Bioactivity of Metallothionein-3 Correlates with Its Novel β Domain Sequence Rather Than Metal Binding Properties†

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Received October 19, 1994; Revised Manuscript Received January 9, 1995[⊗]

ABSTRACT: Human and mouse metallothionein-3 (MT-3) molecules exhibit the same metal binding stoichiometry with Zn(II), Cd(II), or Cu(I) as MT-1 or MT-2 molecules, suggesting that MT-3 consists of two domains enfolding separate polymetallic clusters. The kinetic reactivities of Zn(II) complexes of MT-3 with the chelator ethylenediaminetetraacetic acid (EDTA) or the thiol reagent dithiobis(2-nitrobenzoic acid) (DTNB) resembles the reactivity of ZnMT-1. Furthermore, the candidate α and β domain peptides of human MT-3 are very similar to MT-1 domain peptides in the reactivity of Zn(II) complexes. Zn(II) complexes of human and mouse MT-3 inhibit the survival of rat cortical neurons cultured in the presence of an Alzheimer's disease brain extract. Inhibitory activity is unique to the MT-3 isoform and is a property of the N-terminal β domain. The inhibitory activity of the 32-residue MT-3 β domain is abolished by a double mutation within the β domain resulting in the conversion of the C-P-C-P sequence to either C-S-C-A or C-T-C-T. Thus, the bioactivity arises from a novel structure of the N-terminal β domain of MT-3 and not any unusual metal-binding properties.

Brain extracts from Alzheimer's disease (AD) patients contain enhanced neurotrophic activities that promote cortical neuron survival and dendrite outgrowth (Uchida et al., 1988, 1991; Uchida & Tomogaga, 1989; Erickson et al., 1994). Uchida et al. (1991) purified a novel metallothionein (MT) from normal brain tissue that inhibited the neurotrophic activities of Alzheimer's disease cortical extract. Although this MT, designated growth inhibitory factor (GIF), is 70% identical to MTs found in a variety of tissues, MT molecules from horse kidney, rabbit liver, and rat liver failed to exhibit growth inhibitory activity (Uchida et al., 1991; Erickson et al., 1994). An antipeptide antibody generated to GIF adsorbed GIF activity from the normal brain extract, thereby proving that inhibitory activity was due to GIF (Uchida et al., 1991).

The GIF gene has been cloned from the human, mouse, and rat (Palmiter et al., 1992; Tsuji et al., 1992; Kobayashi et al., 1993). The homology of GIF to the MT family of proteins prompted the redesignation of GIF as MT-3 (Palmiter et al., 1992). The bioassay, specific for the MT-3 class of MT isoforms, is the first assay capable of discriminating between MT isoforms. Thus, MT-3 is the only known MT to exhibit an activity that may be distinct from metal ion buffering.

Quantitation of MT-3 protein by immunoblotting and of MT-3 mRNA by Northern analysis revealed that MT-3 levels were depressed in Alzheimer's disease brain (Uchida et al., 1991; Tsuji et al., 1992). As Alzheimer's brain tissue is characterized by a proliferation of neurofibrillary tangles and curly fibers (Katzman, 1986; Muller-Hill & Beyreuther, 1989), the intriguing possibility was raised that abnormally low concentrations of MT-3 in Alzheimer's brain tissue may be responsible for excessive dendritic sprouting and subsequent neuronal degeneration (Uchida et al., 1991; Tsuji et al., 1992; Uchida, 1993). However, the correlation between reduced MT-3 levels and AD has not been confirmed by our studies (Erickson et al., 1994).

Human and mouse MT-3 resemble other mammalian MTs in containing 20 cysteines in conserved Cys-X-Cys and Cys-X_2-Cys sequence motifs. An alignment of sequences of MT-3 and known MTs reveals two insertions in MT-3 resulting in a polypeptide length of 68 residues as opposed to 61 or 62 residues in the well-characterized mammalian MT-1 and MT-2 isoforms (Kagi, 1993). A one-residue insertion occurs in a region corresponding to the N-terminal β domain of MT-2 and a six-residue insertion is present in a sequence corresponding to the C-terminal α domain (Uchida et al., 1991; Palmiter et al., 1992). Single-residue insertions within the N-terminal β domain occur also in rabbit and avian MT sequences and mouse MT-4 (Kagi, 1993; Quaife et al., 1994). The MT-3 β domain insertion occurs after residue 4, whereas the insertions in rabbit MT-2a and MT-2c sequences and in chicken MT are present after residues 9 and 3, respectively (Kagi, 1993). The six-residue α insertion in MT-3 is present within the only extended loop of the MT-2 structure (Robbins et al., 1991) and its sequence is not well conserved.

We reported previously that the addition of an Alzheimer's disease brain extract to a primary culture of neonatal rat cortical neurons resulted in prolonged neuronal survival that

† This work was supported by Grant ES 03817 from the National Institute of Environmental Health Sciences, National Institutes of Health, to D.R.W. and by Grant CA 61268 to R.D.P. We acknowledge support from the National Institutes of Health (5P30-CA 42014) to the Biotechnology Core Facility at the University of Utah for polypeptide and oligonucleotide syntheses and the assistance of Dr. R. W. Schackmann. Support is also acknowledged to the Utah Regional Cancer Center (Grant 5P30 CA42401) for support of the Electrospray Mass Spectrometry Core Facility and the assistance of Dr. J. Peltier.

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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1995.

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                1         10        20        30        40        50        60
  hMT-3  MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ
  mMT-3  MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAEAEKCSCCQ
β-hMT-3  MDPETCPCPSGGSCTCADSCKCEGCKCTSCKK
α-hMT-3                                  MSCCSCCPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ
  rMT-1  MDPN CSCSTGGSCTCSSSCGCKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKGA      SDKCTCCA
  rMT-2  MDPN CSCATDSGCSCAGSCKCKQCKCTSCKKSCCSCCPVGCAKCSQGCICKEA      SDKCSCCA
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FIGURE 1: Comparison of MT sequences used in these studies.

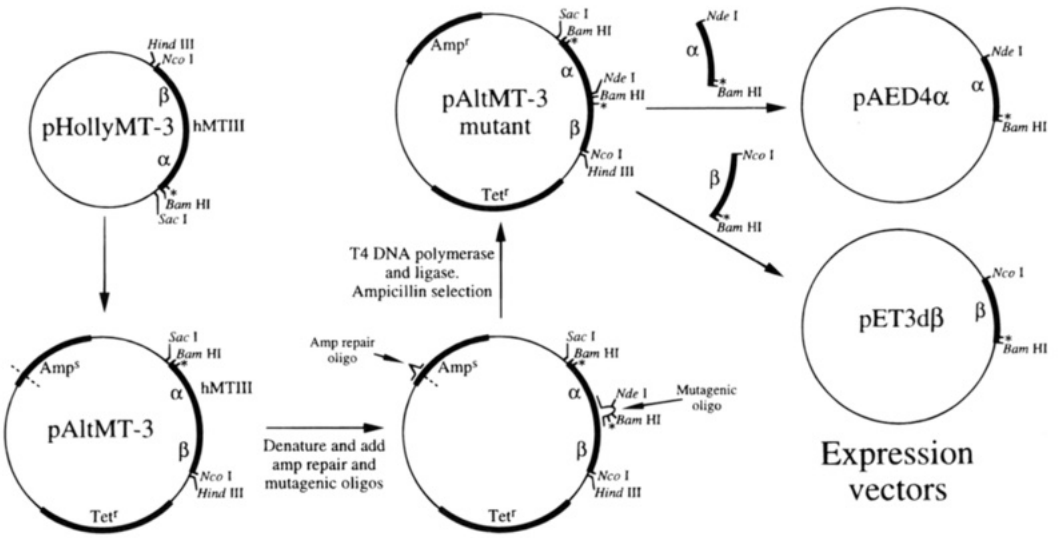

FIGURE 2: Construction of expression vectors for human MT-3 α and β peptides. Details of the construction are presented in Experimental Procedures.

could be antagonized by the addition of purified ZnMT-3 but not ZnMT-1 (Erickson et al., 1994). To elucidate the basis of the novel ability of MT-3 to inhibit the neurotrophic activity of AD brain extract on cultured rat neurons, we purified human and mouse MT-3 molecules as well as human MT-3 domain peptides for analyses of activity and metal binding properties. The inhibitory activity of MT-3 may arise from its metal binding properties or its structure. A structural contribution may arise from a unique tertiary fold or a specific MT-3 sequence. A comparison of the two MT-3 sequences and rat MT-1 is presented in Figure 1. We report that inhibitory activity maps to the N-terminal β domain and is dependent upon two prolyl residues in this domain.

EXPERIMENTAL PROCEDURES

Isolation of MT-3. Human MT-3 was isolated from bacteria after expression of the *MT-3* gene from a pET vector as described previously (Erickson et al., 1994). Mouse MT-3 cDNA (Palmiter et al., 1992) was amplified by polymerase chain reaction (PCR) with oligonucleotides mMT3-1 (5′ GCT GCT GAA CTG GCC ATG GAC CCT GAG ACC TGC CCC 3′) and mMT3-2 (5′ CGC GGA TCC TCA CTG GCA GCA GCT GCA TTT CTC 3′). The resulting product was cleaved with *Nco*I and *Bam*HI and subcloned into pET3d (Novagen) for expression in *Escherichia coli*. The sequence of the MT-3 gene was verified by nucleotide sequencing using Sequenase (U.S. Biochemical Corp.). Purification of mouse MT-3 was carried out as described for human MT-3 (Erickson et al., 1994). Purity was verified by amino acid analysis and by analytical C_{18} reverse-phase HPLC. Amino acid analysis was performed on a Beckman 6300 analyzer following hydrolysis of samples with 5.7 N HCl at 110 °C *in vacuo*. The elution of MT-3 proteins on C_{18} HPLC was achieved with a linear gradient of 0−60% acetonitrile. MT-3 proteins from the human and mouse eluted at 37.8% and 36% acetonitrile, respectively. Masses of human and mouse MT-3 polypeptides were determined by electrospray mass spectrometry on a Fisons Instruments Trio 2000 spectrometer (VG Biotech). The instrument data system used the LAB BASE software provided by the manufacturer. Typical instrument conditions operating conditions were as follows: probe potential, 2.3 kV; counterelectrode potential, 0.25 kV; and sampling cone potential, 46 V. The *m/z* range from 700 to 1700 was scanned at a rate of 10 s/scan, and 10−25 scans were summed to obtain each spectrum. After acquisition, the spectra were smoothed and baseline-subtracted. Transformed spectra were obtained using the subroutine provided with LAB BASE software. Apoprotein samples in 10% glacial acetic acid were infused into the mass spectrometer at a rate of 4−6 μL/min using a Harvard syringe pump to obtain the mass of the metal-free polypeptides.

Construction of Expression Vectors for Human MT-3 α and β Domain Peptides. Human MT-3 cDNA was prepared from cells by reverse transcription followed by PCR. It was cloned into vector pHolly as an *Nco*I/*Bam*HI fragment. The gene was excised as a *Sac*I/*Hin*dIII fragment and subcloned into pAlter-1 (Promega) to create vector pAltMT3 (Figure 2). A double mutation was created in the vector by using two oligonucleotides: one to repair a mutation in the Amp^r gene, the second to create the desired mutation in the MT-3 gene as outlined in Promega's Altered Sites Mutagenesis system. The ampicillin repair oligonucleotide (Promega) and the mutagenic oligonucleotide (5′ GGG CAG CAG GAG CAG CAG CTC ATA TGG ATC CTA CTT CTT GCA GGA GGT GC 3′) were annealed to the heat-denatured circular plasmid and a second strand was synthesized with

T4 DNA polymerase. After ligation with T4 DNA ligase, the resulting DNA was transformed into *E. coli* BMH71-18 *mutS* (Promega). Repaired plasmids were selected for ampicillin resistance. Restriction mapping and sequencing confirmed the inclusion of the mutagenic sequence in 30% of the clones. The mutagenic oligonucleotide added a stop codon after sequences encoding the N-terminal β domain followed by a *Bam*HI site and an ATG start codon within an *Nde*I site (CATATG) in frame with the stop codon already present at the end of the sequence encoding the C-terminal α domain. The MT-3 β domain DNA was removed as an *Nco*I/*Bam*HI fragment and subcloned into pET3d (13) (Novagen) for expression in *E. coli*. DNA encoding the α domain was excised as an *Nde*I/*Bam*HI fragment and cloned into pAED4,[1] a T7-based expression plasmid related to pET-3a (Studier et al., 1990). The constructs should result in the expression of a 32-residue N-terminal β domain peptide and a 37-residue C-terminal α domain peptide. The only sequence change is the addition of an initiator methionine prior to Ser_{33}.

Isolation of Human MT-3 Domain Peptides. The cloned genes for the domain peptides were expressed in *E. coli* strain BL21 (pLysS). Cells at OD_{600nm} of 0.5 were induced with 0.4 mM IPTG for 30 min prior to the addition of 0.4 mM $CdSO_4$. Cells were harvested after an additional 2.5 h as described previously (Erickson et al., 1994). The purification protocol described previously (Erickson et al., 1994) was altered for the purification of MT-3 α peptides. After chromatography on DEAE-cellulose, the Cd(II)-containing fractions were concentrated by partial lyophilization followed by gel filtration on Sephadex G-75 (5 × 90 cm) equilibrated in 20 mM Tris-HCl, pH 8. The Cd-containing α peptides were concentrated by lyophilization and further purified by C_{18} reverse-phase HPLC at pH 2 using a 0−60% acetonitrile gradient. The α peptide eluted at 39% acetonitrile.

The β domain peptide of residues 1−32 was synthesized by standard Fmoc chemistry on an ABI 431A instrument. A 0.25-mmol synthesis was carried out using cleavage conditions recommended by the manufacturer. Only one predominant product was recovered after cleavage. Purification of the β peptide was accomplished by two successive C_{18} reverse-phase HPLC runs on preparative and semipreparative columns. The β peptide eluted at 29.5% acetonitrile. Two 32-residue mutant β peptides were synthesized with either P7T,P9T or P7S,P9A double substitutions using standard Fmoc chemistry. In repeated syntheses of the P7S,P9A mutant, multiple products were recovered with deletions in the sequence. We were unable to isolate adequate quantities of the mutant for biochemical studies, so the P7T,P9T mutant peptide was synthesized. The correct product was isolated from a less complex product mixture. Electrospray MS of the purified peptide was used to verify the existence of the double mutation.

Domain peptides from rat MT-1 and MT-2 were isolated by limited proteolysis as described previously (Winge, 1991).

Protein and Metal Analyses. Protein samples were quantified by amino acid analysis following acid hydrolysis and quantitation of sulfhydryl groups using dithiodipyridine (Grassetti & Murray, 1967). Atomic absorption analysis was carried out on a Perkin-Elmer 305 spectrometer. Apoproteins were prepared by gel filtration on Sephadex G-25 equilibrated with 0.02 N HCl. Metal ion reconstitution was performed by mixing apoproteins in 0.02 N HCl with a set molar equivalency of Zn(II), Cd(II), or Cu(I) followed by neutralization with Tris base to pH 7.6. The concentration of metal stock solutions was verified by atomic absorption spectroscopy. Cu(I) was stabilized as Cu(I)-acetonitrile (Hemmerich & Sigwart, 1963) and the complex was stored anaerobically at −20 °C. For the preparation of mixed Cu(I),Zn(II) MT samples, 6 mol equiv of Cu(I) was added to preformed Zn_7MT anaerobically and the mixture was desalted to isolate CuZnMT complexes.

Ultraviolet absorption spectroscopy was carried out on a Beckman DU spectrophotometer. Cu(I) luminescence measurements were performed on a Perkin-Elmer 650-10S fluorometer with a band-pass filter as described previously (Byrd et al., 1986). Excitation was at 300 nm and the emission peak occurred at 570 nm.

Kinetic Reactivity. The kinetic reactivity of metal complexes of MT-3 and its domain peptides was carried out using DTNB and EDTA (Savas et al., 1991; Shaw et al., 1991).

Neuron Survival Assay. Neonatal rat cortical cell cultures were prepared and maintained as previously described (Erickson et al., 1994). One hour after cells were plated into serum-free, defined medium (MEM N2), cultures were supplemented with one of the following: AD brain extract (240 μg of protein/mL), purified MT (or MT domain peptides) dissolved in sterile phosphate-buffered saline (PBS), or both AD brain extract and MT. The quantity of MT added was restricted to <20 μg/mL to avoid precipitation in the mixtures. The number of neurons surviving after 3 days was quantitated by microscopic examination of four random fields, using immunocytochemically validated morphological criteria (Erickson et al., 1994). Brain extract was freshly prepared as previously described (Erickson et al., 1994) from the frontal cortex of a brain fulfilling all criteria for the diagnosis of Alzheimer's disease. This brain (AD3) had 50% of the normal concentration of MT-3 and contained enhanced neurotrophic activities (Erickson et al., 1994). Although this sample had reduced levels of MT-3, our previous study did not reveal a general correlation between Alzheimer's disease and low levels of MT-3. The same frontal cortex was used for all experiments.

RESULTS

Characterization of Metal Binding Sites in MT-3. Human and mouse MT-3 molecules were produced in *E. coli* by expression of the genes from a T7-based expression vector. Bacterial cultures were induced with IPTG in the presence of 0.4 mM $CdSO_4$. MT-3 proteins were purified from bacterial extracts as described in Experimental Procedures. The proteins were shown to be homogenous by the appearance of a single symmetrical elution peak on C_{18} reverse-phase HPLC and the expected amino acid composition. The molecular masses of purified human and mouse metal-depleted MT-3 molecules were determined to be 6925.4 and 7007.2, compared to predicted masses of 6926.1 and 7007.3, respectively. The close correlation of the observed and predicted masses indicate that both pure proteins retain the unblocked N-terminal Met residue.

The production of MT-3 molecules in bacteria cultured in the presence of $CdSO_4$ resulted in the isolation of CdMT-3

[1] D. Doering and P. Matsudaira, unpublished results.

Table 1: Metal Binding Stoichiometry of MT-3 and Its Domains

MT-3 source	mol equiv of metal ions bound[a]	
	Cd(II)	Zn(II)
human MT-3	7.2 ± 0.1 (n = 2)	7.0 ± 0.2 (n = 3)
mouse MT-3	7.1 ± 0.1 (n = 2)	6.8 ± 0.1 (n = 2)
human α-MT-3	4.0 ± 0.2 (n = 2)	4.3 ± 0.1 (n = 2)
human β-MT-3	3.2 ± 0.1 (n = 2)	3.1 ± 0.1 (n = 3)
human P7T,P9T β	[b]	2.8 (n = 1)

[a] Determined after reconstitution of samples with excess metal ion followed by desalting on Sephadex G-25. [b] Not determined.

complexes. The observed Cd(II) stoichiometry of MT-3 purified from bacterial cultures was 6.5 and 6 mol equiv for human and mouse MT-3, respectively. Both complexes were devoid of bound Zn(II). Human and mouse MT-3 undergo reversible aggregation. Rechromatography of the monomeric MT-3 on Superdex 75 revealed both monomeric and dimeric MT-3. Likewise, rechromatography of dimeric MT-3 resulted in elution of both dimeric and monomeric species.

Metal ion titration studies were carried out to determine the maximal number of metal binding sites in MT-3. Human and mouse MT-3 were depleted of bound Cd(II) ions by gel filtration at pH 2. Reconstitution of MT-3 molecules with excess Cd(II) or Zn(II) followed by desalting gel filtration to remove excess metal ions revealed 7 mol equiv of metal bound for both human and mouse MT-3 (Table 1). Titration of apo-MT-3 molecules with increasing quantities of Cd(II) followed by monitoring the thiol → Cd(II) charge transfer (CT) transitions in the ultraviolet revealed maximal intensity at 7 mol equiv, which is the same maximal number of Cd(II) ions bound to MT-1 and MT-2 (Braun et al., 1986; Robbins et al., 1991; Kagi, 1993). Thus, MT-3 binds the same number of divalent metal ions as the well-characterized MT-1 and MT-2 molecules (Robbins et al., 1991; Kagi, 1993). The less than maximal Cd(II) occupancy in MT-3 purified from bacterial cultures may arise from metal loss during the purification procedures. The seven metal sites in mammalian MT-1 and MT-2 are distributed between two distinct polymetallic clusters with three ions bound in the N-terminal β domain and four ions in the C-terminal α domain (Figure 1) (Braun et al., 1986; Robbins et al., 1991).

Metallothionein binds Cu(I) and Ag(I) ions with a higher stoichiometry than divalent ions (Nielson et al., 1985). To determine whether MT-3 forms analogous Cu(I) complexes, human and mouse apoMT-3 were titrated with increasing quantities of Cu(I) presented as Cu(I)-acetonitrile and the emission of Cu(I)−MT-3 complexes was monitored (Stillman & Gasyna, 1991). Maximal emission was observed with 12 Cu(I) ions for both human and mouse MT-3 (Figure 3A). This is the same maximal stoichiometry as for MT-1 and MT-2 (Nielsen & Winge, 1985).

Metal Ion Reactivity of MT-3. Metal ions bound to MT-1 and MT-2 are known to be kinetically reactive (Shaw et al., 1991; Petering et al., 1992). To determine whether the unique bioactivity of MT-3 molecules correlated with altered kinetic reactivity of MT-bound Zn(II) ions, we evaluated the reactivity of MT metal complexes with EDTA (Shaw et al., 1991). The proteins were incubated with 0.2 mM EDTA at 23 °C and the loss of charge transfer transitions in the ultraviolet was monitored. The kinetics of the reaction are complex, suggesting at least two phases. An initial rate of Zn(II) transfer to EDTA occurs within the time of mixing

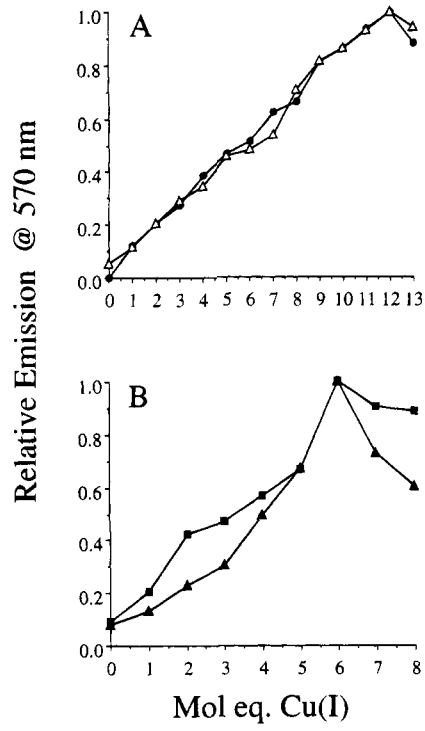

FIGURE 3: Luminescence of Cu(I)MT samples. Apo-MT and domain peptide samples (2 nmol) were titrated with increasing quantities of Cu(I)-acetonitrile. Titrations were carried out anaerobically at room temperature. Following addition of Cu(I), the pH was neutralized to pH 8 with Tris buffer. Excitation was at 300 nm and emission was scanned from 500 to 700 nm. Panel A shows the emission rise for human (●) and mouse (△) MT-3 samples. Panel B shows emission rise of α (■) and β (▲) domain peptides of human MT-3. In each case the relative emission is normalized such that maximal emission is set at 1.0.

and accounts for less than 10% of MT-bound Zn(II). Monitoring the second, slower phase of the reaction revealed similar rates of Zn(II) loss from human and mouse MT-3 and rat MT-1 (Figure 4A). This slower phase of the reaction accounts for over 70% of the MT-bound Zn(II). Likewise, the kinetics of EDTA-mediated Cd(II) loss from human MT-3 and rat MT-1 were similar.

The kinetic reactivity of MT has been suggested to be a result of solvent-accessible thiolates in the rat MT-2 structure (Robbins et al., 1991). We evaluated the thiol reactivity of metal complexes of MT-1 and MT-3. The percentage of thiolates reactive with Ellman's reagent, DTNB, was similar for human and mouse MT-3 and rat MT-1 (Figure 5). Thus, the metal binding properties of MT-3 and perhaps the solvent accessibility of thiolates resemble those of MT-1.

Isolation of MT-3 Domain Peptides. The similar metal binding properties suggested that the structure of MT-3 may resemble MT-2 in having two distinct domains (Robbins et al., 1991; Kagi, 1993). Consequently, the bioactivity of MT-3 may be conferred by either one domain or the combination of two domains. We isolated domain peptides from human MT-3 to determine whether the activity maps to either one of the two candidate domains.

Initially, T7-based expression vectors were constructed with DNA encoding α and β domain peptides. Labeling experiments using ^{35}S revealed that both domain peptides were produced. However, we were only successful in the purification of the α peptide. There was no accumulation of the β peptide from cultures induced with either cadmium or copper salts added to the bacterial medium.

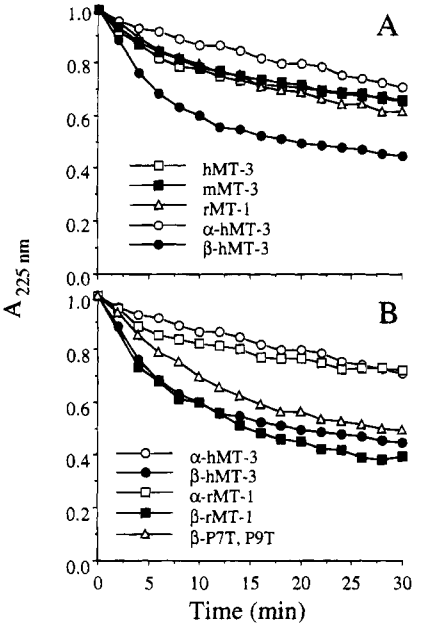

FIGURE 4: Reactivity of MT-bound Zn(II) with EDTA. Zn(II) complexes of MTs and MT domain peptides were prepared by reconstitution of apoMTs with excess Zn(II) followed by desalting on Sephadex G-25. Samples [14 nmol of Zn(II)] were incubated with 0.2 mM EDTA in 20 mM Tris-HCl, pH 8, and the change in absorbance at 225 nm was monitored with time. Human (h), mouse (m), and rat (r) MT samples were used as well as α and β domain peptides from either human MT-3 or rat MT-1.

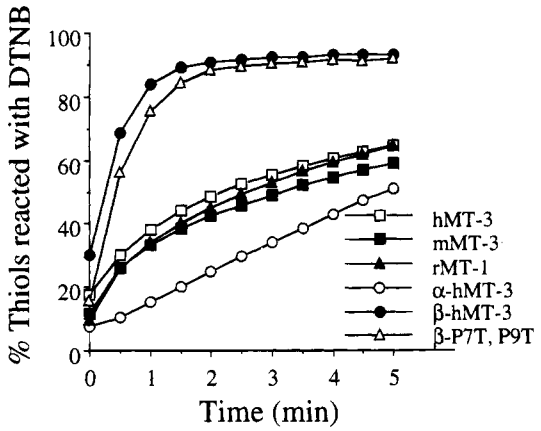

FIGURE 5: Thiol reactivity of MTs with Ellman's reagent (DTNB). Zn(II) complexes of MTs (20 nmol of thiol) were incubated with 1 mM DTNB in 20 mM Tris-HCl, pH 8. The absorbance at 412 nm was monitored as a function of time.

Cd(II) complexes of α-MT-3 domain peptides were purified from *E. coli*. The final step in the purification was C_{18} HPLC, which resolved three α-related components. Amino acid analysis and electrospray MS of each component revealed that heterogeneity in the α samples arose from incomplete processing of the N-terminal Met. The full-length α peptide exhibited a mass of 3811.7, which compares well with the predicted mass of 3811.5 of the Met form of the α peptide. A des-Met form was also evident. A third component resolved by HPLC exhibited a mass consistent with the presence of the N-terminal Met existing as N-formyl-Met.

Since we were unsuccessful in isolating the β domain peptide by bacterial expression of the βMT-3 gene, we synthesized the 32-residue polypeptide by solid-phase peptide synthesis using standard Fmoc chemistry. Electrospray mass spectrometry of the purified β peptide yielded a mass of 3263.3, which agreed well with the predicted mass of 3263.8.

Metal Binding Properties of MT-3 Domain Peptides. Apo domain peptide samples were titrated with increasing quantities of Cd(II) to assess their maximal metal binding stoichiometries. Monitoring the S → Cd(II) charge transfer transition revealed maximal intensity at 3 and 4 mol equiv of Cd(II) for the N-terminal β and C-terminal α domain peptides, respectively. No difference in metal binding was observed among the three α components. Domain peptides reconstituted with excess Zn(II) ions were gel-filtered to remove unbound ions. The resulting metal complexes contained approximately 4 and 3 mol equiv of bound Zn(II) for α and β samples, respectively (Table 1). Each of the two domain peptides exhibited maximal emission at 6 mol equiv in titration studies with Cu(I) (Figure 3B). This is, also, the maximal stoichiometry for Cu(I) binding to MT-1 and MT-2 (Nielson & Winge, 1985). The observed binding of Cd(II), Zn(II), and Cu(I) at stoichiometries expected for the authentic domains of MT-2 supports the prediction that MT-3 exists as a two-domain structure.

The reactivity of metal complexes of each domain was assessed with EDTA and DTNB. Zn(II) complexes of domain peptides were incubated with 0.2 mM EDTA, and the loss of the S → Zn CT transition was monitored (Figure 4A). The kinetics of Zn(II) depletion from the β peptide complex was more rapid than Zn(II) depletion from the α peptide complex (Figure 4A). As expected, the kinetics of Zn(II) depletion from intact MT-3 was intermediate between the rates for α and β. Similar results were obtained for Cd-(II) complexes of each (data not shown). The EDTA reactivity of the Zn(II) complexes of MT-3 domain peptides was indistinguishable from the kinetics of Zn(II) complexes of the corresponding MT-1 domain peptide (Figure 4B). The α domain complex of both MT-3 and MT-1 was less reactive than the β domain complexes of each.

Zn(II) complexes of the β domain of MT-3 were also more reactive with the thiol reagent DTNB than α MT-3 domain complexes (Figure 5). In all cases, the α domain Zn(II) complex was less reactive than the intact protein. The kinetic instability of the β domain may contribute to our inability to purify metal−β-MT-3 complexes from *E. coli*.

Bioactivity of Human and Mouse MT-3. Since the bioassay consists of Alzheimer's disease extract and rat cortical neurons, the question arose whether the activity of human MT-3 was species-specific. Human and mouse MT-3 polypeptides differ in 9 out of 68 possible positions. These sequence variations are distributed in both domains, and three residue changes occur in the unique C-terminal six-residue insertion (Figure 2). The inactive rat MT-1 differs from active human MT-3 in 19 positions excluding the insertion residues. Both human and mouse Zn_7MT-3 complexes antagonized the ability of Alzheimer's disease brain extract to support neuron survival, although the activity of human MT-3 was more pronounced (Figure 6A). Neither MT-1 nor MT-2 from the rat exhibited any activity. In the absence of added AD brain extract, all Zn_7MTs stimulated survival of neurons (Figure 6C; Erickson et al., 1994), demonstrating that the inhibitory activity of MT-3 is not due to a direct toxic effect on neurons.

Cu(I) complexes of MT are structurally distinct from Zn-(II) complexes. To determine whether the growth inhibitory activity was dependent on a specific metalloconformer of

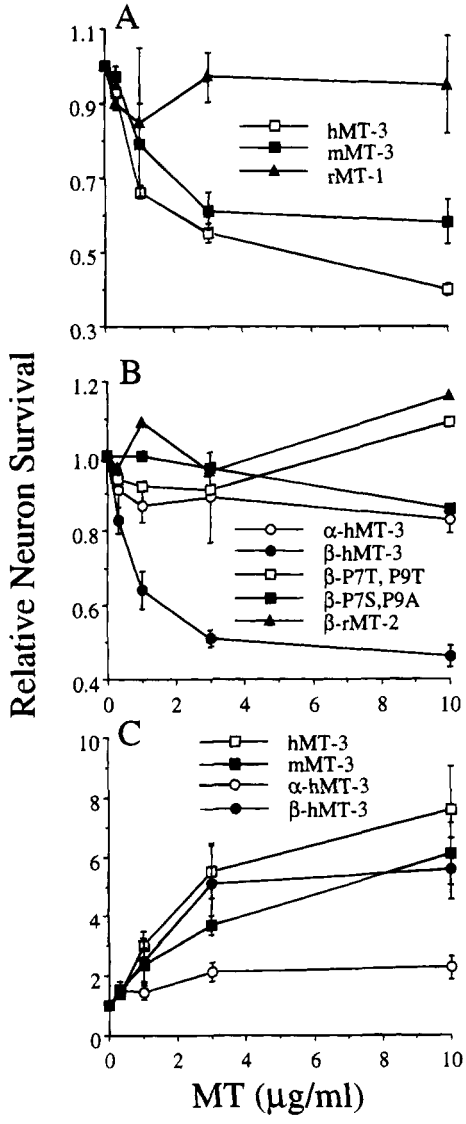

FIGURE 6: Effect of MTs (panel A) and MT domain peptides (panel B) on survival of cortical neurons cultured in the presence of Alzheimer's disease brain extract. Panel C is the effect of MT-3 and constituent domains on survival of neurons cultured in the absence of brain extract. Relative survival was calculated by dividing the average number of neurons per field in the presence of added MT or an MT domain by the average number of neurons per field in the absence of added MT or MT domain peptide. Values are the means ± SEM (error bars are shown only for samples tested in at least 3 independent trials). Samples were tested in duplicate for each independent trial ($n = 7$ for hMT-3, $n = 3$ for mMT-3, rMT-1, and α-hMT-3, $n = 4$ for β-hMT-3, $n = 2$ for β-P7T,P9T βMT-3 and β-rMT-2, and $n = 1$ for P7S,P9A βMT-3). On average, culturing neurons in the presence of brain extract (without added MT) increased survival 8-fold compared to cultures grown in the absence of extract (without added MT).

MT-3, we prepared mixed Cu(I)/Zn(II) complexes for analysis. Cu(I) complexes of either MT-1 or MT-3 prepared by the Cu(I)-dependent displacement of Zn(II) bound to MT resulting in CuZnMT complexes as well as a Cu(I)–glutathione complex were toxic to neurons in culture in the presence and absence of AD extract. Likewise, Cd(II) complexes of any MT were directly neurotoxic, so the growth inhibitory activity could not be assessed for either Cd(II) or Cu(I) complexes of MT-3.

Activity of Zn(II) Complexes of Domain Peptides. Zn(II) complexes of the α and β domains of MT-3 were tested for their ability to inhibit the increased survival of cortical neurons when cultured in the presence of AD brain extract. The β domain Zn(II) complex exhibited a concentration-dependent inhibition, whereas the α domain complex had little effect (Figure 6B). The β domain of rat MT-2 was devoid of bioactivity. In the absence of added AD extract, domain peptide complexes stimulated neuronal survival (Figure 6C).

The growth inhibitory activity of the β domain of MT-3 suggests that structural differences within the β domains of MT-1 and MT-3 are a determinant of bioactivity. In addition to the one-residue insertion in the MT-3 β domains, there are five sequence positions at which significant changes occur between MT-1 or MT-2 and the two MT-3 β peptides (Figure 2). One striking change is the C_6-P-C-P_9 tetrapeptide sequence in MT-3. The C-P-C-P sequence is novel in the MT family of proteins (Kagi, 1993). To explore whether this sequence contributes to the activity of MT-3, we synthesized a β peptide variant in which the C_6-P-C-P_9 sequence was changed to C-T-C-T. Serines, threonines, and alanines are commonly found in these positions in other MTs (Kagi, 1993).

The P7T,P9T β peptide was purified to a single component by C_{18} HPLC. The mass of the mutant peptide was 3271.1, which agreed well with the calculated mass of 3271.8. The mutant β peptide bound 3 Zn(II) ions. The kinetic reactivities of the mutant Znβ MT-3 complex with EDTA and DTNB were similar to those of the wild-type β peptide (Figures 4B and 5).

The survival of neurons cultured in the presence of AD brain extract was unaffected by the P7T,P9T mutant β molecule (Figure 6B). A second β mutant containing the C_6-S-C-A_9 sequence was also devoid of inhibitory activity (Figure 6B). As expected, the mutant β peptides stimulated neuronal survival in the absence of AD extract (data not shown).

DISCUSSION

Zn(II) complexes of both human and mouse MT-3 inhibit the survival of neurons cultured with Alzheimer's disease brain extract. This activity is unique to MT-3 (Uchida et al., 1991; Erickson et al., 1994). The growth inhibitory activity of human MT-3 originally reported by Uchida et al. (1991) was of an ill-defined Cu,Zn complex. In the present study, we observed that activity measurements could only be assessed for Zn(II) complexes, as the presence of Cu(I) or Cd(II) ions were cytotoxic to neurons.

To determine the basis of the growth inhibitory activity of MT-3, we asked whether the MT-3 activity resulted from either unusual metal-binding properties or a distinct structure. We observed that the metal binding stoichiometry and cluster reactivity of MT-3 is similar to that of MT-1 and MT-2 molecules. The similar binding stoichiometries of intact MT-3 and MT-1 molecules as well as their individual domain peptides suggests that MT-3 consists of two domains enfolding separate polymetallic clusters similar to MT-1 and MT-2 (Braun et al., 1986; Robbins et al., 1991). The binding of Cu(I) and Cd(II) to bovine and equine MT-3 is also similar to other MTs (Pountney et al., 1994).

The two metal clusters in MT-3 undergo facile ligand substitution reactions as is known for MT-1 and MT-2 (Shaw et al., 1991; Petering et al., 1992). Equivalent rates of

reactivity with EDTA as a competing ligand were observed for each MT as well corresponding domain peptides. Zn-α complexes of MT-3 and MT-1 exhibit greater stability than Zn-β complexes to reactions with EDTA and a sulfhydryl reagent. We conclude that the metal binding properties of MT-3 are not unique. Hence, any differences in bioactivity are most likely due to differences in protein sequences that allow MT-3 to interact with other molecules preferentially.

The ability of MT-3 to inhibit neuron survival stimulated by the addition of AD brain extract resides in the β domain. The inhibitory activity of the β domain alone is comparable to intact MT-3. Survival was inhibited to the same extent by 10 μg of either the β domain or intact MT-3, indicating that the β domain was fully functional in this assay but slightly less potent on a molar basis. The α domain inhibited less than 15% at any concentration used. In this regard it is similar to other MTs that lack bioactivity. The mutation of the two prolyl residues in the β domain destroyed its inhibitory activity without altering its metal-binding properties, which provides compelling evidence that the structure adopted by this sequence is the primary determinant of its inhibitory activity in this assay.

In the absence of AD extract, Zn(II) complexes of all MTs and all β domains stimulate neuron survival while all α domains are less active. We have discovered that β-mercaptoethanol, dithiothreitol, and glutathione also stimulate survival in this assay at similar sulfhydryl concentrations. Thus, it is likely that the antioxidant properties of MTs and β domains account for the increased survival in the absence of AD extract. In agreement with this suggestion, we observed that changing the medium an hour after neurons were plated increased survival substantially. This medium change may remove various oxidants that were generated during the preparation of neurons for culture. Perhaps the α domains fail to provide significant protection against oxidation because they exhibit more avid Zn(II) binding. The stimulation by all the β domains (MT-1, MT-3, and mutant MT-3) is consistent with the idea that this assay measures a common property of Zn(II) thiolate clusters. The converse of this argument is that the inhibitory assay measures some other property of MT-3. If antioxidant and metal binding properties are not involved, then presumably the β domain of MT-3 preferentially binds to neurons or some molecules in the Alzheimer's brain extract. It could either potentiate the deleterious effect of some factor, inactivate a neurotrophic factor, or alter the response of neurons to the extract. The functional and medical significance of this bioassay is unclear, because we have not been able to confirm the loss of MT-3 in brains of people with AD (Erickson et al., 1994).

The mapping of activity to the β domain implies that the six-residue insertion in the α domain is without consequence. That insertion maps to a flexible loop in the MT-2 structure and its sequence and length are not conserved among species. The differences within the β domain are limited to a single residue insertion, the novel C-P-C-P tetrapeptide sequence, and a few nonconserved amino acid substitutions. In the rat MT-2 structure, the corresponding tetrapeptide sequence is C-S_6-C-A_8. The φ dihedral angles for Ser_6 and Ala_8 are −97° and −71°. The φ angle for Pro bonds is fixed to −60° ± 20° (Schulz & Schirmer, 1979), so the presence of Pro at position 6 would necessitate a limited conformational alteration relative to the known MT-2 structure. Although the magnitude of a structural adjustment to accommodate a C-P-C-P sequence is unclear, there may be enough degrees of freedom in other rotational bonds to accommodate the Pro residues with only a localized change in conformation. The Pro at position 8 may not change the fold as the φ angle of Ala_8 in the MT-2 structure is within the acceptable range for Pro. The C-P-C tripeptide sequence is rare in MT sequences; pigeon MT-1 is the only known animal MT containing this sequence.

Sequences within the β domain in addition to the C-P-C-P tetrapeptide may also contribute to the growth inhibitory activity of MT-3. Sequence differences between the β domains of MT-3 and the common MT-1, MT-2 isoforms are limited. Residue changes at positions 4 and 24 may be significant (numbers relative to MT-3 sequence). The Glu at position 4 in MT-3 is unique to MT-3 molecules; in other MT isoforms that residue is a conserved Asn. The Gly at position 24 is unique to MT-3, but this position shows high variability among MT isoforms and thus variability may not be significant.

We suggest that a conformation unique to MT-3 within the β domain may provide an interface for an interaction that may be the basis for the inhibitory activity of MT-3. The prolyl residues within the C-P-C-P sequence in MT-3 may provide either the scaffolding or the interacting surface for a physical interaction. A direct binding role for prolyl residues was shown recently to be important in the interaction of SH3 domains and their proline-rich target polypeptides (Lim & Richards, 1994; Yu et al., 1994). The side chains of two Pro residues within a P-X-X-P turn of a polyproline helix protrude from the helix and intercalate with aromatic groups in the SH3 domain (Yu et al., 1994). If the pyrrolidone rings of the MT-3 prolyl residues constitute a direct macromolecular binding site, additional nonprolyl residues are likely to confer specificity as with SH3 domains.

REFERENCES

Braun, W., Wagner, G., Worgotter, E., Vasak, M., Kagi, J. H. R., & Wuthrich, K. (1986) *J. Mol. Biol. 187*, 125−129.

Byrd, J., Berger, R. M., McMillin, D. R., Wright, C. F., Hamer, D., & Winge, D. R. (1988) *J. Biol. Chem. 263*, 6688−6694.

Erickson, J. C., Sewell, A. K., Jensen, L. T., Winge, D. R., & Palmiter, R. D. (1994) *Brain Res. 649*, 247−253.

Grassetti, D. R., & Murray, J. F. (1967) *Arch. Biochem. Biophys. 119*, 41−49.

Hemmerich, P., & Sigwart, C. (1964) *Experientia 19*, 488−489.

Kagi, J. H. R. (1993) in *Metallothionein III* (Suzuki, K. T. Suzuki, Imura, M., & Kimura, M., Eds.) p 29, Birkhauser Verlag, Basel, Switzerland.

Katzman, R. (1986) *New Engl. J. Med. 314*, 964−973.

Kobayashi, H., Uchida, Y., Ihara, Y., Nakajima, K., Kohsaka, S., Miyatake, T., & Tsuji, S. (1993) *Mol. Brain Res. 19*, 188−194.

Lim, W. A., & Richards, F. M. (1994) *Struct. Biol. 1*, 221−225.

Muller-Hill, B., & Beyreuther, K. (1989) *Annu. Rev. Biochem. 58*, 287−307.

Nielsen, K. B., & Winge, D. R. (1985) *J. Biol. Chem. 260*, 8698−8701.

Nielsen, K. B., Atkin, C. L., & Winge, D. R. (1985) *J. Biol. Chem. 260*, 5342−5350.

Palmiter, R. D., Findley, S. D., Whitmore, T. E., & Durnam, D. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6333−6337.

Petering, D. H., Krezoski, S., Chen, P., Pattanaik, A., & Shaw, C. F., III (1992) in *Metallothioneins* (Stillman, M. J., Shaw, C. F., III, & Suzuki, K. T., Eds.) p 165, VCH Publishers, New York.

Pountney, D. L., Fundel, S. M., Faller, P., Birchler, N. E., Hunziker, P., & Vasak, M. (1994) *FEBS Lett. 345*, 193−197.

Quaife, C. J., Findley, S. D., Erickson, J. C., Froelick, G. J., Kelly, E. J., Zambrowicz, & Palmiter, R. D. (1994) *Biochemistry 33*, 7250−7259.

Robbins, A. H., McRee, D. E., Williamson, M., Collett, S. A., Xuong, N. H., Furey, W. F., Wang, B. C., & Stout, C. D. (1991) *J. Mol. Biol. 221*, 1269−1293.

Savas, M. M., Petering, D. H., & Shaw, C. F., III (1991) *Inorg. Chem. 30*, 581−583.

Schulz, G. E., & Schirmer, R. H. (1979) *Principles of Protein Structure*, Springer-Verlag, New York.

Shaw, C. F., III, Savas, M. M., & Petering, D. H. (1991) *Methods Enzymol. 205*, 401−414.

Stillman, M. J., & Gasyna, Z. (1991) *Methods Enzymol. 205*, 540−555.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendoff, J. W. (1990) *Methods Enzymol. 185*, 60−89.

Tsuji, S., Kobayashi, H., Uchida, Y., Ihara, Y., & Miyatake, T. (1992) *EMBO J. 11*, 4843−4850.

Uchida, Y. (1993) in *Metallothionein III* (Suzuki, K. T., Imura, M., & Kimura, M., Eds.) p 315, Birkhauser Verlag, Basel, Switzerland.

Uchida, Y., & Tomonaga, M. (1989) *Brain Res. 481*, 190−193.

Uchida, Y., Ihara, Y., & Tomonaga, M. (1988) *Biochem. Biophys. Res. Commun. 150*, 1263−1267.

Uchida, Y., Takio, K., Titani, K., Ihara, Y., & Tomonaga, M. (1991) *Neuron 7*, 337−347.

Winge, D. R. (1991) *Methods Enzymol. 205*, 438−447.

Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., & Screiber, S. L. (1994) *Cell 76*, 933−945.

BI942447O