Disruption of copper and zinc homeostasis in the brain plays a critical role in Alzheimer disease (AD). Copper binding to amyloid-β peptide (Aβ) is linked with the neurotoxicity of Aβ and free radical damage. Metallothionein-3 (MT-3) is a small cysteine- and metal-rich protein expressed in the brain and found down-regulated in AD. This protein occurs intra- and extracellularly, and it plays an important role in the metabolism of zinc and copper. In cell cultures Zn7MT-3, by an unknown mechanism, protects neurons from the toxicity of Aβ. We have, therefore, used a range of complementary spectroscopic and biochemical methods to characterize the interaction of Zn7MT-3 with free Cu2+ ions. We show that Zn7MT-3 scavenges free Cu2+ ions through their reduction to Cu+ and binding to the protein. In this reaction thiolate ligands are oxidized to disulfides concomitant with Zn2+ release. The binding of the first four Cu2+ is cooperative forming a Cu(I)4-thiolate cluster in the N-terminal domain of Cu4,Zn4MT-3 together with two disulfides bonds. The Cu4-thiolate cluster exhibits an unusual stability toward air oxygen. The results of UV-visible, CD, and Cu(I) phosphorescence at 77 K suggest the existence of metal-metal interactions in this cluster. We have demonstrated that Zn7MT-3 in the presence of ascorbate completely quenches the copper-catalyzed hydroxyl radical (OH·) production. Thus, zinc-thiolate clusters in Zn7MT-3 can efficiently silence the redox-active free Cu2+ ions. The biological implication of our studies as to the protective role of Zn7MT-3 from the Cu2+ toxicity in AD and other neurodegenerative disorders is discussed.

Essential transition metals like copper and zinc play a critical role in neurobiology. The homeostasis of both metals is tightly regulated and essential for brain physiology (1). In vitro evidence suggests that in physiological conditions micromolar concentrations of zinc and copper are actively released from neurons during neurotransmission processes into pre- and postsynaptic clefts. However, at present it is not known whether the released copper is present in a chemically exchangeable form (2). Dysregulated metal metabolism (zinc and copper) occurs in neurodegenerative disorders such as Alzheimer (3, 4), Parkinson (5), and prion (6) diseases. In these diseases increased extracellular copper concentrations and free-radical production have been found. There is now direct evidence that copper is bound to amyloid-β peptide (Aβ) in senile plaque of Alzheimer disease (AD) (7). Copper is also linked with the neurotoxicity of Aβ and free radical damage (8).

Because of its redox-active nature (Cu2+/Cu+) its reactivity with molecular oxygen (O2) generates the reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals (9, 10). The redox cycling of copper requires its reduction by biological components, including ascorbate, which actively accumulates in the brain at concentrations between 0.5 and 10 mM (11). Hence, copper chelation and its redox-silencing may represent critical events in preventing the progression of neurodegenerative diseases. In recent years a metal chelation therapy has emerged as a promising tool to attenuate abnormal metal-protein interactions that lead to increased free-radical toxicity (10).

The natural metal chelator metallothionein-3 (MT-3), also known as the growth inhibitory factor, is a small non-inducible cysteine- and metal-rich protein mainly expressed in the brain. In the brain its expression was found in zinc-enriched neurons. Like other mammalian metallothioneins (MTs), MT-3 binds with a high affinity essential monovalent and divalent d10 metal ions Cu(I) and Zn(II). Recently, the interaction of Zn7MT-3 with the small-GTPase Rab3a, which is strictly linked to the exo-endocytic cycle of synaptic vesicles, has been demonstrated. It has been suggested that Zn7MT-3 actively participates in synaptic cycle of zinc vesicles (12). However, besides intraneuronal localization, cell culture studies revealed that the protein also occurs in comparable amounts extracellularly. This suggests that MT-3 may play an important role not only in neuronal metal homeostasis, but also in extracellular metal-related neurochemistry (12–16). As isolated, this brain protein contains both Cu(I) and Zn(II) ions bound in a Cu(I)4,Zn3–4MT-3 species. The important role for MT-3 in zinc and copper metabolism in the brain has been suggested based on its down-regulation in AD patients, where altered copper homeostasis is
linked to extracellular amyloid pathology (17, 18). Therefore, understanding of the mechanisms underlying a high affinity and specificity of this physiological metal chelator appear to be essential for rational drug design of metal-protein-attenuating compounds (19).

The structural studies revealed that MT-3, like other mammalian MTs, binds seven Zn(II) ions through the array of 20 conserved cysteines into two metal-thiolate clusters, i.e. Zn$_{10}$(CysS)$_9$ and Zn$_{12}$(CysS)$_{11}$. In these clusters the metals are tetrahedrally coordinated by both bridging and terminal cysteine thiolates. Less detailed structural information is currently available regarding the structure of Cu(I)-thiolate clusters in this protein. The spectroscopic characterization of native Cu(I)$_2$Zn$_{10}$MT-3 isolated from human and bovine brains and that of in vitro prepared Cu(I)$_2$Zn$_{10}$MT-3 revealed the presence of two homometallic metal-thiolate clusters located in two protein domains: a Cu(I)$_2$-thiolate cluster in the N-terminal B-domain and a Zn(II)$_4$-thiolate cluster in the C-terminal α-domain (20-25). A digonal and trigonal Cu(I) coordination by cysteine sulfur in the Cu(I)$_2$-thiolate cluster of MT-3 has been inferred from the extended x-ray absorption fine structure (EXAFS) (20) and other spectroscopic studies of Cu(I) containing mammalian MTs (21, 26, 27). Similar coordination geometries have also been found in the crystal structure of the Cu(I)$_2$(CysS)$_{10}$ cluster present in copper MT from yeast (28). To play a role in the metabolism of copper in extracellular space, MT-3 would have to interact with Cu$^{2+}$ ions. However, information regarding the reactivity of zinc-thiolate clusters in Zn$_n$MTs with Cu$^{2+}$ is currently not available, being limited to information obtained on Cu$^{2+}$ reactivity with Cd$_2$MT-2 from rabbit liver (29). Due to the redox nature of the coordinating thiolate ligands in Zn(II)-thiolate clusters and their documented high nucleophilicity, the sulfur oxidation would represent the source of electrons for d$^9$ Cu$^{2+}$ reduction to Cu$^+$ and the consequent Cu(I) binding to the protein. The ability of MT-3 structure to accommodate widely different coordination geometries such as those for Cu(I) (digonal and trigonal) and Zn(II) (tetrahedral) in its metal-thiolate clusters may afford the structural bases for such a reaction. This process would be of great importance under neuropathological conditions in which a dysregulation of copper homeostasis is well documented. In this context it is worth noting that, in metal-linked neurodegenerative disorders such as Alzheimer, Parkinson, and prion diseases, the down-regulation of MT-3 has been reported (16, 30). However, the role of this protein in these diseases is unknown.

In the present study we show that the thiolate/disulfide couple in MT-3 represents an efficient system linking zinc-thiolate cluster reactivity to Cu$^{2+}$/Cu$^+$ homeostasis. By detailed spectroscopic and biochemical investigations of Zn$_n$MT-3 reactivity toward free Cu$^{2+}$ ions, we demonstrate that Zn$_n$MT-3 in a well defined process efficiently scavenges Cu$^{2+}$ ions. In this reaction thiolate ligands are oxidized to disulfides concomitant with Zn$^{2+}$ release. The binding of the first four Cu$^{2+}$ ions is cooperative forming a Cu(I)$_4$-thiolate cluster in the N-terminal domain of Cu$_4$Zn$_{10}$MT-3 together with two disulfides bonds. The Cu$_4$-thiolate cluster exhibits an unusual stability toward air oxygen. In addition, we show that this reaction completely quenches the copper-driven free hydroxyl radical production. Thus, Zn$_n$MT-3 through the silencing of redox-active Cu$^{2+}$ ions may play an important protective role in Cu$^{2+}$ toxicity in the brain under physiological and pathological conditions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Media for protein expression were purchased from BD Biosciences. Subtilisin from *Bacillus licheniformis* and monobromobimane were obtained from Fluka. All other standard reagents were of the highest purity available from commercial sources. All solutions were rendered metal-free by passing through a Chelex 100 (Bio-Rad) column to prevent adventitious metal contamination.

**Recombinant Human MT-3 Expression, Purification, and Metal Reconstitution**—A pet-3d (Novagen) plasmid encoding for human MT-3 sequence was used for recombinant protein expression. Expression in *Escherichia coli* strain BL21(DE3)pLys and purification was performed as described previously (31). The metal-free protein (apoprotein) was generated with the method of Vasak (32). Correctness of the expressed protein was confirmed by ESI-MS. The fully Zn$^{2+}$-loaded form was prepared by metal reconstitution (32). Zinc-to-protein ratios were determined by measuring protein concentration photometrically in 0.1 M HCl (ε$_{220}$ = 53,000 M$^{-1}$ cm$^{-1}$) and metal content by flame atomic absorption spectroscopy (SpectraAA-110, Varian Inc.). Cysteine-to-protein ratios was determined via photometric sulphydryl groups (CysSH) quantification upon their reaction with 2,2'-dithiopyridine in 0.2 M sodium acetate/1 mM EDTA (pH 4) using ε$_{760}$ = 7600 M$^{-1}$ cm$^{-1}$ (33) and that of zinc by atomic absorption. In all cases a zinc-to-protein ratio of 7.0 ± 0.3 and a CysSH-to-protein ratio of 20 ± 2 were obtained.

**Reaction of Zn$_n$MT-3 with Cu$^{2+}$ followed by UV-visible and CD Spectroscopy**—Zn$_n$MT-3 samples (5 µM) were titered with increasing Cu$^{2+}$/protein ratios (from 0 to 10 equivalents) and incubated for 1 h at room temperature. Free and loosely bound metals were removed by the addition of Chelex 100 slurry (10 mg/ml) to samples, and the resin was removed by centrifugation (14,000 × g, 2.5 min). UV-visible spectra were recorded on a Cary 3 spectrophotometer (Varian Inc.). CD measurements were performed using a Jasco spectropolarimeter (model J-810), and the spectra were smoothed and expressed as molar ellipticity, [θ], in units of deg dmol$^{-1}$ cm$^{-2}$.

**Determination of Metal Binding Ratios in Cu,ZnMT-3 upon the Reaction with Cu$^{2+}$**—Samples for metal content determination were prepared as described for UV-visible and CD measurements. Sample aliquots were diluted 10-times into 15 mM HNO$_3$ and copper and zinc concentrations were determined by flame atomic absorption spectroscopy.

**Stability of Cu$_2$ZnMT-3 to Air followed by Size-exclusion Chromatography**—500-µl aliquots of Zn$_n$MT-3 and Cu$_2$ZnMT-3 samples (5 µM) were prepared as described for UV-visible and CD measurements. 200-µl aliquots were applied to a size-exclusion column (Superdex 75) connected with an Äkta chromatographic system (Amersham Biosciences). The protein was eluted with 10 mM Tris/HCl, 100 mM NaCl (pH 8.0) buffer at a flow rate of 1 ml/min. The samples...
were then incubated in air for 24 h at 25 °C, and the size-exclusion analysis was repeated. Protein peaks were integrated using the Unicorn version 4.00 software (Amersham Biosciences), and the elution volume and peak integrals were compared to assess that the species generated were stable to air.

**Cu(I) Luminescence Characterization of Cu,Zn-MT-3 Species at 77 K—**Luminescence spectra and emission decay lifetimes measurements of Cu,Zn-MT-3 species at 77 K were obtained using a SPEX FLUOROLOG spectrofluorometer equipped with a 1934C phosphorimeter accessory employing a 22.5° detection geometry. Measurements of the emission (excitation wavelength: 300 nm) and excitation (emission wavelengths: 425 nm and 565 nm) spectra were performed in quartz tubes, inner diameter of 2 mm, immersed in a cylindrical quartz Dewar filled with liquid nitrogen. Spectra were recorded at 77 K on microcrystalline frozen samples using a 55-µs delay and a 300-µs sample window. Lifetime measurements were performed on the same samples using 10-µs and 15-µs acquisition delay increments, 300- and 800-µs acquisition window for emissive bands at 425 nm and 565 nm, respectively.

**Nano-ESI-MS Characterization of Cu,Zn-MT-3 and ApoMT-3 Species upon Reaction of Zn7MT-3 with Cu2+**—To determine the mixed metalloforms of MT-3 obtained in the titration of Cu2+ into Zn7-MT-3 and the extent of thiolate oxidation in this process, nano-ESI-MS was performed at pH 7.5 and 2.5, respectively. At pH 7.5 both metal ions are bound to the protein, whereas at pH 2.5 the oxidized metal-free form obtained upon metal depletion was analyzed. The Zn7-MT-3 and Cu,Zn-MT-3 samples (100 µM protein) were prepared as described above (ESI-MS at pH 7.5). 100-µl samples were concentrated to a final volume of 25 µl using Microcon YM10 (cut-off: 10 kDa, Amicon, Millipore), and the buffer was exchanged by three washing cycles (3 × 500 µl) with 10 mM 4-ethylmorpholin buffer (pH 7.2) to remove NaCl contaminations and concentrated to a final concentration of ~120 µM. Immediately prior to ESI-MS analysis, protein samples were diluted into 5 mM ammonium acetate/acetonitrile/methanol/water (10:12.5:37.5:50, pH 7.5–8, ESI-MS at pH 2.5). To avoid any further oxidation of thiolate ligands during the generation of the metal-depleted samples, they were rendered oxygen-free by three freeze-pump-thaw cycles on a vacuum line, and all further steps were carried out in a nitrogen-purged glove box. Metals were removed by incubation of samples (100 µl) in 2 mM guanidinium hydrochloride and 1 M HCl for 15 min; they were then diluted to a final volume of 500 µl with 0.1 M HCl, exchanged into 0.1 M HCl by three washing cycles (3 × 500 µl) on Microcon YM3 (cut-off: 3 kDa, Amicon, Millipore), directly frozen in the glove box, and stored in liquid nitrogen. Immediately prior to ESI-MS analysis protein samples were diluted with acetic acid, pH 2.5. In all cases the samples were infused through a fused silica capillary (inner diameter, 75 µm) at a flow rate of 0.5 µl/min into a nano-ESI-MS quadrupole time of flight Ultima API mass spectrometer (Micromass). Electrospray Pico-TIPS (inner diameter, 30 µm) were obtained from New Objective (Woburn, MA). MS spectra were recorded in a positive mode at a capillary exit voltage of 2.1 kV, cone voltage of 50 V, and Rf lens energy of 50 V. Mass spectra were deconvoluted using MaxEnt-1 software (Micromass).

**Localization of the Cu(I)Thiolate Cluster in the Structure of Cu₆Zn₄MT-3 by Immunochemical Analysis**—Our previous studies using fully reduced protein established that Cu(I) ions are preferentially bound to the β-domain of the protein (23). Moreover, we could show that the α-domain of Zn₇-MT-3 is susceptible to digestion by subtilisin. Therefore, to obtain information as to the location of the Cu(I)₄-thiolate cluster in Cu₆Zn₄-MT-3 subtilisin digestion was performed. The solution of Cu₆Zn₄-MT-3 generated in the reaction with Cu²⁺ ions described above was digested by subtilisin from B. licheniformis at a 10:1 (w/w) ratio. The proteolytic digestion was performed under agitation at 37 °C for 24 h. Aliquots of the digested and not-digested proteins were analyzed by dot blot, and the immunochemical detection was performed with domain-specific antibodies. The antibodies were raised against short peptides of MT-3 with unique sequences specific for the α- and β-domain of human MT-3 (23). Partial protein digestion was confirmed by SDS-PAGE analysis of MT-3-bimane derivatives upon cysteine modification with monobromobimane (34). The presence of the Cu(I)-thiolate cluster in the undigested domain was confirmed by its characteristic luminescence spectrum at 77 K (see above).

**Determination of Copper-catalyzed Hydroxyl Radical Production Using 3-Coumarincarboxylic Acid**—Detection of the ascorbate-driven copper-catalyzed hydroxyl radical production has been monitored by fluorescence spectroscopy of the product of the reaction of 3-CCA with hydroxyl radicals, i.e. 7-hydroxy-3-coumarin carboxylic acid (7-OH-CCA, excitation wavelength: 395 nm; emission wavelength: 450 nm) (35). Hydroxyl radical production was measured upon the addition of ascorbate (300 µM) to a solution containing CuCl₂ (1 µM) or upon the reaction of CuCl₂ (1 µM) with Zn₇-MT-3 (250 nM) in the presence of 3-CCA (100 µM) in 20 mM phosphate buffer (pH 7.4). The formation of the product 7-OH-CCA was followed on a SPEX FLUOROLOG spectrofluorometer over a time period of 60 min.

**RESULTS**

**Formation and Stability of Cu₆Zn₄MT-3 in Air**—The thiolate ligands of mammalian MTs bind Zn(II) and Cu(I) ions with a high affinity (Cu(I) ≫ Zn(II)), forming metal-thiolate clusters. The absorption spectrum of Zn₇-MT-3 is characterized by a metal-induced shoulder at ~235 nm. In the first experiments, following the reaction of Cu²⁺ with Zn₇-MT-3 by absorption spectroscopy, we observed a decrease of the Zn₇-MT-3 absorption profile below 230 nm concomitant with a development of prominent shoulder at ~260 nm originating from CysS-Cu(I) LMCT transitions (23, 26). The latter indicates that Cu²⁺ reduction to Cu⁺ took place (see below). Inspection of the reaction kinetics obtained by monitoring the absorption at 262 nm revealed that, with a Cu²⁺/Zn₇-MT-3 ratio up to six, the reaction is completed already during the sample mixing. With more than 6 Cu(II) equivalents (up to 10) the initial fast phase was followed by a slow phase reaching a plateau in ~1 h (data not shown).

3 B. Roschitzki and M. Vasak, unpublished observation.
The Cu(I)-thiolate clusters are present in a number of proteins with different cellular functions like transcription factor Ace1 (36), Amt1 (37), and the intracellular domain of copper transporter Ctr1 (38). The Cu(I)-thiolate clusters in these proteins and in MT-1/-2, obtained upon the anaerobic titration of Ace1 (36), Amt1 (37), and the intracellular domain of copper transporter Ctr1 (38). The Cu(I)-thiolate clusters in these proteins and in MT-1/-2, obtained upon the anaerobic titration of Ace1 (36), Amt1 (37), and the intracellular domain of copper transporter Ctr1 (38).

Therefore, to learn more about the homogeneity and stability of the reaction products formed in the titration of Zn$_7$MT-3 with Cu$^{2+}$, the protein samples were studied by means of size-exclusion chromatography and absorption spectroscopy. In gel-filtration study the samples were analyzed after 1- and 24-h aerobic incubation in 10 mM Tris/HCl, pH 8.0, 100 mM NaCl. The elution profiles of the products were monitored at 220 nm (black line) and 250 nm (light gray line). For comparison the elution profile of Zn$_7$MT-3 monitored at the same wavelengths is also shown. The number of Cu$^{2+}$ equivalents added is indicated in each panel.

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Reactivity of Metallothionein-3 with Cu$^{2+}$

shows a family of absorption envelopes characteristic of Cu(I) binding to thiolate ligands at >250 nm. The plot of the intensity of the shoulder at 262 nm as a function of Cu$^{2+}$ equivalents added reveals that these features increase in intensity linearly with the first 8 Cu$^{2+}$ equivalents (Fig. 2, inset). At this stoichiometry, a clear break point is seen. With more than 8 Cu$^{2+}$ equivalents, the spectral changes characterized by an increasing absorption >275 nm and decreasing absorption <275 nm occurred. On standing in air these species showed a progressive decrease of the absorption profile between 240 and 400 nm with time consistent with a thiolate oxidation and consecutive metal release from the protein. This behavior is in agreement with the instability of these forms with time seen also in gel filtration (see above).

The development of the Cu(I)-induced spectral features was paralleled by the disappearance of CysS-Zn(II) LMCT absorption envelope in the high energy region of the spectra resulting in an isosbestic point at 230 nm. That this behavior indicates a Zn$^{2+}$ release from the Zn(II)-thiolate cluster(s) is supported by the metal determination. The atomic absorption data in Fig. 2 (inset) show that the binding of up to 8 Cu(I) equivalents is paralleled by the corresponding release of Zn$^{2+}$ ions. At this titration point nearly all Zn$^{2+}$ ions were released from the protein, and no additional copper binding occurred.

The corresponding CD spectra better document this binding process (Fig. 3). The CD spectrum of Zn$_7$MT-3 is characterized by a biphasic CD profile with bands at (+)1247 and (−)1228 nm with a crossover point at 239 nm. These CD features, which are observed in all mammalian Zn$_7$MTs, have been assigned to an excitonic splitting of the first CysS-Zn(II) LMCT transition at 235 nm in the cluster structure (31). With increasing Cu$^{2+}$ equivalents, and upon their reduction by the protein to Cu$^{+}$, new intense dichroic bands developed mainly in the low energy region of the spectra (above 240 nm). Upon the binding of the first 4 Cu(I) equivalents to the protein, a progressive increase of the CD profile characterized by two strong CD bands at (+)1255 and (−)285 nm and two isodichroic points at 242 and 276 nm was seen. The presence of isodichroic points indicates that two distinct species are concomitantly present in solution, i.e., Zn$_7$MT-3 and Cu$_4$Zn$_7$MT-3. This suggests that the first 4 Cu(I) equivalents are bound cooperatively to the protein.

Further Cu(I) binding introduces CD changes, which point to the development of new Cu(I)Zn$_7$MT-3 complexes. Thus, upon the binding of 5–6 Cu(I) equivalents the isodichroic point at 242 nm was abolished, the (+)1255 nm CD band underwent a 5 nm red shift, and the negative CD band at 285 nm increased in intensity. The binding of more than 6 Cu(I) equivalents resulted in a progressive minor red shift and a decreased magnitude of both CD bands at (+)255 and (−)285 nm. The plot of the intensity of the positive CD band at 254 nm as a function of Cu(I) equivalents better documents the binding process (Fig. 3, inset). A clear break point with the first 4 Cu(I) equivalents and the preservation of the corresponding CD profiles provides evidence for the cooperative formation of a Cu$_4$Zn$_7$MT-3 species. Evidence for the formation of Cu$_4$Zn$_7$MT-3 was obtained from the ESI-MS studies presented below. Overall, the absorption and CD studies showed that Zn$_7$MT-3 can reduce up to 8 Cu$^{2+}$ concomitantly with the Cu(I) binding into the protein structure. At this point it should be noted that throughout the Cu$^{2+}$ titration very weak dichroic bands developed above 300 nm, i.e., at (+)340, (−)390 nm, and a shoulder at ∼320 nm. The spectral origin of these bands is discussed in the next paragraph.

Organization of Cu(I) Binding Sites in Cu(I)$_4$Zn$_7$MT-3—Information regarding the Cu(I) binding sites and their interactions can be obtained from the analysis of the absorption and CD features of Cu(I)$_4$Zn$_7$MT-3 in the spectral range between 250 and 400 nm (Fig. 4). The observed spectral features are similar in shape and intensity to those reported for two Cu(I)$_{4(CysS)}_{6−7}$ clusters in Cu(I)$_n$MT-1, generated through filling of the apoprotein with Cu(I) ions under strictly anaerobic conditions (23). In these studies, a detailed interpretation of the origin of the underlying transitions was given. By analogy with these studies the intense shoulder at ∼260 nm with the corresponding CD band at ∼255 nm can be assigned to the first CysS-Cu(I) LMCT transition. Although the 285 nm CD band was not previously assigned, the corresponding intensity in the absorption spectrum suggests an electric dipole allowed transition. However, above 300 nm Cu(I)$_4$Zn$_7$MT-3 as well as Cu(I)$_8$MT-1 show CD bands occurring in the tailing of the absorption spectra. Whereas Cu(I)$_4$Zn$_7$MT-3 showed CD
bands at \(\sim (-)315, (+)340\) nm, and a very week CD band at \((-)390\) nm, Cu(I)\textsubscript{8}MT-1 showed CD bands at \((-)325\) and \((+)357\) nm. These bands occur at very low \(e\) values. The \((+340)\) nm band in Cu(I)\textsubscript{4}Zn\textsubscript{4}MT-3 has a dissymmetric factor \(g (\Delta e/e)\) of \(2.5 \times 10^{-3}\) indicating that this transition is magnetic dipole-allowed and electric dipole-forbidden (26). In mononuclear Cu(I)-halide complexes besides strong electric dipole-allowed charge-transfer bands, no other absorption bands at lower wavelength are observed, e.g. in the Cu(I)Cl\textsubscript{3} complex the lowest energy band occurs at 273 nm (41). However in polynuclear Cu(I) complexes, including the Cu(I)\textsubscript{8}MT-1 complex, which exhibit relatively short Cu...Cu distances (<2.8 Å) an intramolecular \(d^{10}-d^{10}\) interaction of adjacent Cu(I) ions leads to a number of excited states with a largely metal character. These weak low energy bands have been assigned to formally spin forbidden \(3d \rightarrow 4s\) metal cluster-centered (CC) transitions (42–45). Thus, the occurrence of similar transitions in Cu(I)\textsubscript{4}Zn\textsubscript{4}MT-3 suggest the presence of a Cu(I)\textsubscript{4}-thiolate cluster. The assignment of the low energy transitions as essentially singlet-triplet in nature is consistent with the phosphorescence spectra presented below.

Characterization of Cu(I)\textsubscript{4}Zn\textsubscript{4}MT-3 Species by Cu(I) Luminescence at 77 K—Low temperature luminescence proved to be a powerful tool in the structural investigation of Cu(I)-MT's because of the characteristic luminescence properties of the Cu(I)-thiolate clusters. The low temperature luminescence spectra of Cu(I)\textsubscript{4}Zn\textsubscript{4}MT-3 obtained upon the titration with up to 8 Cu(II) equivalents were recorded on microcrystalline, frozen solutions using a front face detection at 77 K. The representative emission and excitation spectra of Cu(I)\textsubscript{4}Zn\textsubscript{4}MT-3 obtained upon the reaction of 4 Cu(I) and 8 Cu(II) equivalents with Zn\textsubscript{7}MT-3. Emission lifetimes of both emissive bands in Cu(I)\textsubscript{4}Zn\textsubscript{4}MT-3 were obtained from the decay fit (inset). Spectra were recorded at 77 K using MT-3 samples (18 \(\mu\)M) in 20 mM Tris/HCl, 100 mM NaCl, pH 8.0. The emission spectra were recorded with excitation at 300 nm and the excitation spectra using the emissive bands at 565 nm (dashed line) and 425 nm (full line).
The observation of two emissive bands for a Cu(I)$_4$-thiolate cluster in Cu(I)$_4$Zn$_4$MT-3 is striking. The luminescence properties of inorganic tetra- and hexanuclear Cu(I) clusters and similar clusters in Cu(I)-MTs have been reported (26, 43, 44, 46). Although the Cu(I)$_6$ clusters exhibit only a single low energy band at ~700 nm, high and low energy bands at ~430 and 620 nm are observed in the luminescence spectra of Cu(I)$_4$ clusters. For the latter species two distinct triplet-excited state manifolds have been suggested. One of which, referred to as “cluster centered” (CC) is delocalized over the metal core with contributions from both d-s and charge-transfer components, whereas the other is mainly of charge-transfer character. The presence of two emissive bands in Cu(I)$_4$ clusters has been correlated with the intranuclear Cu...Cu distances in these clusters (<2.8 Å) (43). However, in Cu(I)$_6$ clusters with larger Cu...Cu distances (>2.8 Å) only one emissive band at low energy was detected. In this case, the CC emission is less likely to be observed. Moreover, the charge-transfer emissions are dependent on the nature of the coordinating ligand but are unaffected by intranuclear Cu...Cu distances. Accordingly, the high energy emissive band in Cu(I)$_4$Zn$_4$MT-3 has been assigned to $^3$CC origin and that at low energy to $^3$LMCT. This assignment is supported by the obtained excitation spectra of Cu(I)$_4$Zn$_4$MT-3 monitored at the maxima of the respective emissive bands (Fig. 5). The excitation spectrum of Cu(I)$_4$Zn$_4$MT-3 monitored at 565 nm emission wavelength shows an unresolved excitation envelope starting around 350 nm and extending to the high energy region suggests that these features are predominantly of LMCT origin. However, the excitation spectrum monitored at 425 nm emission wavelength show two partially resolved bands at ~265 and 300 nm. The correspondence of the 265 nm excitation maximum with the position of the first CysS-Cu(I) LMCT transition and that at 300 nm with the cluster-centered transition in the absorption spectrum strongly suggests its $^3$CC origin with contributions from both d-s and charge-transfer components (26, 43, 47). Thus, the luminescence properties of Cu(I)$_4$Zn$_4$MT-3 are in accordance with a Cu(I)$_4$-thiolate cluster being present in this species.

**Nano-ESI-MS Characterization of Cu,ZnMT-3 and ApoMT-3**—The nano-ESI-MS characterization of the metalloforms formed upon the titration of Zn$_n$MT-3 with Cu$^{2+}$ and their metal composition at pH 7.5 is shown in Fig. 6 (right panels). The results of the first four titration steps reveal the progressive disappearance of the mass peak of Zn$_n$MT-3 (7368 Da) concomitant with the occurrence of the mass peak of the Cu$_4$Zn$_4$MT-3 species (7424 Da), confirming its cooperative formation. At higher Cu$^{2+}$/protein stoichiometries, the simultaneous presence of a number of different mass peaks was detected (~5 major mass peaks) (data not shown).

The oxygen-dependent redox cycling of copper in the Fenton-type Haber-Weiss reaction is responsible for the metal-catalyzed production of ROS leading to the oxidation of cysteine thiolate ligands. In this process disulfides or higher oxidation states of sulfur are formed. For example, the oxygen- and time-dependent cysteine sulfur oxidation to disulfide, sulphenic acid, and sulfonic acid has been observed by x-ray photoelectron spectroscopy of Cu(I)$_6$-MT isolated from yeast (39). To determine the sulfur oxidation state we have performed nano-ESI-MS analysis of the apoprotein (pH 2.5) upon the metal depletion of the Cu(I)$_4$Zn$_4$MT-3 form. Due to the high binding affinity of Cu(I) to cysteine-thiolate...
ligands a special treatment of samples was required to achieve its release prior to nano-ESI-MS analysis (see “Experimental Procedures”). To prevent adventitious sulfur oxidation in the process of metal release, all preparation steps have been performed in a nitrogen-purged glove box. The ESI-MS spectrum of the fully reduced apoprotein (Fig. 6, left panels) shows a mass peak at 6925.9 Da. The comparison of ESI-MS spectra of the apoprotein obtained upon the metal removal from Cu(I)ZnMT-3 species containing up to 3 Cu(I) equivalents bound (composed of an increasing ratio of Cu(I)\(_2\)Zn\(_4\)MT-3 to Zn\(_4\)MT-3) and from Cu(I)\(_4\)Zn\(_4\)MT-3 showed a small progressive mass shift to lower masses. The obtained mass of 6920.9 Da for the latter species suggests the presence of two intramolecular disulfide bonds in the Cu(I)\(_4\)Zn\(_4\)MT-3 structure. In this context it may be noted that common colorimetric assays for thiolate determination are redox-sensitive and, therefore, could not be used for an indirect quantification of disulfide content. However, the resolution of the ESI-MS measurements at pH 2.5 (indirect quantification of disulfide content). However, the obtained MS data rule out the MT-3 species containing sulfur in higher oxidation states. This indicates that the source of electrons for Cu\(_{2+}\) reduction is furnished by CysS ligands in a process concomitant with the formation of disulfide bridges. However, their location in the protein structure is currently unknown. The obtained data do not provide a deeper insight as to the disulfide formation process, in particular, whether their formation occurs cooperatively together with that of the Cu\(_{4+}\)-thiolate cluster. However, in the course of the formation of the final species Cu\(_2\)Zn\(_4\)MT-3 at pH 7.5 a progressive shift to lower masses of ~4 Da was observed. This behavior would be consistent with a model in which Cu(II) binding and its reduction would be a kinetically controlled process resulting in a non-cooperative formation of disulfide bonds. This would imply that, upon the Cu(II) reduction, a rearrangement of Cu(I) to form a thermodynamically stable Cu\(_{4+}\)-thiolate cluster occurs.

**Localization of the Cu(I) Cluster-containing Domain**—To identify the domain localization of the Cu\(_{4+}\)-thiolate cluster in Cu\(_2\)Zn\(_4\)MT-3, a subtilisin digestion of the protein was performed followed by low temperature luminescence measurements of the digested product(s). The presence of the intact Cu\(_{4+}\)-thiolate cluster in the digestion product is evident from the conserved luminescence features of this cluster seen also with Cu\(_2\)Zn\(_4\)MT-3, i.e., luminescence bands at 425 and 565 nm (see Figs. 5A and Fig. 7). In Fig. 7, inset, the immunoenzyme dot-blot analysis of the partial digested Cu\(_{4+}\)Zn\(_4\)MT-3 using domain-specific antibodies is presented. The results show that, although the \(\alpha\)-domain was digested, the \(\beta\)-domain remained intact. Therefore, the \(\beta\)-domain of the protein harbors the Cu\(_{4+}\)-thiolate cluster.

**Determination of Copper-catalyzed Hydroxyl Radical Production**—In the presence of transition metals, which can undergo a redox cycling like Cu\(_{2+}\) ions, ascorbate can act as a source of electrons for the reduction of molecular oxygen with a concomitant production of ROS species. It has been demonstrated that Cu(II)-catalyzed oxidation of ascorbate (HA\(^-\)) to dihydroascorbate (DHA) occurs through the formation of a Cu(II)-ascorbate complex, which binds molecular oxygen. In this complex Cu(II) mediates the electron transfer from ascorbate to the oxygen molecule in a series of reactions that leads to the production of peroxyl radical (HO\(_2\)), superoxide anion radical (O\(_2\)\(^-\)), and hydrogen peroxide (H\(_2\)O\(_2\)). These reactions can be summarized as follows (35).

\[
\begin{align*}
\text{HA}^- + \text{Cu}^{2+} + \text{O}_2 &\rightarrow \text{HA}^+ + \text{Cu}^{2+} + \text{HO}_2^- \quad \text{REACTION 1} \\
\text{HA}^+ + \text{Cu}^{2+} &\rightarrow \text{DHA} + \text{Cu}^+ \quad \text{REACTION 2} \\
\text{HA}^+ + \text{O}_2 &\rightarrow \text{DHA} + \text{O}_2^- \quad \text{REACTION 3} \\
\text{Cu}^+ + \text{HO}_2^- + \text{H}^+ &\rightarrow \text{Cu}^{2+} + \text{H}_2\text{O}_2 \quad \text{REACTION 4} \\
\text{Cu}^+ + \text{H}_2\text{O}_2 &\rightarrow \text{Cu}^{2+} + \text{OH}^- + \text{OH}^+ \quad \text{REACTION 5}
\end{align*}
\]

In these reactions Cu\(_{2+}\) ion is reduced to Cu\(^+\) and oxidized back in a Fenton-type of reaction, which represents the source of 1 electron equivalent required for the reduction of hydrogen peroxide. The final product of these reactions is the hydroxyl radical (OH\(^\cdot\)) (48, 49). 3-CCA has often been used to detect hydroxyl radicals. This compound through a specific OH\(^\cdot\) addition leads to the predominant formation of the fluorescent derivative 7-OH-CCA. Because of the non-fluorescent nature of 3-CCA and its high hydroxylation rate constant, the fluorescent detection of 7-OH-CCA enables real-time measurements of the kinetics of hydroxyl radical generation (35).

In our studies the generation of hydroxyl radicals in the reac-
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The concentration of Zn$_7$MT-3 with Cu$^{2+}$ was studied in phosphate buffer to avoid partial quenching of hydroxyl radicals by common organic buffers like Tris or Hepes. The ascorbate concentration (300 μM) is similar to that reported to be present in the cerebrospinal fluid and in the extracellular space of the brain (11). As a blank this concentration was added to the solution containing 1 μM CuCl$_2$ in the presence of 3-CCA (0.1 mM). The observed hydroxyl radical production was characterized by an almost linear increase of the fluorescence signal of 7-OH-CCA at 450 nm (Fig. 8). However, the same experiments performed with increasing Cu$^{2+}$/Zn$_7$MT-3 ratios (up to 4) resulted in a complete quenching of the hydroxyl radical production. It is well established that thiolate ligands in MTs are a highly efficient scavenger of free radicals. In this process, the thiolate ligands are oxidized to disulfides concomitant with metal release (50–53). Therefore, the stability of Cu(I)$_5$Zn$_4$MT-3 under the same conditions was also assessed by monitoring the intensity of Cys$_2$-Zn(II)/Cys$_2$-Cu(I) LMCT transitions at 230 nm in the absorption spectrum. The preservation of the absorption intensity with time indicates that no metal release occurred. Overall, the demonstrated reduction of Cu$^{2+}$ ions to Cu$^+$ by the protein and their concomitant binding into the N-terminal domain of Cu(I)$_5$Zn$_4$MT-3 results in a “redox-silent” Cu(I)$_4$-thiolate cluster that is stable toward ascorbate-induced copper-catalyzed redox cycling thus preventing the ROS production from molecular oxygen.

**DISCUSSION**

Copper is an essential element needed for a number of essential enzymes, but the ion is potentially toxic, because it is a potent generator of free radicals. Several lines of evidence support the concept that the copper-induced cellular toxicity is strictly linked to the participation of copper in the formation of ROS (54). Although in intracellular space the “free pool” of copper is tightly regulated and reported to be lower than 1 atom per cell (55), in the extracellular space and under conditions of dysregulated homeostasis, concentrations of free and loosely bound copper are expected to be significantly increased leading to extra- and intracellular oxidative stress. Dysregulated copper homeostasis and copper-mediated oxidative stress have been suggested to play a role in a number of metal-linked degenerative disorders such as AD (3), Parkinson disease (5), prion diseases (6), and in amyotrophic lateral sclerosis (56).

The ROS generation through redox cycling of copper requires its reduction during this process. This can be accomplished by biological components such as ascorbate, glutathione, dopamine, and cholesterol to name just a few. The central nervous system has the highest concentration of ascorbate of all tissues. Ascorbate is actively transported from plasma into cerebrospinal fluid where it equilibrates with the brain extracellular fluid to a concentration of 200–400 μM and is transported further into neurons where it accumulates to a concentration of ~10 mM (11). The reduction of Cu$^{2+}$ ions to Cu$^+$ in the presence of oxygen catalyzes the production of ROS such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH) through Fenton-type reactions (49, 54). The ROS production leads then to DNA base oxidation and strand brakes (57), lipid peroxidation (58, 59), and oxidative modification of proteins. The occurrence of these oxidative stress markers, together with altered energy metabolism, increased production of advanced glycosylation end product, aldehydes, carbonyls, and peroxynitrite, has been reported to occur in AD (60).

In the central nervous system, the natural metal chelator MT-3 represents one of the major players in copper and zinc homeostasis. This protein occurs intra- and extracellularly and was found down-regulated in AD (13–16, 17). Although the MT-3 role in zinc homeostasis in zinc-enriched neurons is slowly emerging, its role in extracellular space is poorly understood. In neuronal cultures the protein, by an unknown mechanism, rescues the cells from the toxicity of Aβ peptides (61), which has been linked to abnormal binding of metals like Cu(II) and Zn(II). In the present work we have addressed the question as to the reactivity of Zn(II)-thiolate clusters in Zn$_7$MT-3 with free Cu$^{2+}$ ions. The Zn(II) coordination appears to be essential in stabilizing thiolate groups in their reduced state. However, at the same time, the charge distribution in the Zn(II)-SCys bond leads to a significant degree of nucleophilicity of thiolate sulfurs, which facilitates the Cu$^{2+}$ reduction. We have shown that Zn$_7$MT-3 reduces Cu$^{2+}$ to Cu$^+$ concomitant with Cu(I) binding and the generation of an oxygen stable Cu(I)$_4$-thiolate cluster in a cooperative manner together with the formation of two disulfide bridges in the Cu(I)$_5$Zn$_4$MT-3 structure. This is in agreement with the reduction of four Cu$^{2+}$ ions. The Cu$^{2+}$ reduction and binding were also observed with higher Cu$^{2+}$/protein stoichiometries, but these metalloforms are oxygen-sensitive and undergo a copper-catalyzed protein oxidation. Moreover, we showed that the formation of an oxygen-stable Cu(I)$_4$-thiolate cluster results in a complete quenching of the ascorbate-induced copper-catalyzed hydroxyl radical production. This suggests that free Cu$^{2+}$ ions can be efficiently “redox-silenced” through their reduction and binding to MT-3. It is well documented that mammalian M$^{10}$MTs, including MT-3, protect the cells from free radical-induced toxicity (15). In this process the free radical attack occurs at metal-bound thiolates
leading to the protein oxidation and/or modification concomitant with metal release (50–53). However, in contrast to the free radical scavenging effect of MTs, the reaction of Zn7MT-3 with Cu2+ occurs in a well defined process. The demonstrated inhibition of hydroxyl radical production by free Cu2+ ions through their redox silencing in the protein structure represents a novel protection mechanism.

The obtained results are closely related to the properties of the MT-3 structure and its reactivity. The preferential binding of Cu(I) to the β-domain of MT-3 may originate from its structural property enabling an accommodation of the digonally and/or trigonally coordinated Cu(I) ions. The NMR structure of MT-3 with divalent metal ion reveals that the 3-metal cluster in the β-domain, which can be approximated to a cyclohexane ring, is structurally less constrained and more flexible compared with the adamantane-like 4-metal cluster in the α-domain (31, 62, 63). It has been shown, moreover, that the conserved T2CPCP2 motif in the β-domain of MT-3 is important for increased dynamics of this domain (64, 65). Therefore, the accessibility of thiolate ligands to electrophilic attack could play an important role in preferential reactivity of the β-domain with Cu2+. However, the stability of the Cu(I)4-thiolate cluster in air oxygen is striking and not understood. We suggest that the structural constraints and the short Cu(I)...Cu(I) distances (<2.8 Å) in the Cu(I)4-thiolate cluster, leading to peculiar metal-metal interactions, may be important for its stability.

In the structure of Cu(I)4Zn4MT-3 two disulfide bridges are present. Although the location of both disulfides in the protein structure is currently unknown, we favor the β-domain for the following reason. Because the Cu(I)4-thiolate cluster in Cu(I)4Zn4MT-3 is located in the β-domain, the remaining 4 Zn(II) ions ought to be bound to the α-domain. It is expected that the formation of disulfides in this domain would substantially destabilize the 4-metal cluster resulting in metal release. The obtained data do not exclude a direct involvement of disulfide sulfur(s) in Cu(I) coordination. In inorganic model complexes a direct Cu(I) coordination by a disulfide bridge sulfur has been reported (66, 67). The presence of two disulfides in the β-domain, leaving five reduced free thiolate ligands, would be sufficient to coordinate 4 Cu(I) ions through terminal and bridging sulfurs in digonal or trigonal coordination.

Increasing experimental evidence supports a critical role of abnormal Cu(II)-protein interactions in neurodegenerative diseases. The Cu(II) binding to β-amyloid in AD, to α-synuclein in Parkinson disease, and to the prion protein and the production of ROS has been reported to play a significant role in the progression of these diseases (6, 10). In all these metal-linked neurological disorders the down-regulation of MT-3 has been reported (16, 30). Thus, MT-3 may play a so far unrecognized role in brain pathology. The potentially protective mechanism of Zn7MT-3 from Cu(II)-mediated toxicity in the brain under physiological and pathological conditions may not be limited to the reduction of free Cu2+ ions and their binding to the protein. The protein may also, through Cu(II) removal and reduction to Cu(I), control abnormal Cu(II)-protein interactions and free radicals production described for these neurodegenerative diseases. The release of redox-inert Zn2+ from Zn7MT-3 during this process and its subsequent binding to such binding site(s) may represent another protective effect. Studies currently in progress in our laboratories are directed toward investigation of the reactivity of Aβ-Cu(II) complexes with Zn7MT-3 to understand at a molecular level the mechanism underlying the protective effect of Zn7MT-3 from Aβ toxicity observed in neuronal cell cultures (61). These studies may provide the basis for a common protective role of MT-3 in different Cu2+-linked neurological disorders.

Acknowledgments—We thank Audrey Gillet and Tamara Delaïne for preliminary experiments. We also thank Dr. S. Chesnov (Functional Genomic Center, Zürich, Switzerland) for recording nano-ESI-MS spectra.

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