Redox Labile Site in a Zn_4 Cluster of Cu_4,Zn_4−Metallothionein-3[†]

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ABSTRACT: Human metallothionein-3 (MT-3) is a neuronal inhibitory factor mainly expressed in brain and downregulated in Alzheimer's disease. The neuroinhibitory activity has been established for native Cu_4,Zn_3−MT-3 and recombinant Zn_7−MT-3. However, there is only limited knowledge about the structure and properties of the former metalloform. We have now generated native-like MT-3 through direct Cu(I) and Zn(II) incorporation into the recombinant apoprotein. Its characterization revealed monomeric Cu_4,Zn_4−MT-3 containing metal−thiolate clusters located in two mutually interacting protein domains, a Cu_4 cluster in the β-domain and a Zn_4 cluster in the α-domain. Using the PC12 cell line, the nontoxic nature of the protein was demonstrated. The results of electronic absorption and Cu(I) luminescence at 77 K showed that the Cu_4 cluster possesses an unprecedented stability in air. In contrast, the Zn_4 cluster is air sensitive. Its oxidation results in the release of one Zn(II) and the formation of a Zn_3 cluster, i.e., Cu_4,Zn_3−MT-3. This process can be prevented or reversed under reducing conditions. The determined apparent stability constant for the Zn_4 cluster of 2.4×10^{11} M^{-1} is similar to that obtained for other zinc-containing MTs. This suggests that a substantially increased nucleophilic reactivity of specific thiolate ligands is responsible for this effect. Thus, the Zn_4 cluster in MT-3 may play a redox-dependent regulatory role.

Alzheimer's disease (AD)[1] is a complex cerebral disorder characterized by a progressive dysfunction, dystrophy, and death of neurons (*1*). Many of the remaining neurons display an aberrant neuritic sprouting (*2*). The underlying relationship between the various pathological factors has yet to be fully resolved, but several potential contributors to the disease process have been identified. For example, presenilins mediate the processing of the amyloid precursor protein to yield the amyloid peptide (Aβ), which is the principal component of neuritic plaques (*3*, *4*). In addition to the obvious pathological proteins, other elements have been examined with regard to their association with AD. One potential factor is metallothionein-3 (MT-3). This third isoform of mammalian metallothioneins, also termed neuronal growth inhibitory factor (GIF), has been identified as an inhibitory factor deficient in Alzheimer's disease (AD) brains (*5*). Recent astrocytic cell culture studies established that MT-3 occurs intracellularly and extracellularly in comparable amounts (*6*). This protein exhibits neuroinhibitory activity *in vitro* that makes it unique from the MT-1 and MT-2 isoforms, which are expressed in almost all tissues, including brain. Thus, MT-3, but not MT-1/-2, antagonizes the ability of AD brain extract to stimulate survival and neuritic sprouting of cultured neurons (*5*). The protein is mainly expressed in hippocampal glutamatergic neurons that release zinc from synaptic terminals (*7*). Other studies showed that only MT-3, but not MT-1/-2, protects cultured neurons from the toxic effect of Aβ peptides (*8*) and that the protein is not induced by the typical inducers of MT-1/-2 biosynthesis such as metal ions (Zn, Cd, and Cu), hormones, inflammation-related stimuli, and stressful agents (*9*, *10*). Since the discovery of MT-3, there have been several conflicting reports with regard to MT-3 levels in AD. However, a recent comprehensive study at both the protein and mRNA level provides clear evidence of its downregulation in AD brains (*11*). In AD, the absence of MT-3 may be responsible for the initiation of an aberrant neuritic sprouting. At present, the underlying biological mechanism leading to the inhibitory activity of MT-3 is not clear. Besides the inhibitory function, a protective role of exogenous MT-3 against free radical damage has also been suggested (*6*, *12*). Independently, changes in MT-3 mRNA levels in response to CNS injury have been taken to indicate its important role in brain repair (*13*, *14*).

Structural data have shown that, like mammalian MT-1/-2 (*15*, *16*), M^{II}_7−MT-3 possesses two-protein domains each encompassing a metal−thiolate cluster: a three-metal cluster, $M^{II}_3Cys_9$, located in the N-terminal β-domain (residues 1−31) and a four-metal cluster, $M^{II}_4Cys_{11}$, in the C-terminal α-domain (residues 32−68) (*17*, *18*). However, markedly increased structural flexibility and cluster dynamics were observed in the biologically active MT-3 compared to MT-1/-2 (*18*, *19*). Consequently, whereas the solution structure of the α-domain of mouse Cd_7−MT-3 could be determined from NMR data, the dynamic disorder encountered in the β-domain prevented the direct determination of its structure (*20*). Growth inhibitory activity in neuronal cell cultures has been reported for isolated Cu_4,Zn_3−MT-3 as well as for

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[1] Abbreviations: 5F-BAPTA, 1,2-bis(2-amino-5-fluorophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AChE, acetylcholine esterase; AD, Alzheimer's disease; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ESI-MS, electrospray ionization mass spectroscopy; EXAFS, extended X-ray absorption fine structure; FFT, fast Fourier transform; LMCT, ligand-to-metal charge transfer; MT, metallothionein; NGF, nerve growth factor; XAS, X-ray absorption spectroscopy.

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recombinant Zn_7−MT-3 (*21*). Other studies have established that the observed bioactivity is confined to the N-terminal β-domain of Zn_7−MT-3, i.e., Zn_3−β (1−31) (*22*), and that this activity is abolished by the mutation of the conserved C(6)-P-C-P(9) motif to that found in MT-1/-2 (*23*). Recent mutational analysis revealed that besides the C(6)-P-C-P(9) motif the insertion of the unique Thr5 was also required to engineer MT-3-like bioactivity into the inactive mouse MT-1. The corresponding ^{113}Cd NMR studies on Cd_7−MT-1 showed that the introduction of the T(5)-C-P-C-P(9) motif resulted in an increase in conformational flexibility and intersite metal exchange in the Cd_3−thiolate cluster, suggesting that structure/cluster dynamics are critical for the biological activity of MT-3 (*24*).

Although the inhibitory activity of isolated Cu_4,Zn_3−MT-3 is well-established, there is only limited knowledge about its structure. Further studies of this metalloform are important for gaining a better understanding of the MT-3 function. However, because of the inherent difficulties in isolating large amounts of this factor from the brain, we have developed the following strategy aimed at the generation of native-like structure through the correct incorporation of Cu(I) and Zn(II) ions into recombinant apo-MT-3. First, it was necessary to accumulate sufficient structural and spectroscopic information about isolated Cu_4,Zn_3−MT-3 (*25*). In this respect, Cu and Zn K-edge EXAFS of Cu_4,Zn_3−MT-3 established the presence of homometallic Cu_4− and Zn_3− thiolate clusters located in two independent protein domains, with Cu(I) ions being primarily trigonally and Zn(II) tetrahedrally coordinated (*26*). Second, because of the high affinity of Cu(I) ions for thiolate ligands, the formation and localization of the Cu_4 cluster in the protein structure were considered crucial for the generation of Cu_4,Zn_3−MT-3 and the further characterization of this form. Evidence for the specific formation of the Cu_4 cluster in the N-terminal β-domain of MT-3 was obtained by a combination of spectroscopic and immunological techniques (*27*).

In this work, we report the generation, characterization, and highly unusual properties of the native-like structure of Cu(I)- and Zn(II)-containing MT-3. The analytical data revealed the formation of Cu_4,Zn_4−MT-3. Its spectroscopic characterization by electronic absorption, circular dichroism, and Cu(I) luminescence spectroscopy at 77 K demonstrated the domain-specific formation of two homometallic metal−thiolate clusters. A highly unusual property of the Cu_4 cluster in the β-domain of the protein is its stability to air oxidation. In contrast, the Zn_4 cluster in the α-domain was found to be air sensitive, resulting in a limited cluster oxidation accompanied by a metal release yielding Cu_4,Zn_3−MT-3. This process could be reversed under reducing conditions, thus indicating a redox sensitivity of the Zn_4 cluster. Independently, the nontoxic nature of the generated MT-3 form in neuronal cell cultures was also demonstrated.

MATERIALS AND METHODS

Expression and Purification of MT-3. Human Zn_7−MT-3 was expressed in *Escherichia coli* and purified as described previously (*19*). The apo form of MT-3 was generated by the method of Vašák (*28*). The concentration of the apoprotein was determined spectrophotometrically (ϵ_{220} = 53 000 M^{-1} cm^{-1}), and the free cysteine content (20 Cys residues per protein) was assessed by the sulfhydryl reaction with 2,2′-dithiopyridine in 0.2 M sodium acetate and 1 mM EDTA (pH 4), using an ϵ_{343} of 7600 M^{-1} cm^{-1} (*29*). The molecular mass of the apoprotein, 6926 Da, was determined by ESI-MS (SCIEX API III$^+$). This mass corresponds well to the calculated mass of 6927 Da. The protein was shock-frozen in liquid nitrogen and stored at −20 °C until it was used.

Preparation of Cu(I)- and Zn(II)-Containing MT-3. All solutions used in the preparation of Cu(I)- and Zn(II)-containing MT-3 were rendered oxygen-free by three freeze−pump−thaw cycles on a vacuum line, and all preparation steps were carried out in a nitrogen-purged glovebox. The metal content of the freshly prepared $[Cu(CH_3CN)_4]ClO_4$ stock solution in 5 mM HCl and 20% acetonitrile and that of $ZnCl_2$ in 0.01 M HCl were determined by atomic absorption spectroscopy (Varian AA-110). The Cu(I)- and Zn(II)-containing MT-3 was prepared as follows. First, 4 equiv of Cu(I) was added to a 15 μM apo-MT-3 (10 mM HCl and 5% acetonitrile) in the presence of Chelex-100 resin (1 mg/mL) and the sample incubated for 5 min. In the next step, 6 equiv of Zn(II) was added and the pH was increased to 8.0 with 0.5 M Tris base. Following the 90 min incubation, Chelex-100 resin was removed by centrifugation (5 min at 15000*g*). Subsequently, the metal-containing protein samples were equilibrated at 37 °C overnight. The metal-to-protein ratio was calculated using the metal concentrations determined by atomic absorption and the protein concentration determined by quantitative amino acid analysis. A metal-to-protein ratio of 4 was obtained for both Cu(I) and Zn(II), indicating that the Cu_4,Zn_4−MT-3 form was prepared.

Spectroscopic Measurements. Absorption spectra were recorded on a Cary 3 spectrophotometer. Circular dichroism (CD) measurements were taken using a Jasco spectropolarimeter (model J-715). The CD spectra are expressed as molar circular dichroism ($\Delta\epsilon$) in units of M^{-1} cm^{-1}. The final CD spectra were smoothed using the FFT algorithm of the Microcal Origin 5.0 software. Luminescence spectra were obtained on a SPEX fluorolog spectrofluorometer fitted with a 1934 C phosphorimeter accessory using 45° detection geometry. The excitation wavelength was 300 nm. Luminescence spectra were recorded at 77 K on the microcrystalline frozen samples using a 50 μs delay and a 0.3 ms acquisition window with excitation at 300 nm. They were smoothed using the FFT algorithm mentioned above. The EPR spectrum was recorded with a Bruker (ESP 300) spectrometer at 10 K cooled with liquid helium.

Determination of the Zn Binding Constant by 5F-BAPTA ^{19}F NMR. The ^{19}F NMR method of Hasler *et al.* (*30*) was used in the determination of the apparent stability constant for Zn(II) in Cu_4,Zn_4−MT-3. In this case, the ^{19}F NMR spectrum of a mixture of a metal chelator 5F-BAPTA (Molecular Probes Inc.) and Cu_4,Zn_4−MT-3 was measured. All preparative steps were performed in a nitrogen-purged glovebox. Briefly, the pH of a 36 mM stock solution of 5F-BAPTA in 10 mM Tris was adjusted to 8.0 with 0.1 M HCl and degassed. The NMR samples typically contained 2 mM 5F-BAPTA and 119 μM Cu_4,Zn_4−MT-3 in 10 mM Tris-HCl (pH 8.0) and 10% D_2O. This protein concentration corresponds to a Zn-binding site concentration of 475 μM, the binding sites being considered independent and equal. To ensure that thermodynamic equilibrium was reached, the samples were allowed to equilibrate for 3 h at room

temperature before the ^{19}F NMR spectra were acquired. Note that in rabbit Cd_7−MT-2a the half-life for metal exchange ranges between 0.5 s and 16 min for the β- and α-domain, respectively (*31*). The well-separated ^{19}F signals of free 5F-BAPTA and Zn-5F-BAPTA were integrated using the Bruker WIN-NMR software package. The apparent binding constant of Zn-5F-BAPTA was corrected for the conditions applied in this measurement (pH, salt concentration, buffer, and temperature) as described previously (*32*), using the program *Chelator* (*33*). In the calculation, an initial binding constant K for the Zn−5F-BAPTA complex of 1.3×10^8 M^{-1} (30 °C, 40 mM HEPES, pH 7.1, ionic strength of 0.138 M) (*32*) was used and recalculated for the conditions of the NMR measurements (25 °C, 10 mM Tris-HCl, pH 8.0, ionic strength of 4 mM). The resulting value of K was 4.3×10^9 M^{-1}. This stability constant was used to determine the apparent binding constants for Cu_4,Zn_4−MT-3.

Stability of Cu_4,Zn_4−MT-3 in Air. The 50 μL aliquots of Cu_4,Zn_4−MT-3 (60 μM) were added to 450 μL of air-saturated buffer [25 mM Tris-HCl and 50 mM NaCl (pH 8.0)] in the presence and absence of 2 mM 2-mercaptoethanol. The metal release was followed over a time period of 36 h using independent samples. The amount of free metal was determined by atomic absorption upon sample ultrafiltration (Centricon Millipore, molecular mass cutoff of 3000 Da). The data of the Zn(II) release from Cu_4,Zn_4−MT-3 were fitted using an exponential function (Microsoft Origin 5.0).

PC12 Cell Cultures. PC12 cells were kindly provided by S. Gloor (University of Zürich). Cells were grown at 37 °C in 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 1% donor heat-inactivated horse serum (DHS) and 1 mM L-glutamine (all from Gibco). Cells were plated at a density of 5×10^4 cells/mL on 24-well C^+ culture dishes (Sarstedt). All cell cultures (0.8 mL) containing 100 mg/mL NGF 75 (Roche) were grown for 8 days in the presence of 1 or 50 μg/mL Cu_4,Zn_4−MT-3, Zn_7−MT-3, or Zn_7−MT-2. Each second day, the cultures were fed by exchanging 0.4 mL of the medium with new medium, which contained corresponding amounts of NGF and MT. After 8 days, cells were harvested.

Acetylcholinesterase (AChE) Assay. Cells were washed twice with ice-cold phosphate-buffered saline (pH 7.5) and resolubilized in 10 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$, and 1% Triton X-100. Esterase activity was measured following the method described by Rieger *et al*. (*34*). Briefly, samples were analyzed for total AChE content using 0.5 mM acetylthiocholine and 0.4 mM DTNB in 100 mM Tris-HCl buffer (pH 7.8). The increasing absorption was recorded at 412 nm over the course of 10 min. The protein concentration was determined with the protein assay kit (Bio-Rad). The specific activities of AChE were compared.

RESULTS AND DISCUSSION

Preparation and Characterization of Cu,Zn−MT-3. The preparation of Cu,Zn−MT-3, as described in Materials and Methods, relied on our previous studies in which the specific formation of the Cu_4 cluster in the N-terminal β-domain of MT-3 was established (*27*). In these studies, we could show that all nine Cys residues of this domain are involved in metal binding, giving rise to the Cu_4Cys_9 cluster. The previous Cu K-edge EXAFS studies revealed that the Cu(I) ions in the

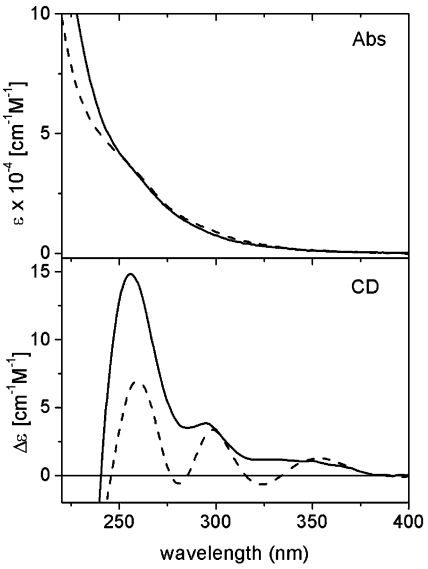

FIGURE 1: Electronic absorption and circular dichroism spectra of human Cu_4,Zn_4−MT-3 and the corresponding Cu_4 cluster in MT-3. Cu_4,Zn_4−MT-3 (—) and the sole Cu_4 cluster in MT-3 (- - -) (*27*) at pH 8.0. For details about the sample preparation, see Materials and Methods.

Cu_4 cluster of native Cu_4,Zn_3−MT-3 are predominantly trigonally coordinated by cysteine thiolates (*26*). We suggested, therefore, that a similar structure architecture of the Cu_4 cluster also exists in the recombinant protein. The generation of Cu,Zn−MT-3 described in this work required additional preparative steps favoring domain selectivity, such as a competition with a chelating resin and the subsequent temperature equilibration of the sample. The structural studies of Zn_7- and Cd_7−MT-3 showed that the 11 Cys residues of the C-terminal α-domain of the protein bind four divalent metal ions in a $M^{II}_4Cys_{11}$ cluster, with each metal ion being tetrahedrally coordinated by both bridging and terminal thiolates. To ascertain the full metal occupancy of the α-domain in Cu,Zn−MT-3, ∼6 equiv of Zn(II) was added at low pH to the protein sample containing the Cu_4 cluster and the metal chelating resin Chelex-100. Following the pH increase, the Cu,Zn−MT-3 sample was equilibrated at 37 °C (see Materials and Methods). As judged by the Cu(I) luminescence at 77 K, obtained on small aliquots of the sample, this time period was sufficient for the protein to reach a thermodynamic minimum. At this stage, the sample was removed from the nitrogen-purged glovebox. The analytical data revealed the formation of Cu_4,Zn_4−MT-3. Gel filtration and ion exchange chromatography of the sample revealed a single chromatographic peak indicating the sample homogeneity. The possible reason for the isolation of Cu_4,Zn_3−MT-3 from brains of various species is discussed below.

Spectroscopic Characterization of Cu_4,Zn_4−MT-3. In the past, the structural features of Cu(I)−thiolate clusters in proteins, e.g., transcription factors Ace1 and Amt1, MTs, and related proteins have been studied by various spectroscopic techniques, including electronic absorption, CD, luminescence, X-ray absorption spectroscopy (XAS), and EXAFS (*31*, *35*−*43*). The former three techniques have also been used in the studies presented here. The absorption and CD spectra of Cu_4,Zn_4−MT-3 are characterized by the appearance of metal-dependent features, starting at ∼380 nm and extending into the far-UV region (Figure 1). The spectral

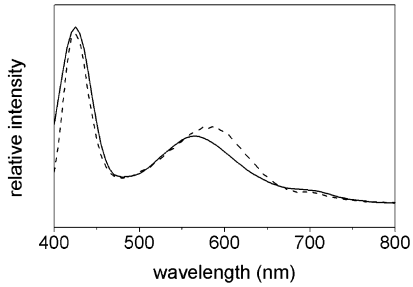

FIGURE 2: Luminescence spectra of human Cu_4,Zn_4−MT-3 and the Cu_4 β-domain of MT-3. Cu_4,Zn_4−MT-3 (—) and the Cu_4 cluster in the β-domain (- - -) (*17*) at 77 K, with excitation at 300 nm. The high- and low-energy emission in both samples decayed according to a single-exponential function with lifetimes (τ) of approximately 30 and 105 μs, respectively.

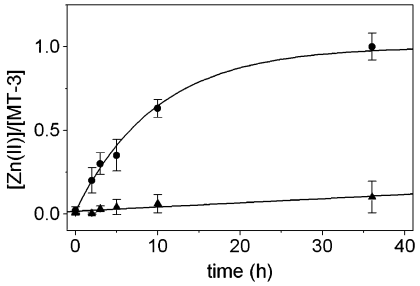

FIGURE 3: Release of Zn(II) from Cu_4,Zn_4−MT-3. The rate of Zn(II) release was measured as a function of time in the presence (▲) and absence (●) of 2 mM 2-mercaptoethanol at pH 8.0. For details, see the text.

contribution of CysS−Zn(II) LMCT bands and the peptide backbone transitions occurs below 250 nm (*31*). Consequently, the spectra that are presented are dominated by electronic transitions of the Cu_4 cluster. For illustration, the absorption and CD spectra of the partially metal occupied Cu_4−MT-3 species are also shown (*27*). The absorption spectrum of this species shows a broad absorption envelope characterized by a prominent shoulder at 262 nm and a weak shoulder at ∼300 nm (Figure 1). By analogy with mammalian Cu(I)−MTs (*42*), the bands in the high-energy region (below 280 nm) originate predominantly from CysS−Cu(I) LMCT transitions, whereas those in the low-energy region with very weak molar absorptivities but rather strong CD bands originate from formally spin-forbidden 3d → 4s metal cluster-centered transitions brought about by Cu(I)−Cu(I) interactions in polynuclear Cu(I) complexes (*42*, and references therein). The formation of the Zn_4 cluster in Cu_4,Zn_4−MT-3 results in spectral changes also in the range above 250 nm, being consistent with a minor structural alteration of the Cu_4 cluster brought about by a mutual interaction of both protein domains. In both Zn_7− and Cd_7−MT-3, such interactions are well-documented (*19*).

The low-temperature (77 K) luminescence spectrum of Cu_4,Zn_4−MT-3 shows two emissive bands at 420 and ∼600 nm (Figure 2). For comparison, the previously obtained luminescence spectrum of the Cu_4 cluster in the C-terminal β-domain is also presented (*17*). The high-energy as well as the low-energy emission in both samples decayed according to a single-exponential function with lifetimes (τ) of approximately 30 ± 2 and 105 ± 8 μs, respectively. Both the large Stokes shift (defined as the energy difference between the excitation at 300 nm and the position of emission bands) and the lifetimes suggest that both emissive bands are spin-forbidden; hence, the relevant excited states have triplet spin multiplicity. In the previous detailed studies of the mammalian Cu(I)−MT complexes, closely similar low- and high-energy emissive bands with similar lifetimes have been reported (*42*). These emissions have been assigned to two distinct triplet excited-state manifolds of $Cu(I)_4$−thiolate clusters (*37*, *42*). The lower-energy emission at 600 nm was assigned to a triplet LMCT excited state and the higher-energy band at 420 nm to a triplet cluster-centered (^{3}CC) excited state, being of mixed LMCT/d-s character (*42*). Two emissive bands at the same position and with similar lifetimes have also been observed in our previous studies of isolated Cu_4,Zn_3−MT-3, which contained two homonuclear clusters (*25*, *26*). These results support the correctness of the reconstituted Cu_4 cluster structure present in the β-domain of MT-3.

Stability of Cu_4,Zn_4−MT-3 to Air Oxidation. All Cu(I)−thiolate clusters in inorganic models and proteins such as Ace1, Amt1, and MT-1/-2 studied thus far are susceptible to oxidation in air (*42*, *44*). In our previous studies on native Cu_4,Zn_3−MT-3, a remarkable stability of the Cu_4 cluster to oxidation was observed (*45*). To examine the stability of Cu_4,Zn_4−MT-3, in particular of its Cu_4 cluster, spectroscopic techniques such as electronic absorption, Cu(I) luminescence, and EPR at 10 K were used. The preservation of the absorption and luminescence features over the course of 48 h indicated that no copper oxidation or sample precipitation occurred. This conclusion is supported also by the absence of the Cu(II) EPR signal after exposure of the sample to air for 48 h (data not shown). Although the subsequent gel filtration of the sample confirmed the preservation of monomeric MT-3 with four Cu(I) ions bound, the number of bound Zn(II) ions dropped to three, i.e., Cu_4,Zn_3−MT-3, suggesting that the exposure of the sample to air resulted in a release of one metal ion from the α-cluster. However, the incubation of the sample with 2 mM 2-mercaptoethanol or 5 mM GSH followed by gel filtration under reducing conditions resulted in the recovery of Cu_4,Zn_4−MT-3, a behavior consistent with disulfide formation. DTNB is routinely used when monitoring the Cys residues in both the apo-MT and Zn_7−MT forms (*46*). Although in this reaction the Zn(II) ions are released from the structure, the Cu(I) ions are usually not. Therefore, we have applied this method in examining the nature of the Zn_4Cys_{11} cluster in the α-domain of Cu_4,Zn_4−MT-3 under various conditions. However, the finding of 16 ± 2 cysteines in freshly prepared Cu_4,Zn_4−MT-3 suggested that under these conditions the Cu(I)-occupied β-domain influences the measurements. Nevertheless, the need for reducing conditions in reversing the process suggests an oxidation of the cluster, presumably the formation of disulfide bond(s). To learn more about this oxidation process, aliquots of the freshly prepared Cu_4,Zn_4−MT-3 sample were mixed with air-saturated buffer in the presence and absence of 2-mercaptoethanol and the amount of released metal was determined upon ultrafiltration as a function of time (Figure 3). Whereas under reducing conditions no free metal was found, in air-saturated samples approximately one Zn(II) was released with a half-time of ∼6 h. Preliminary results obtained in similar studies of recombinant human Zn_7−MT-3 and native Zn_7−MT-2 from rabbit liver revealed that while under reducing conditions no metal was released,

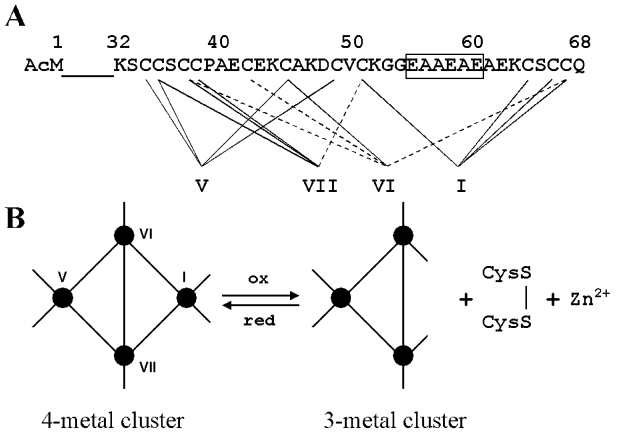

FIGURE 4: Proposed redox behavior of the Zn_4 cluster in Cu_4,Zn_4−MT-3. (A) Metal−thiolate connectivities within the α-domain of MT-3. The metal ions are labeled with Roman numerals (*20*). (B) Proposed oxidation process occurring in the Zn_4 cluster of MT-3.

under nonreducing conditions approximately one Zn(II) was released from MT-3 and none from MT-2. Thus, Cu_4,Zn_4−MT-3 shows, on one hand, unprecedented stability of the Cu_4Cys_9 cluster to oxidation and, on the other, the easy and reversible oxidation of the Zn_4Cys_{11} cluster. Both properties have not been encountered in the studies of corresponding MT-1/-2 metallo forms.

In the NMR solution structure of Cd_7−MT-3, only the structure of the Cd_4 cluster could be directly determined (*20*). The metal organization and the metal−cysteine connectivities in the Cd_4Cys_{11} cluster (Figure 4A) were found to be identical to that reported for a number of MT-1/-2 structures from different mammalian species (*16*). The four-metal clusters in mammalian MTs can be viewed as two fused three-metal clusters possessing a cyclohexane type of geometry with a common edge (*47*). Major structural differences in the α-domain of MT-3 compared to other mammalian MTs exist in the C-terminal 16-amino acid peptide (53−68), which also includes the unique acidic hexapeptide insert (Figure 4A). In this region, a major dynamic disorder occurs (*20*). The two terminal cysteine ligands and one bridging cysteine ligand of this peptide coordinate a single Zn(II) ion [designated in the three-dimensional structure as I (*20*)], which we propose to be released upon exposure to air. In this case, a Zn_3 cluster (Zn_3Cys_9) and a single disulfide could be easily formed. The proposed tentative scheme for this process is shown in Figure 4B.

Measurement of the Apparent Zn(II) Binding Constant. In view of the unusual properties of the Zn_4 cluster in Cu_4,Zn_4−MT-3, its apparent stability constant was determined. We employed our recently developed ^{19}F NMR method, which is based on the competition between the metal chelator 5F-BAPTA and a metalloprotein (*30*). The presence of two homotropic ^{19}F nuclei in the structure enables the concomitant detection of free 5F-BAPTA and its 1:1 complexes with various metal ions, due to a change in the ^{19}F chemical shift position of the chelator upon metal binding. A knowledge of the equilibrium concentrations of free 5F-BAPTA and its zinc complex, the starting concentrations of 5F-BAPTA, and the protein-bound Zn(II) as well as the Zn(II)−5F-BAPTA binding constant enables the apparent metal binding constant of Zn(II)-containing proteins to be determined (*30*). The ^{19}F NMR spectrum obtained after 3 h following the anaerobic sample preparation showed two ^{19}F signals that were assigned to the zinc-bound and free 5F-BAPTA on the basis of the comparison with the ^{19}F NMR spectra of two independently prepared samples. The spectrum of the same sample acquired after incubation for additional 20 h established that the equilibrium had been reached. The apparent binding constant for the Zn_4 cluster in Cu_4,Zn_4−MT-3 of 2.4×10^{11} M^{-1} at 25 °C was obtained, assuming equivalent and independent metal binding sites (for details, see ref *30*). It may be noted that no copper release was detected by this method. The obtained apparent stability constant is between 3.1×10^{11} and 6.2×10^{10} M^{-1} determined for Zn_7−MT-3 and Zn_7−MT-2, respectively, using the same ^{19}F NMR method (*30*). These results show that all apparent binding constants are rather similar. This suggests that the release of one Zn(II) from Cu_4,Zn_4−MT-3 is not due to decreased thermodynamic stability but rather reflects a substantially increased reactivity of the thiolate ligands involved in its coordination.

PC12 Cell Culture Studies. In a number of neuronal cell culture studies and in the presence of AD brain extract, the neuroinhibitory activity of isolated Cu_4,Zn_3−MT-3 has been demonstrated (*5*, *48*). In these studies, the neurotoxicity of this form could be ruled out. However, in other studies, an attempt to generate Cu(I)- and Zn(II)-containing MT-3 resulted in the sample neurotoxicity (*49*). The toxicity of copper ions in proteins is usually connected with uncontrolled redox cycling leading to free radical production. Therefore, the effect of Cu_4,Zn_4−MT-3 on neuronal PC12 cells was also examined and compared with those of Zn_7−MT-3 and Zn_7−MT-2. Neuronal differentiation and growth arrest of PC12 cells were induced by nerve growth factor (NGF). While NGF was being added, varying concentrations of MT-3 or MT-2 (1 and 50 μg/mL) were also added with the aim of examining their influence on cell differentiation. As a measure of the extent of cell differentiation, the enzymatic activity of AChE was determined. On day 8, cells were harvested and both morphological changes and changes in AChE activity investigated. Compared to controls, no differences in these parameters with both MT-3 samples were observed. In the case of MT-2, an ∼2-fold activity increase was seen (data not shown). This observation would be consistent with a promoting effect of MT-2 on PC12 differentiation. A similar observation has been reported in recent cell culture studies in which MT-1/-2 had a promoting effect on laminin-1-induced acinar differentiation (*50*). Therefore, it can be concluded that like native Cu_4,Zn_3−MT-3 the generated Cu_4,Zn_4−MT-3 (or Cu_4,Zn_3−MT-3) is not neurotoxic.

CONCLUSIONS

The results presented here allow a number of conclusions to be drawn. First, the data clearly indicate that we have succeeded in the generation of a native-like structure of MT-3 through a selective metal filling of both protein domains. These results further suggest that the protein domains possess a widely different metal selectivity. The most surprising finding was the fact that while the Cu_4 cluster of Cu_4,Zn_4−MT-3 is resistant to air oxidation, the Zn_4 cluster is not. This and the unprecedented stability of the Cu_4−thiolate cluster in air make this MT isoform unique. At present, we ascribe the air stability of the Cu_4 cluster to the geometrical

constraints imposed by the protein which stabilize the Cu(I) oxidation state. The release of one Zn(II) from the Zn_4 cluster of the C-terminal α-domain resulted in the formation of a Zn_3 cluster and thus in the Cu_4,Zn_3−MT-3 form. This process would account for the isolation of Cu_4,Zn_3−MT-3 from brains of different mammalian species (*5*, *45*, *51*, *52*). The affinity of Zn(II) in Cu_4,Zn_4−MT-3 that is closely similar to that found in Zn_7−MT-3 and Zn_7−MT-2 (*30*) suggests that the metal release is due to differences in nucleophilic reactivity of thiolate ligands and not to decreased metal affinity. Overall, it would appear that one Zn(II) is released from a specific binding site in the α-domain. Although under the conditions that were used no metal release from the zinc-containing MT-1/-2 isoforms was observed, in the presence of the GSH/GSSG redox pair the release of Zn(II) ions from MT-1/-2 has been reported (*53*). Clearly, depending on the metal loading of the α-domain of MT-3, its overall structure must differ substantially. Since this structural transition can be prevented or reversed under reducing conditions, a partial protein oxidation, most likely a disulfide formation, must also occur. Recent cell culture studies established that MT-3 is present intracellularly and extracellularly in comparable amounts (*6*). In view of this finding, the reversal of the Zn(II) release from MT-3 under reducing conditions may suggest that Cu_4,Zn_4−MT-3 represents the intracellular form of the protein whereas extracellularly Cu_4,Zn_3−MT-3 would be present. The latter form could also exist intracellularly under various conditions, including oxidative stress. At present, a number of zinc finger proteins exist which act as a redox switch depending on the redox status of the cell (*54*−*57*). In this context, it would appear that the zinc cluster in the C-terminal α-domain of Zn_4,Cu_4−MT-3 might play a similar regulatory role.

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