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Evidence of Native Metal–S^{2-}–Metallothionein Complexes Confirmed by the Analysis of Cup1 Divalent-Metal-Ion Binding Properties

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Abstract: It has previously been shown that recombinant synthesis, under metal-supplemented conditions, of diverse metallothioneins (MTs) results in the recovery of a subpopulation of S^{2-}-containing complexes in addition to the S^{2-}-devoid canonical metal–MT species. Further significance of this finding has remained veiled by the possibility of it being a mere consequence of synthesis in a heterologous bacterial system. Herein, we present definitive evidence that S^{2-} ligands are also constituents of native metal–MT complexes. Because, although practically universal, the highest S^{2-} content is incorporated by copper-thioneins when coordinating divalent metal ions, we adapted the *Saccharomyces cerevisiae* Cup1 protein, which is the most paradigmatic copper-thionein, as an experimental model. Most significantly, native Cd–Cup1 complexes were purified and fully spectroscopically and spectrometrically characterized from the 301N mutant yeast strain, which allows Cup1 synthesis even in the absence of copper. These results undoubtedly revealed the presence of a Cd–S^{2-}–Cup1 species in native preparations, which were only recovered when carefully avoiding the use of ion-exchange chromatography in the purification protocol. Furthermore, complete analysis of recombinant (*Escherichia coli*) Zn–Cup1, Cd–Cup1, and Cu–Cup1 and those complexes that result from Zn/Cd and Zn/Cu replacements in vitro and acidification/renaturalization processes yielded a comprehensive and comparative overview of the metal-binding abilities of Cup1. Overall, we consider the main conclusions of this study to go beyond the mere study of the particular Cup1 MT, so that they should be considered to delineate a new point of view on the interaction between copper-thioneins and divalent metal ions, still an unexplored aspect in MT research.

Keywords: bioinorganic chemistry • circular dichroism • metalloproteins • metallothioneins • sulfide ligands

Introduction

The study of the relationships between metals and biological entities, from structural aspects to homeostasis and transport mechanisms, holds a prominent position in current bioinorganic chemistry and molecular systems biology.[1–3]

In this framework, metallothioneins (MTs), the ubiquitous, small, universal, cysteine-rich metal-binding peptides, have been the subject of thorough investigations in the past and still pose stimulating questions in the field.[4] MTs are a family of small, non-homologous, metal-binding proteins that exhibit distinct preferences for divalent or monovalent metal ions. This behavior allows differentiation of them into extreme Zn- and Cu-thioneins[5] and a stepwise intermediate gradation of MTs that holds a continuum of metal-binding behaviors. Genuine Zn-thioneins, which are optimized for divalent metal binding, render unique species when synthesized as Zn–MT complexes and mixed Zn,Cd–MT complexes when folded in the presence of high amounts of Cd^{II} ions. Furthermore, in regard to Cu^{I} binding, they yield mixtures of several heteronuclear Zn,Cu–MT complexes of different stoichiometries when biosynthesized in a Cu-rich environment. Contrarily, strict Cu-thioneins, optimized for the

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intracellular chelation of Cu^{I} ions, can render unique, homometallic Cu species in the presence of high amounts of Cu ions, whereas these Cu-thioneins produce a mixture of species of different stoichiometry if folded as Zn or Cd complexes. Mammalian MT1 isoform and the yeast Cup1 protein are the paradigmatic representatives of Zn- and Cu-thioneins, respectively.[6] Although the metal–MT complexes are well known as a normal consequence of the high affinity of the abundant MT cysteines for heavy metal ions, which lead to the formation of multiple metal–thiolate bonds, detailed 3D structural data are very scarce due to specific problems in X-ray studies and NMR spectroscopic analysis.[7] Therefore, any information about the structural aspects of metal–MT coordination is highly significant in shedding light on MTs.

We unexpectedly discovered in 2005 the presence of S^{2-} ligands in most of the recombinantly constituted metal–MT complexes.[8] Our results clearly showed that the highest S^{2-} content was incorporated by Cu-thioneins when coordinating divalent metal ions, significantly Cd^{II} ions. However, the importance of this finding remained obscured by the possibility that the presence of S^{2-} ligands in metal–MT complexes could be a mere consequence of their synthesis in a heterologous bacterial host. Therefore, we undertook a study of the complexes rendered by Cup1, the paradigmatic *Saccharomyces cerevisiae* Cu-thionein when coordinating divalent metal ions both under native (yeast) and heterologous (*Escherichia coli*) cell environments to not only obtain their full description but to also finally tackle the question about the existence or not of native metal–S^{2-}–MT ternary species. Protein Cup1 (see ref. [9] for a recent review), first isolated in the 1970s from yeast grown under copper overload,[10,11] is a 61-residue polypeptide, post-translationally processed into a 53-residue mature form that binds up to eight Cu^{I} ions through the metal–thiolate bonds contributed by 12 cysteine residues.[12] The highly mobile N- and C-terminal tails determine that Cu–Cup1 binding does not render a unique, stable metal–MT species but different Cu^{I}–MT stoichiometries, eventually in dimeric forms.[13–18] As a consequence, elucidation of the Cu–Cup1 3D structure was only possible for a truncated Cu-thiolate core cluster, which encompasses six trigonally and two digonally coordinated Cu^{I} ions.[19]

The fact that Cup1 is synthesized as Cu^{I} complexes in yeast in response to an overload of Cu ions is not caused by the inability of the polypeptide to coordinate other metal ions, but is rather a consequence of their inability to induce its synthesis because *CUP1* transcription only occurs after Cu-dependant Ace1 activation of the gene promoter.[20,21] However, it has been reported that in some strains Cup1 also mediates enhanced Cd^{II} resistance both through *CUP1* copy number amplification[22] and/or altered patterns of gene-expression regulation that allow Cup1 synthesis in the absence of copper.[23] One of the best characterized Cd^{II}-hypertolerant *S. cerevisiae* strains is 301N,[24] in which a mutation in the heat-shock transcription factor Hsf1 provokes an increased binding affinity to heat-shock elements (HSE) that finally results in high constitutive transcription of *CUP1*.[25] When the 301N cells grow in Cd-supplemented media, they accumulate Cd–Cup1 complexes in the cytoplasm, thus preventing Cd^{II} binding to vital molecular targets.[26] Therefore, these are naturally occurring complexes as products of the coordination of Cd^{II} ions by a Cu-thionein and therefore the optimum material to look for native metal–S^{2-}–MT complexes. Although purification of 301N Cd–Cup1 complexes has been reported,[27] they were only slightly characterized.

We present herein the characterization of native Cd–Cup1 complexes together with Zn–Cup1 and Cd–Cup1 complexes synthesized in *E. coli* cells. Additionally, the comparison of the Cu–Cup1 features of the complexes we obtained in bacteria with those of native species reported previously not only validated our recombinant system, but also allowed us to consider some further aspects of the Cu–Cup1 binding process. Most importantly, we can confirm now beyond all doubt that S^{2-} ligands are also constituents of native metal–MT complexes and, on the whole, data included herein define the interaction between copper-thioneins and divalent metal ions, thus going beyond the mere study of the paradigmatic Cup1 MT.

Results and Discussion

Native Cd–Cup1 complexes: The availability of the *S. cerevisiae* 301N strain was decisive in this study to investigate the putative presence of S^{2-} ligands in native Cd–Cup1 complexes and compare them with the corresponding recombinant preparations. Spot-colony tests of this strain in Cd^{II}-supplemented plates, using two wild-type backgrounds (BY4741 and YPH499) as controls, confirmed the capacity of this strain to grow even in 0.5 mM $CdSO_4$ (Figure 1).

Subsequently, we utilized cultures (10 L) of 301N in 0.5 mM $CdSO_4$-supplemented media to obtain the corresponding protein extracts, which were first fractionated

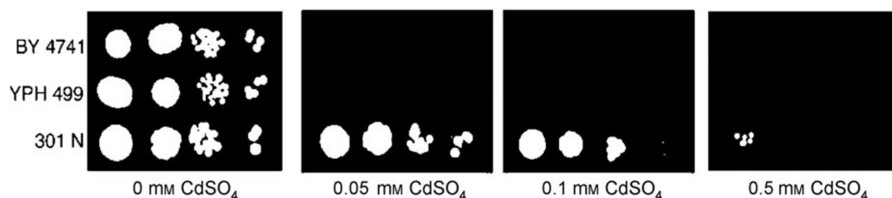

Figure 1. Growth phenotype of three *S. cerevisiae* strains in a Cd^{II}-supplemented medium. Cadmium tolerance (0–0.5 mM) was assayed in yeast-extract peptone dextrose (YPD) agar plates containing serial tenfold dilutions of two metal sensitive wild-type strains (BY4741 and YPH499) and the resistant 301N strain. Colonies from the highest Cd^{II}-supplemented plate were used to inoculate yeast cultures for all the native purification experiments.

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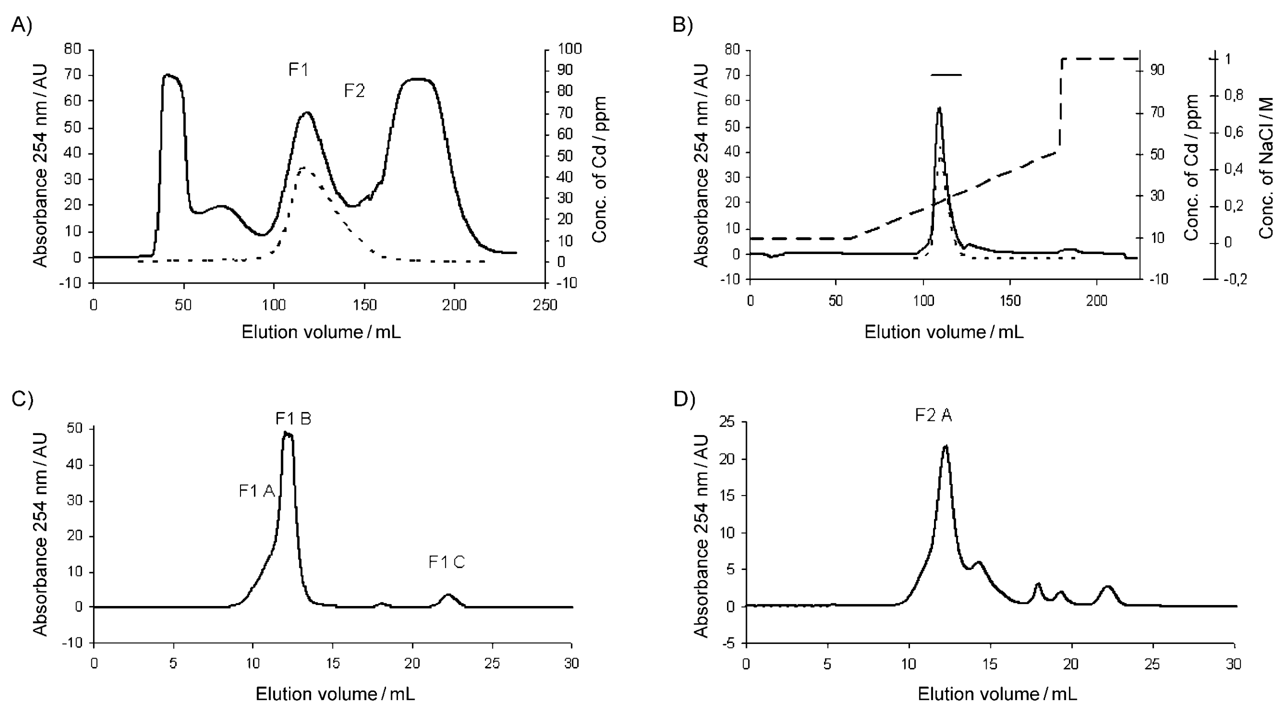

Figure 2. Elution profiles of native Cd–Cup1 complexes. A) The crude extract of *S. cerevisiae* cells was fractionated in a Sephadex G75 column and the content of Cd^{II} ions in each fraction (6 mL) was assessed by ICP-AES (dotted lines). Cd^{II}-containing fractions were pooled as F1 and F2. B) The subsequent purification of F1 and F2 by anion exchange on a DEAE-Sepharose A50 column rendered a single protein and Cd^{II}-containing peak at a saline concentration between 200 and 300 mM. C) The Cd^{II}-containing fractions were pooled in F1A and F1B after the elution of the Superdex 75 column. D) The F2 pool from the elution of the Superdex 75 column revealed that Cd^{II} ions were only present in fraction F2A.

through conventional size-exclusion chromatography on Sephadex G75 columns (see the Experimental Section). This procedure yielded a main peak that included almost all the Cd in the sample (F1 in Figure 2A) and a minor Cd-containing set of fractions, which were also kept for further analysis (F2 in Figure 2A). Because we hypothesized that Cd–S^{2-}–Cup1 complexes could be eliminated from the final preparation by the ion-exchange matrices routinely used for native MT purification,[27–29] we designed two different second purification steps, either by using an anion-exchange chromatography diethylaminoethyl (DEAE) Sepharose A50 column or a further gel-filtration step on a Superdex 75 fast-performance liquid chromatography (FPLC) column.

In the first case, the Sephadex G75 F1 and F2 pools were loaded together on the DEAE column and eluted with a saline gradient. All the Cd^{II}-containing material, which had remained bound to the column, was eluted in a single peak with 250–300 mM NaCl (Figure 2B). These fractions were pooled and spectrometrically and spectrophotometrically characterized.

ESI-MS analysis after acidification of the sample rendered only one peak with a calculated MW value of 5668.6 Da, which evidenced the purity of this native Cd–Cup1 preparation purified by exchange chromatography (n1Cd–Cup1), as the theoretical MW value of native apo-Cup1 is 5668.2 Da. The coincidence between the results of the conventional and acidic inductively coupled plasma atomic emission spectroscopy (ICP-AES) analyses of n1Cd–Cup1 already suggested the exclusive presence in this preparation of S^{2-}-devoid Cd–Cup1 complexes, which was fully confirmed by the absence of dihydrogen sulfide, detectable by gas chromatography–flame photometric detection (GC–FPD) upon acidification of the preparation (Table 1).

Concurrently, ESI-MS analysis revealed that the major species present in n1Cd–Cup1 was Cd_5–Cup1 together with a minor Cd_6–Cup1 species and, surprisingly, Cd_4Zn_1–Cup1 (Figure 3A). This preparation exhibited a spectropolarimetric fingerprint (Figure 4A), also typical of S^{2-}-devoid Cd–MT complexes due to the absence of absorption bands at approximately $\delta = 280$ nm.[8] Taken together, these results evidenced that this preparation consisted of a major canonical Cd_5–Cup1 species and completely lacked S^{2-}-containing complexes.

For the second purification strategy, the Cd-containing material recovered from the Sephadex G75 column (F1 and F2 pools in Figure 2A) were refractionated through the Superdex 75 FPLC column. All the protein-containing fractions were analyzed for Cd^{II} ions both by standard and acidic ICP-AES. Fraction F1 rendered a main Cd^{II}-containing peak (F1B; named as n2Cd–Cup1 for the native Cd–Cup1 preparation purified by avoiding anion-exchange chromatography; Figure 2C). The ESI-MS analysis at acidic pH values identified a unique protein component, namely, the native apo-Cup1 peptide (MW = 5667.5 Da). The ICP-AES results strongly suggested that this preparation should enclose a considerable proportion of S^{2-}-containing com-

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Table 1. Analytical characterization of recombinant and native metal–Cup1 complexes.

Metal–MT complex	Metal–MT (ICP-AES)	Metal–MT (acidic ICP-AES)	S^{2-}–MT[a] (GC–FPD)	Metal–MT species[b] (ESI-MS)	MW_{exp} [Da][c]	MW_{th} [Da][d]
n1Cd–Cup1[e]	4.27 Cd	4.20 Cd	u.d.l.	**Cd_5–Cup1**	6221.5 ± 0.5	6220.3
				Cd_4Zn_1–Cup1	6174.0 ± 0.8	6173.3
	0.65 Zn	0.60 Zn		Cd_6–Cup1	6331.1 ± 0.7	6330.7
n2Cd–Cup1[f,g]	2.93 Cd	5.27 Cd	2.3	**Cd_5–Cup1**	6221.6 ± 0.4	6220.3
				Cd_6S_1–Cup1 ≈ Cd_4–Cup1	6367.6 ± 1.2 ≈ 6106.8 ± 0.9	6364.7 ≈ 6109.8
				Cd_6S_4–Cup1	6473.6 ± 1.6	6466.9
				Cd_4Zn_1–Cup1	6170.9 ± 0.6	6173.3
rCd–Cup1[g]	2.68 Cd	5.93 Cd	1.7	**Cd_5–Cup1**	6364.5 ± 0.5	6364.5
				Cd_6S_1–Cup1	6512.1 ± 1.2	6509.0
				Cd_6S_4–Cup1	6616.0 ± 1.85	6611.2
rZn–Cup1	2.82 Zn	2.81 Zn	u.d.l.	**Zn_4–Cup1**	6066.1 ± 0.4	6064.6
				Zn_5–Cup1	6129.5 ± 0.5	6129.1
				Zn_3–Cup1	6002.7 ± 0.4	5999.7

[a] u.d.l. = under detection limits. [b] Species proposed according to the mass difference between the holo- and apo-proteins. The species in bold are the major components of the preparation. [c] Experimental molecular mass from ESI-MS measurements, which were always performed in duplicate. All the corresponding standard deviations were always less than 1%. [d] Theoretical molecular mass of corresponding species. [e] Native Cd–Cup1 preparation purified by using anion-exchange chromatography. [f] Native Cd–Cup1 preparation purified by avoiding anion-exchange chromatography. [g] The spectra of these preparations vary with time (see text).

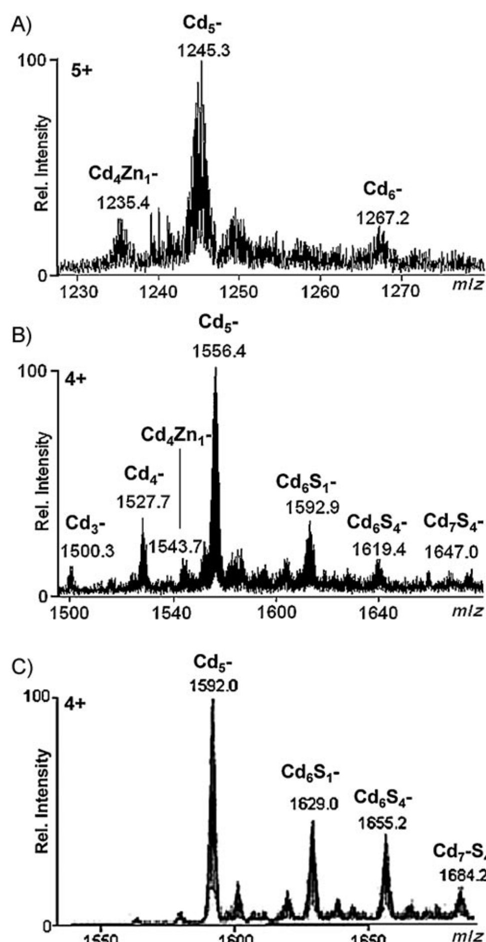

Figure 3. ESI-MS spectra of A) native n1Cd–Cup1, B) native n2Cd–Cup1, and C) recombinant Cd–Cup1 preparations. Only one charge state is shown for the sake of clarity.

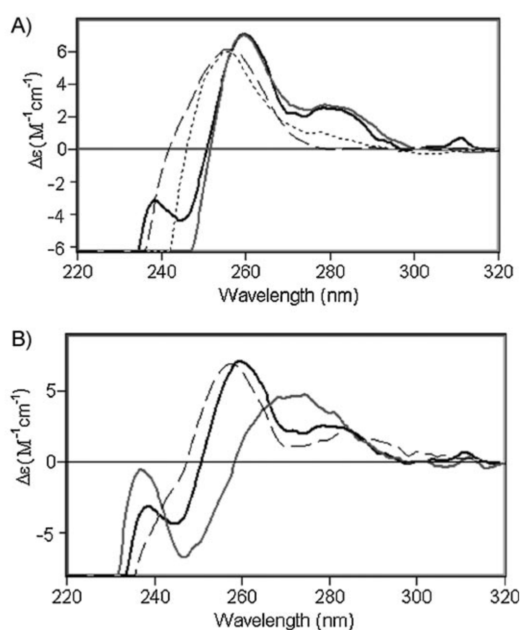

Figure 4. A) CD spectra of Cd–Cup1 preparations: n1Cd–Cup1 (dashed line), n2Cd–Cup1 (solid gray line), rCd–Cup1 (solid black line), and Zn–Cup1 after the addition of three equivalents of Cd^{II} ions (dotted line). B) Comparison of the CD envelopes of the recombinant Cd–Cup1 preparation before (solid black line) and after (solid gray line) the denaturation/renaturation process achieved by lowering and raising the pH value between 7.0 and 2.0 and after the addition of three equivalents of S^{2-} ions to the reneutralized sample (dashed line).

plexes because the mean ratios of Cd/MT obtained under normal conditions (2.93:1) increased to 5.27:1 upon sample acidification (Table 1). GC–FPD analysis showed a ratio of 2.3:1 for acid-labile S^{2-}/MT. Coincidently, ESI-MS analysis at pH 7.0 revealed a mixture of several Cd–Cup1 complexes (Figure 3B), in which the Cd_5–Cup1 species still was the major complex, but a significant presence of Cd_6S_1–Cup1 and Cd_6S_4–Cup1 species could be detected (Table 1). Among other minor components, it is worth noting the presence of Zn_1Cd_4–Cup1, a species also identified in the DEAE-eluted sample. The Cd-containing peak from the Sephadex F2 pool fractions (F2A, Figure 2D) totally lacked

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Cd–S^{2-}–Cup1 complexes and exhibited a composition basically equivalent to that of the DEAE-eluted major Cd_5–Cup1 and minor Zn_1Cd_4–Cup1 complexes (data not shown). Therefore, we considered n2Cd–Cup1 (F1B peak) to be representative of this preparation strategy. Significantly, the CD fingerprint of this sample exhibited a positive absorption around $\delta = 280$ nm, to which Cd–S^{2-}–Cup1 chromophores contributed,[8] with the expected signals at approximately $\delta = 250$ nm (Figure 4 A).

Up to this point, the results of the purification of native Cd–Cup1 complexes clearly demonstrated that by avoiding ion-exchange chromatography we could obtain a preparation with a main Cd_5–Cup1 species and, outstandingly, minor S^{2-}-containing Cd–S^{2-}–Cup1 complexes (i.e., Cd_6S_1–Cup1 and Cd_6S_4–Cup1). Contrarily, S^{2-}-containing complexes were totally undetectable in the native preparations obtained through ion-exchange chromatography, which indicates that they were most probably lost during purification. Subsequently, we undertook characterization of the Zn^{II}- and Cd^{II}-binding abilities of recombinant Cup1 to fully interpret these results and to validate those results obtained from the recombinant synthesis of a considerable number of MTs.[8]

Cloning, synthesis, and purification of recombinant metal–Cup1 complexes: DNA sequencing confirmed that amplification by polymerase chain reaction (PCR) on VC-sp6 DNA yielded as the product a complementary DNA (cDNA) strand that encoded a protein with the sequence of the native mature (N-term processed) form of Cup1, that is, starting at Gln9 (Gln = glutamine) of the translated polypeptide.[12] Small-scale expression assays with the pGEX-*CUP1* construct enabled the analysis of the recombinant apo-Cup1 peptide by ESI-MS measurements after acidification at pH 1.5 of the complexes obtained from Zn^{II}-supplemented cultures. The results confirmed the identity and integrity of the recombinant product after a unique peak of MW = 5811.88 Da was detected (Figure 5 A), which is consistent with a theoretical MW value of 5812.35 Da calculated for a recombinant Cup1 peptide that includes glycine–serine (Gly–Ser) N-terminal residues derived from the glutathione-S-transferase (GST) fusion construct. This small tag has been shown to be inert in terms of metal binding[30] and, accordingly, the CD spectrum of this preparation was completely silent in the region that corresponds to metal–MT chromophores (Figure 5 C). The features of recombinant Cu^{I}–Cup1 complexes prepared from this expression system have also been analyzed as part of our validation system. The corresponding results and comparison with native forms are available in the Supporting Information.

Recombinant Zn–Cup1 complexes: Cup1 was biosynthesized in *E. coli* Zn-supplemented cultures as a mixture of homometallic species, mainly major Zn_4–Cup1 and minor Zn_5–Cup1 and Zn_3–Cup1 species (Figure 5 B and Table 1), in concordance with the stoichiometry of four Zn^{II} ions deduced several years ago from metal-reconstitution experiments.[12] The recombinant Zn–Cup1 (rZn–Cup1) preparation exhibited extremely low chirality comparable to that of the apo-form (Figure 5 C), which suggested that Zn^{II} coordination, although effectively taking place, induces a low degree of folding of the Cup1 peptide. All these features are a good reflection of the Cu-thionein character of Cup1 and is fully coincident with other MTs of the same type, notably metallothionein A (MtnA)[30] and metallothionein B (MtnB)[31] of *Drosophila* and the mammalian MT4.[32]

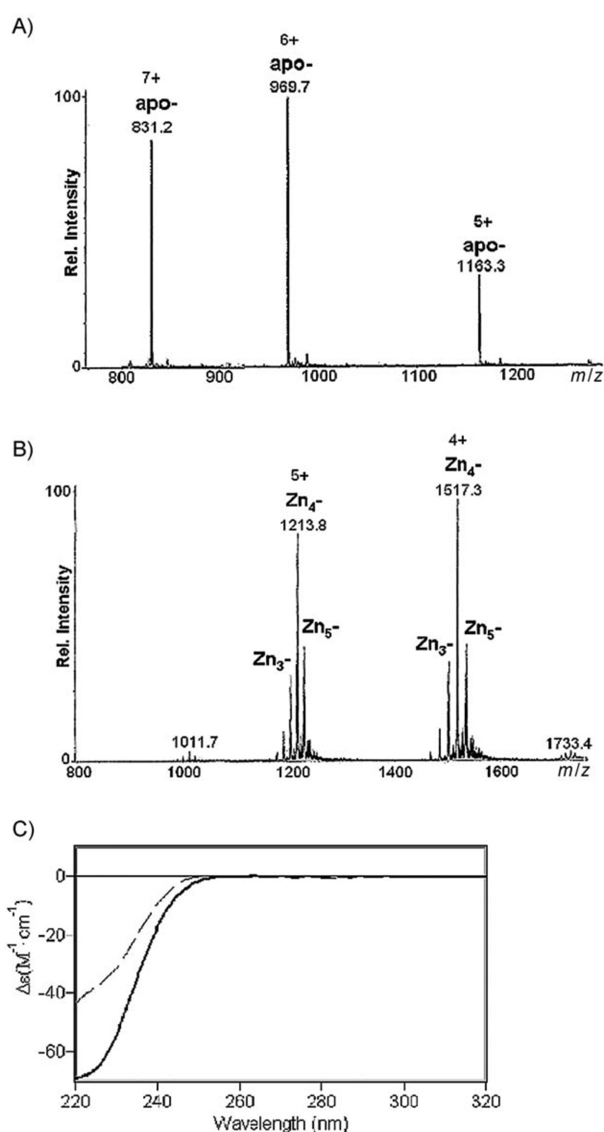

Figure 5. ESI-MS and CD spectra of the recombinant apo-Cup1 (A and C, solid black line) and Zn–Cup1 (B and C, dashed line) preparations.

Recombinant and Cd–Cup1 complexes generated in vitro: Expression of *CUP1* in Cd^{II}-supplemented *E. coli* cultures yielded a mixture of homometallic Cd–Cup1 complexes of variable stoichiometry - major Cd_5–Cup1 - that significantly included several minor S^{2-}-containing species (Cd_6–S_1–Cup1, Cd_6–S_4–Cup1, and Cd_7–S_7–Cup1; Figure 3 C and Table 1). The presence of labile S^{2-} ligands was confirmed not only

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by the discrepancy between normal and acidic ICP-AES measurements but also by GC–FPD analyses (Table 1). Unlike rZn–Cup1, the recombinant Cd–Cup1 (rCd–Cup1) preparation was chiroptically active, thus exhibiting a CD spectrum mainly composed of two bands: the exciton coupling centered at approximately $\delta = 250$ nm, attributable to the Cd–Scys (cys = cystein) chromophores, and the Gaussian band at approximately $\delta = 280$ nm, which corresponds to the presence of Cd–S^{2-} entities[8] (Figure 4). Strikingly, we observed that the CD and UV/Vis spectra of both types of fresh Cd–Cup1 preparation (native n2Cd–Cup1 and rCd–Cup1, denoted by [g] in Table 1) varied in shape over time so that two main different CD fingerprints were identified. The first one remained unaltered roughly until three days after purification and then evolved into a second shape that exhibited more intense positive and negative Cotton effects, which remained unaltered until after 15 days at room temperature (Figure 6 A).

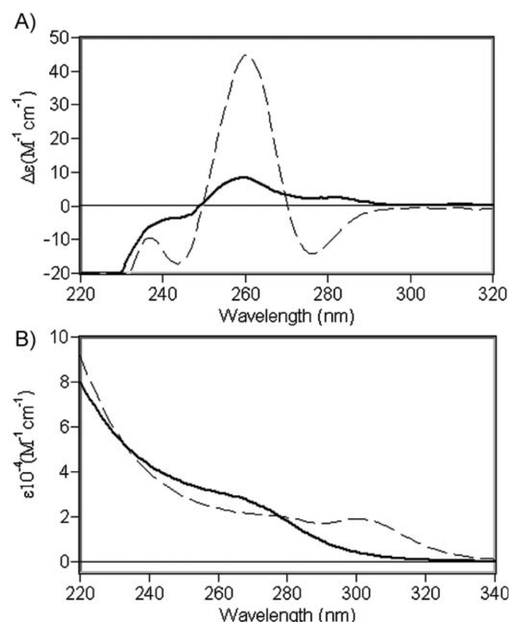

Figure 6. Evolution of the CD (A) and UV/Vis (B) spectroscopic features of rCd–Cup1 with time. The solid lines correspond to the spectra recorded with fresh samples, whereas the dashed lines show the spectra of the same sample allowed to stand for more than three days.

UV/Vis absorptions suggested that these spectropolarimetric changes were associated with a variation in the relative amount of the Cd−SCys and Cd−S^{2-} bonds in the complexes (Figure 6 B), whereas ESI-MS (data not shown) revealed that these changes led to a higher proportion of high-nuclearity S^{2-}-containing Cd–Cup1 complexes over time, in detriment to the canonical Cd_5–Cup1 species predominant just after purification. This phenomenon, which we invariably observed for all the Cd^{II} complexes formed by Cu-thioneins and which is associated with the presence of S^{2-}-labile ligands, is currently under extensive investigation in our laboratories.

Cd–Cup1 complexes were generated in vitro by two different procedures that always started from recombinant material: Zn^{II}/Cd^{II} replacement in Zn–Cup1 and Cd–Cup1 denaturation/reneutralization. Both types of resulting complexes were compared to those formed recombinantly and natively. The titration of the Zn–Cup1 complexes with Cd^{II} ions yielded final species with spectropolarimetric features evidently different to those of rCd–Cup1, mainly because the absence of S^{2-} ligands in the initial Zn–Cup1 sample determines that the final species of the Zn^{II}/Cd^{II} replacement lack S^{2-} ligands (Figure 7). The titration process was accompanied by a significant increase in the chirality of the progressively redshifted CD bands until five equivalents of Cd^{II} ions were added. At this point, no further coordination of Cd^{II} ions took place, even with an excess of metal ions, according to the saturation of both the CD and UV/Vis signals (Figure 7). This result, fully concordant with Cd_5–Cup1 as the major product of the recombinant synthesis, points to this species as the most favored Cd–Cup1 complex. Concomitantly, Cd_5–Cup1 was also the major species obtained after acidification/reneutralitzation of the rCd–Cup1 preparation, which caused the loss of the acid-labile S^{2-} ligands. Significantly, the addition of three equivalents of S^{2-} ions both at the end of the Zn^{II}/Cd^{II} replacement and denaturation/reneutralization processes led to the incorporation of S^{2-} ions as new ligands in the Cd–Cup1 complexes, as could be detected by CD spectroscopic analysis (Figure 4 B) and confirmed by UV/Vis spectroscopic and ESI-MS analyses (data not shown), with concomitant reproduction of the features of the initial, recombinant Cd–Cup1 complexes. Overall, it could be established that the diverse types of Cd–Cup1 complex prepared so far in this study were linked by the relationships represented in Scheme 1, in which their occurrence basically depends on the availability of S^{2-} ions in the media (intracellular or in vitro) in which they were constituted.

But most significantly, stoichiometric correspondences between the native and recombinant Cd–Cup1 complexes could be readily drawn (Scheme 1). Furthermore, these equivalences were reinforced by the comparison of the CD spectra of the corresponding complexes. In the one hand, the S^{2-}-devoid n1Cd–Cup1 preparation exhibited a spectropolarimetric fingerprint similar to that observed in the Zn^{II}/Cd^{II} replacement process after three equivalents of Cd^{II} ions were added to rZn–Cup1 (Figure 4 B). This result is compatible with the null absorbance at approximately $\delta = 280$ nm of the corresponding spectra. Besides, further differences between this CD spectrum and that of the recombinant Cd–Cup1 preparation in the low-wavelength region can be rationalized by considering the minor presence of the Cd_4Zn_1–Cup1 species. In particular, the CD fingerprint of Cd_4Zn_1–Cup1 resembles that recorded after the addition of three equivalents of Cd^{II} ions to Zn–Cup1 when mixed Zn/Cd–Cup1 complexes are still significantly present in solution. On the other hand, the CD fingerprint of this sample, which includes a positive absorption around $\delta = 280$ nm to which the Cd–S^{2-}–Cup1 chromophores contributed,[8] perfectly

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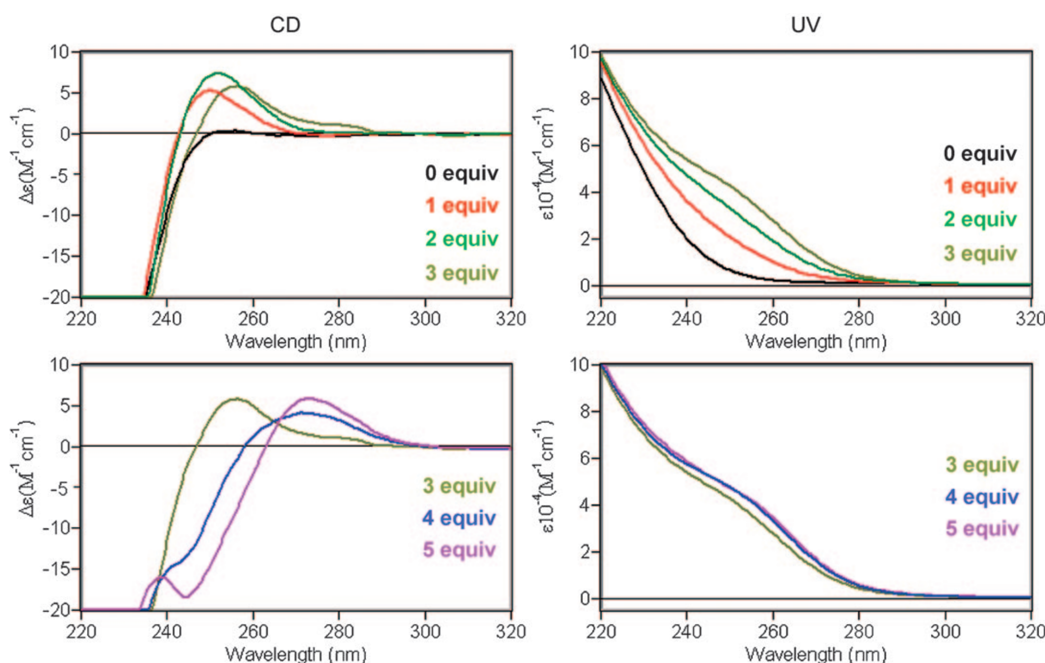
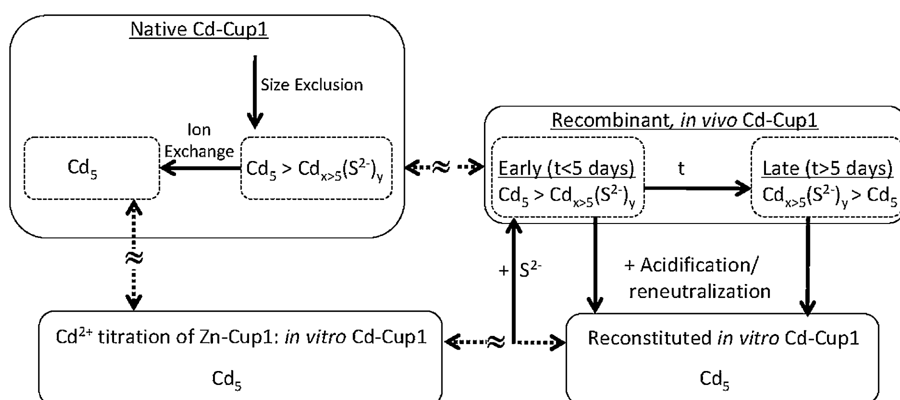


Figure 7. CD and UV/Vis spectra of the titration of a 20 μM rZn–Cup1 preparation, with Cd^{II} ions.



Scheme 1. Relationship among the Cd–Cup1 complexes characterized herein, with indication of the stoichiometry of the major species recovered in each case. For native samples, the type of chromatography used for purification from the total yeast extract is indicated. Recombinant Cd–Cup1 (obtained as described in the Experimental Section) was characterized (CD and ESI-MS) both as fresh or late preparations according to the time (t) elapsed after purification, that is, a maximum of five days or more than five days, respectively. The rCd–Cup1 complexes that result from denaturalization and refolding were analyzed both directly and after the further addition of S^{2-} ions to the preparation. Finally, the result of the Zn/Cd exchange in rZn–Cup1 allowed the characterization of this type of Cd–Cup1 complex prepared in vitro.

matched that of the fresh rCd–Cup1 preparation in the $\delta =$ 250–320 nm interval (Figure 4 A).

Conclusion

As a consequence of the findings reported herein we can now answer two long-standing questions about the presence of S^{2-} ligands in native metal–MT complexes, logically raised after our report of their presence in recombinant preparations.[8] First, we provide clear evidence that ternary metal–S^{2-}–MT species are also natively built and that this phenomenon is significantly highlighted when Cu-thioneins coordinate Cd^{II} ions. Second, we demonstrate that the recovery of this type of ternary complex, certainly not the main component in most of the preparations, is totally dependent on the kind of chromatography used for purification of the MT complex so that they are lost after ion-exchange chromatography. It is therefore not surprising that native metal–S^{2-}–MT complexes have so far gone unreported because MT purification protocols are traditionally based on ion-exchange matrices;[33,34] moreover, divalent-metal-ion complexes formed by Zn-thioneins, unlike those formed by Cu-thioneins, may yield an extremely low proportion of S^{2-}-containing species.[8]

All the types of Cd–Cup1 complex studied herein are compared in Scheme 1. The analysis of their relationships shows that it is the availability of S^{2-} ions in the medium that determines the synthesis of ternary (Cd–S^{2-}–Cup1) complexes and the conventional binary (Cd–Cup1) species. Hence, the composition of the Cd–Cup1 complexes assembled in an in vivo environment, either native or heterolo-

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gous and which have been subsequently purified by avoiding ion-exchange strategies, is a mixture of major Cd_5–Cup1 and minor Cd_x–S^{2-}–Cup1 species (x = number of Cd^{II} ions, $x>5$). The subpopulation of S^{2-}-containing complexes is absent in all the Cd–Cup1 samples obtained in vitro and in the native material eluted from ion-exchange matrices; therefore, all these preparations are almost exclusively composed of the Cd_5–Cup1 complexes. Different reasons account for the absence of the S^{2-} ligands in each case. It seems clear that ion-exchange chromatography eliminates S^{2-} ions from the native sample. Besides, unfolding is achieved in the denaturalization experiments by the loss of metal–thiolate bonds caused by sample acidification, which also leads to the release of S^{2-} ions as gaseous H_2S. Finally, it is sensible to find that no detectable amount of Cd–S^{2-}–Cup1 species are produced when the Zn^{II} ions of the Zn-loaded form are replaced by Cd^{II} ions by titration in vitro because the initial Zn^{II}–Cup1 preparation almost completely lacks S^{2-} ions. It is worth noting how the addition of S^{2-} ions to all types of Cd–Cup1 complex constituted in vitro leads to the recovery of all the features of the S^{2-}-containing preparations prepared in vivo.

Because the features reported herein for Cup1 can be extended to the other Cu-thioneins—for which we have demonstrated parallel behavior when they are recombinantly synthesized—the presence of S^{2-} ions has to be considered to be a key factor in determining the features of the Cd–MT complexes, and to a minor extent the Zn–MT complexes also, rendered by these types of MT. The determination of the conditions under which the naturally assembled metal–MT complexes would most probably include S^{2-} ligands and the possible structural and physiological significances and consequences of this phenomenon remain a matter of further investigation.

Experimental Section

Recombinant synthesis and purification of metal–Cup1 complexes in *E. coli*: The *CUP1* coding region was amplified by direct PCR on genomic *Saccharomyces cerevisiae* DNA extracted from the VC-sp6 strain (MATa trp1–1 ura3–52 ade- his- CANR gal1 leu2–3,112 cup1S)[1] by using the following primers: upstream 5′-ATTGGATCCCAAAATGAAGGT-3′ and downstream 5′-AGACTAGTCGACTCAT TTCCCAGA-3′. The former introduced a BamHI site immediately before the CAA codon (Gln), whereas the latter created a SalI site immediately after the translation stop codon. Thus, the amplified cDNA coded for a polypeptide equivalent to the mature, N-term processed form of Cup1. The 35-cycle PCR amplification (30 s at 94 °C (denaturing), 30 s at 58 °C (annealing), and 30 s at 72 °C (extension)) was carried out in a total volume of 100 µL that comprised a deoxyribonucleotide triphosphate (dNTP) mixture (2 µL, 25 mm), primer solution (2 µL, 20 µm), GoTaq Flexi DNA polymerase (1 U; Promega), and the template DNA (100 ng). The PCR product was isolated from a 2 % agarose gel, digested with BamHI–SalI (New England Biolabs), and directionally inserted in the pGEX-4T-1 (GE Healthcare) expression vector for the synthesis of a glutathione-S-transferase fusion protein (i.e., GST–Cup1). Ligations were performed with the TAKARA DNA ligation kit (v2.1), and the ligation mixture was used to transform first *E. coli* DH5α cells. The GST–Cup1 construct was prepared from these transformants to be automatically sequenced (Applied Biosystems Abiprism 310, PerkinElmer) with the BigDye terminator v3.1 kit (ABI Biosystems). The expression plasmid was transformed into the *E. coli* BL21 protease-deficient strain for recombinant overexpression. Cultures for preparative purposes were grown in 1.5 L Erlenmeyer flasks. Overnight, saturated cultures of transformed BL21 cells served to inoculate fresh lysogeny broth (LB) medium (10 % v/v, 3 or 5 L; 0.5 % bacto yeast extract, 1 % bacto tryptone (Becton, Dickinson, and Co.), 1 % NaCl (Merck); pH 7.3) supplemented with ampicillin (100 mgL^{-1}; Roche). Induction with isopropyl β-D-thiogalactopyranoside (IPTG; Promega) at a final concentration of 200 µm was performed at OD_{600} = 0.6, and cultures were grown for 30 min before the addition of either $ZnCl_2$ (300 µm), $CdCl_2$ (300 µm), or $CuSO_4$ (500 µm; Merck). After 3 h of incubation at 37 °C, cells were harvested by centrifugation for 7 min at 9644 × *g* (Sorvall RC5C). For protein purification, cells were resuspended in 5 % of the original volume of ice-cold phosphate buffered saline (PBS) (1.4 m NaCl, 27 mm KCl, 101 mm Na_2HPO_4, and 18 mm KH_2PO_4) and β-mercaptoethanol (0.5 % v/v; Sigma) was added to avoid protein oxidation. Cells were lyzed by sonication (Branson Sonifier 250; 0.6 Hz) at 4 °C with pulses of 0.6 s for 8 min. From this step onward, all the procedures were carried out in argon (pure-grade 5.6)-saturated buffers. After sonication, the cell debris was pelleted by centrifugation (45 min at 17 212 × *g*) and the supernatant was recovered to purify the GST–Cup1 fusion by batch affinity chromatography with glutathione Sepharose 4B (GE Healthcare) at a 1:10 ratio of matrix/sample volume. The mixture was gently agitated at room temperature for 1 h and washed three times in PBS. A solution of thrombin in PBS (10 UmL^{-1}) was added to the matrix bed to separate the Cup1 portion from the fusion protein, and digestion was allowed to occur overnight at 22–25 °C. Because the GST portion of the fusion protein remained bound to the matrix, the supernatant contained the metal–Cup1 complexes and thrombin. This solution was concentrated with centriprep concentrators (Amicon) with a cut-off point of 3 kDa, and the Cup1 complexes were finally purified to homogeneity by FPLC on a Superdex75 10/300 GL column (GE Healthcare) equilibrated with 50 mm 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)·HCl (pH 7.0) and run at 0.7 mLmin^{-1}. The eluate absorbance was monitored at λ = 254 nm, protein-containing fractions were pooled, and aliquots of (200 µL) were kept at −80 °C until further use. More details on recombinant MT synthesis can be found in our previous report on the copper-resistant suppressor Crs5, the second yeast MT peptide.[34]

Purification of native Cd–Cup1 complexes: The Cd-resistant 301N *S. cerevisiae* strain (MATa, ura1, CUP1r)[24] was used to obtain native Cd–Cup1 complexes suitable for spectroscopic and spectrometric characterization. In this strain, *CUP1* expression is not restricted to Cu induction, and therefore cultures in Cd^{II}-supplemented medium allow native Cd complexes, which accumulate in the yeast cytoplasm to be synthesized. Hence, 301N was streaked on YPD plates (1 % bacto yeast extract, 2 % bacto tryptone, 2 % bacto agar, 5 % glucose (Panreac); pH 5.7) with $CdSO_4$ (500 µm; Merck), and one of the grown colonies was used to inoculate liquid YPD cultures (100 mL). After growth for 48 h at 30 °C, these cultures were used to inoculate a fresh YPD medium (10 L), also supplemented with $CdSO_4$ (500 µm). This culture was also grown for 48 h in a Microferm fermentor (New Brunswich) coupled to a Westfalia CSA1-06-475 centrifuge controlled by a TVE-OP 76/0 program (Braun Biotech) at constant temperature (30 °C), pH (5.7), and O_2 saturation (76–87 %). The cells (≈100 g, wet weight) were recovered after centrifugation, washed in Mili-Q water (3×), and centrifuged (Sorvall RC5C) at 10 000 × *g* for 5 min at 4 °C. Aliquots (20 g) were stored at −20 °C until use. For protein purification, the cells (20 g) were resuspended in argon-saturated Tris·HCl (60 mL, 20 mm), β-mercaptoethanol (0.5 mm), buffer (pH 8.0) and disrupted by 10 cycles of vortexing in glass beads for 1 min followed by 1 min on ice. Complete cell lysis was monitored under the microscope. The total protein cell extract was clarified by centrifugation at 47 800 × *g* for 1 h at 4 °C, and the final volume recovered was approximately 50 mL. All further steps were performed at 4 °C in argon-saturated buffers. The initial homogenate (10 mL) was fractionated by size-exclusion chromatography on a Sephadex G75 column (Amersham GE Healthcare; 2.5 × 40 cm, equilibrated with 20 mm Tris·HCl, 5 mm β-mercaptoethanol, 100 mm NaCl buffer; pH 8.0). The column was eluted at 0.6 mLmin^{-1} with continuous monitoring of absorbance at δ = 254 nm. Fractions

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(6 mL) were collected and submitted to ICP-AES analysis to measure the Cd content (see below). Two alternative strategies followed on from this step. The first option was a second fractionation by means of ion-exchange chromatography of the G75 fraction pool that exhibited the highest Cd content. Half of this pool was concentrated with centriprep concentrators to a final volume of 5 mL and loaded onto a DEAE-Sepharose A50 column (GE Healthcare; 1.5×13 cm, equilibrated with 20 mM Tris·HCl, 5 mM β-mercaptoethanol buffer; pH 8.0) with a flow rate of 0.6 mL min^{-1}. After two void volume washings, elution was performed with a linear gradient of 20–500 mM NaCl in the same buffer and fractions (3 mL) were collected and analyzed. The second purification option was designed to avoid the use of ion-exchange chromatography. Consequently, the other half of the G75 Cd^{II}-rich pool was fractionated by subsequent FPLC. Aliquots of 15 mL were concentrated to a final volume of 0.5 mL and loaded into a Superdex 75 column integrated into Äkta equipment (GE Healthcare) controlled by a computer running the Unicorn program (v5.1), equilibrated with 20 mM Tris·HCl and 5 mM β-mercaptoethanol buffer at pH 8.0. Fractionation was run at a flow rate of 0.8 mL min^{-1} at 20°C, the fractions were pooled according to absorbance at δ = 254 nm, and the Cd^{II} content was measured by ICP-AES analysis.

Preparation by metal replacement of "in vitro-folded" Cd–Cup1 and Cu–Cup1 complexes: The samples herein denominated "in vitro complexes" correspond to those obtained by titration at pH 7.0 or 2.0 of recombinant Zn–Cup1 with Cd^{II} ions ($CdCl_2$ in Mili-Q purified water) or Cu^{I} ions ($[Cu(CH_3CN)_4]ClO_4$ in CH_3CN/H_2O (30% v/v) at equivalent molar ratios. A detailed description of this procedure can be found in references [35] and [36]. All the titrations were carried out under an argon atmosphere and the pH value remained constant throughout all the experiments without the addition of any extra buffer. The complexes prepared in vitro were spectroscopically and spectrometrically characterized following the same methodology explained below for the complexes conformed in vivo.

Acidification/reneutralization experiments: Aliquots of the Cd–Cup1 preparations (20 μM) were acidified from neutral to acid (pH 7.0 to 2.0) with HCl (10^{-3}–1 M). CD and UV/Vis spectra were recorded at pH 7.0, 4.5, 4.0, 3.0, and 2.0 immediately after acidification and at different incubation times at pH 2.0. For reneutralization, the pH value was restored to 7.0 with NaOH (10^{-3}–1 M). CD and UV/Vis spectra were recorded at several pH intervals during reneutralization. Once the solution reached neutral pH, different equivalents of $Na_2S·9H_2O$ were added stepwise to test if the S^{2-} ions had been incorporated into the reconstituted Cd–Cup1 complexes. All the experiments were performed under strictly oxygen-free conditions by saturating the solutions with argon.

Analytical characterization of metal–Cup1 preparations: The S, Zn, Cd, and Cu content of the samples was analyzed by ICP-AES on a Polyscan 61E (Thermo Jarrell Ash, Franklin, MA, USA) spectropolarimeter at suitable wavelengths (S: δ = 182.040; Zn: 213.856; Cd: 228.802; Cu: 324.803 nm). The samples were routinely prepared as described in reference [37], and thus treated with HNO_3 (2% v/v). Alternatively, preincubation in HCl (1 M) at 65°C for 5 min prior to the ICP measurements guaranteed elimination of the acid-labile S^{2-} ligands putatively present in the sample.[8] Therefore, all the sulfur remaining in the sample corresponded to Cys and methionine (Met) residues, thus meaning that the ICP-AES measurements of the sulfur content could be used to quantify the Cup1 concentration in the preparations. In addition, the average ratios of metal/Cup1 were also calculated from these ICP-AES measurements.

When necessary, the mean S^{2-}/protein content was quantified by GC–FPD on an HP-5890 series II gas chromatograph coupled to an FPD80 CE detector (Thermo Finnigan). The calibration curve was determined with diluted standards of S^{2-} ions from 0.25 to 3 ppm. Sample aliquots were transferred to airtight vials and after highly concentrated acidification with H_2SO_4 (pH 0.0), the H_2S generated was allowed to evolve in the gaseous phase for 2 h. Equilibrated head-space gas was subjected to chromatography as reported before.[8]

Electronic absorption measurements were performed on an HP-8453A diode array UV/Vis spectrometer, and the temperature was kept at 25°C by means of a Peltier PTC-351S apparatus. A Jasco spectropolarimeter (Model J-715) interfaced to a computer (GRAMS 32 Software) was used for CD spectroscopic determination. All the spectra were recorded in 1 cm capped quartz cuvettes and finally corrected for dilution effects.

Mass-spectrometric analysis of metal–Cup1 species: Determination of the MW values were performed by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) on a Micro Tof-Q instrument (Bruker) interfaced with a Series 1100 HPLC Agilent pump equipped with an autosampler, all of which were controlled by using Compass Software. Calibration was attained with NaI (0.2 g) dissolved in water/isopropanol (1:1, 100 mL). Samples containing Cup1 complexes with divalent metal ions were analyzed under the following conditions: the protein solution (20 μL) was injected through a polyether–heteroketone column (1.5 m×0.18 mm i.d.) at 40 μL min^{-1}; capillary counter electrode: 5 kV; desolvation temperature: 90–110°C; dry gas: 6 L min^{-1}; spectra collection: *m/z* 800–2000. The carrier buffer was an acetonitrile/ammonium acetate–ammonia mixture (5:95, 15 mM, pH 7.0). Alternatively, the Cu–Cup1 samples were analyzed as follows: the protein solution (20 μL) was injected at 30 μL min^{-1}; capillary counter electrode: 3.5 kV; lens counter electrode: 4 kV; dry temperature: 80°C; dry gas: 6 L min^{-1}. The carrier buffer was an acetonitrile/ammonium acetate–ammonia mixture (10:90, 15 mM, pH 7.0). For the analysis of both the recombinant and native apo-Cup1 molecular mass, the corresponding sample (20 μL) was injected under the same conditions described before using an acetonitrile/formic acid mixture (5:95, pH 2.5) as the liquid carrier, which caused the complete demetallation of the peptide.

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