the relatively large negative charge of the proposed Cd_9S_7-cicMT2 structure of −12 assuming the presence

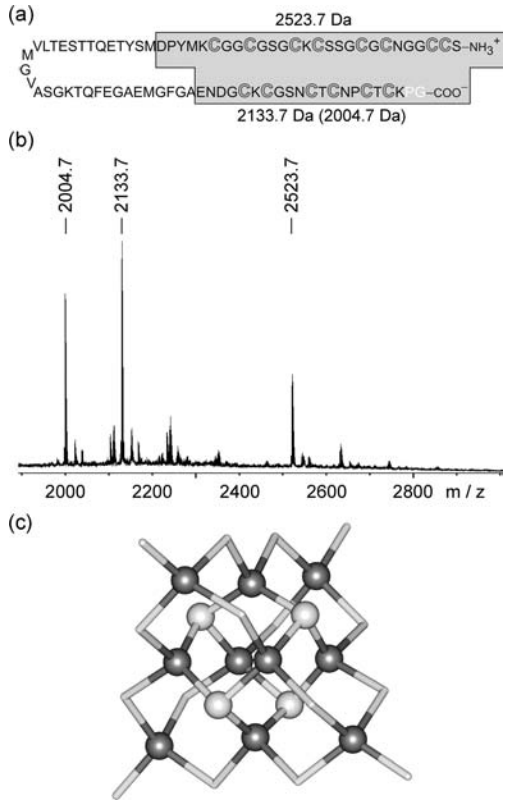

Figure 4. (a) Amino acid sequence of cicMT2 with the Cys residues highlighted. The vector-derived C-terminal ProGly residues are indicated with white letters. The two Cys-rich parts of the protein coordinating the Cd^{II} ions after proteolytic digestion with proteinase K are enclosed by a gray box, and the respective molecular weight of the fragments is given. 2004.7 kDa is the weight of the C-terminal fragment after additional cleavage or the N-terminal Glu. (b) MALDI-TOF analysis of the peak fraction at 12.5 mL elution volume (Figure 2b) of the proteinase K digested Cd_9S_7-cicMT2 sample. (c) Core structure of $[S_4Cd_{10}(SPh)_{16}]^{4-}$.[47] Cd^{II} ions are depicted as dark-gray spheres, S^{2-} ions as light-gray spheres, and the thiolate groups of the ligands as sticks. The phenyl rings are omitted for clarity.

of exclusively S^{2-} ions, which would require some sort of charge compensation. The overall charge would be significantly smaller supposing protonation of the sulfide ions to hydrogen sulfide, HS^-. This assumption would be in line with the observation during the pH titration of the complex (Figure 3 and Table 4), which shows that the first protonation step assigned to protonation of the Cys residues leads to a $^2/_3$ absorptivity decrease while the second protonation step might correspond to protonation of the sulfide ions or possibly HS^-. Accordingly, it is tempting to say that while protonation of the Cys residues requires 14 protons, protonation of the (hydrogen) sulfide ions requires only 50% of this value, i.e., seven protons in line with seven HS^- ions. However, the exact charge and, hence, the protonation states of the different ligands need to be established.

CONCLUSIONS

While PCs are known as efficient Cd^{II} chelators in plants,[48] the results presented here provide evidence that also the Cd^{II} binding capacity of plant MTs such as cicMT2 can be enhanced in the presence of sulfide ions. The ratio of sulfur ligands, i.e., Cys thiolates and sulfide ions, to coordinated Cd^{II} ions in the here-described Cd_9S_7-cicMT2 species is 2.3 and, hence, lower than ratios usually observed for MTs from various species, e.g.,

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2.9 for mammalian MTs or 2.8 for the sulfide-free Cd_5-cicMT2 form. For the sulfide-containing complexes of the fission yeast *S. pombe*, i.e., $Cd_{5.4}S[(\gamma\text{-EC})_3G]_4$, and the in vitro reconstituted complex, as well as for the sulfide-containing high-molecular-weight complex from the plant *R. serpentina*, ratios of 2.4, 2.2, and 1.9, respectively, are observed.[4,5,13] Hence, the here-proposed stoichiometry is well in line with the composition of other cadmium/sulfur complexes previously described. In addition to the observation that the incorporation of sulfide ions increases the Cd^{II} binding capacity of the plant MT cic-MT2, it also leads to significantly higher pH stability and, hence, the recruitment of sulfide ligands might indeed be a relatively inexpensive way for the cell to enhance the effectiveness of Cd^{II} detoxification.

Further experiments will have to show whether the Cd^{II} binding capacity of cic-MT2 can be even further increased if higher initial sulfide concentrations are provided.

■ ASSOCIATED CONTENT

Supporting Information

Results of AAA for the proteinase K digested Cd_9S_7-cicMT2 species. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This work is dedicated with best wishes to Professor Helmut Sigel on the occasion of his 75th birthday.

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