The third isoform of mammalian metallothioneins (MT-3), mainly expressed in brain and down-regulated in Alzheimer's disease, exhibits neuroinhibitory activity in vitro and a highly flexible structure that distinguishes it from the widely expressed MT-1/-2 isoforms. Previously, we showed that two conserved prolyl residues of MT-3 are crucial for both the bioactivity and cluster dynamics of this isoform. We have now used genetic engineering to introduce these residues into mouse MT-1. The S6P,S8P MT-1 mutant is inactive in neuronal survival assays. However, the additional introduction of the unique Thr5 insert of MT-3 resulted in a bioactive MT-1 form. Temperature-dependent and saturation transfer 113Cd NMR experiments performed on the 113Cd-reconstituted wild-type and mutant Cd5-MT-1 forms revealed that the gain of MT-3-like neuronal inhibitory activity is paralleled by an increase in conformational flexibility and intersite metal exchange in the N-terminal Cd5-thiolate cluster. The observed correlation suggests that structure/cluster dynamics are critical for the biological activity of MT-3. We propose that the interplay between the specific Pro-induced conformational requirements and those of the metal-thiolate bonds gives rise to an alternate and highly fluctuating cluster ensemble kinetically trapped by the presence of the 2TPCCTP motif. The functional significance of such heterogeneous cluster ensemble is discussed.

Alzheimer's disease (AD) is a complex neurological disorder characterized by progressive dysfunction, dystrophy, and death of neurons. The neuronal loss is accompanied by the formation of neurofibrillary tangles and senile (neuritic) plaques (1) and by a massive dendritc sprouting in AD brain (2). The underlying relationship between the various pathological factors has still to be fully resolved, but several potential contributors to the disease process have been identified. For instance, presenilins mediate the altered cleavage of the amyloid precursor protein to yield the amyloid peptide, which is the principal component of neuritic plaques (3). Other bio-molecules have also been examined with regard to their association with AD. One potential factor is metallothionein-3 (MT-3), a member of the family of mammalian metallothioneins (4–6). This protein, also termed neuronal growth inhibitory factor, is expressed almost exclusively in the central nervous system and possesses neuroinhibitory properties in vitro that distinguish it from the MT-1 and MT-2 isoforms, also expressed in the central nervous system (7). Thus, MT-3, but not MT-1/2, antagonizes the ability of AD brain extract to stimulate survival and neuritic sprouting of cultured neurons. Independently, MT-3 was also found to affect significantly the migration of cultured astrocytes (8). Neuronal inhibitory activity, which maps to the N-terminal domain (residues 1–30) of the protein (9, 10), has been established for Cu4,Zn3-MT-3 isolated from human (7) and bovine brains (11) and for recombinant human (9, 12, 13) and mouse Zn7-MT-3 (9). In these studies, the effect of free zinc on the bioactivity could be excluded. In addition, changes in MT-3 mRNA levels in response to central nervous system injury have been taken to indicate an important role of the protein in brain repair (14, 15). In this context, it should be noted that MT-3-deficient mice showed no neuropathology under normal conditions but a higher susceptibility to seizures induced by kainic acid (16). The latter has been associated with the ability of MT-3 to prevent glutamate neurotoxicity in vitro (17).

MT-3 displays several other unique properties not found for MT-1/2. Thus, it protects cultured cortical neurons from the toxic effect of amyloid β peptides (18) and is not induced by the typical inducers of MT-1/-2 biosynthesis such as metal ions (zinc, cadmium, and copper), hormones, inflammation-related stimuli, and stressful agents (19, 20). MT-3 was originally identified as an astrocytic component, but subsequent studies have demonstrated its expression in hippocampal glutaminergic neurons that release zinc from synaptic terminals (21). Moreover, constitutive expression of MT-3, but not MT-1, inhibits the growth of cultured kidney cells under zinc-deficient conditions (22). The characteristic physiological function of MT-3 is further supported by in vivo studies, in which mice overexpressing MT-3 in most organs died as a result of pancreatic atrophy, whereas expression of similar amounts of MT-1 had no effect (23). In AD, the absence or a significant decrease of MT-3 has been linked to the initiation of neuritic sprouting extension in an attempt to re-establish synaptic connections (7). In this instance, a potential cascade may cause the affected neurons to overextend themselves and eventually die. Since the discovery of MT-3 as a factor reduced in AD brains, there have been several conflicting reports (7, 8, 13, 24) with regard to AD-related changes of MT-3 levels. However, a recent comprehensive study (25) at both the protein and RNA level on a large sample size provides clear evidence of its down-regulation in AD brains.

Compared with the amino acid sequences of MT-1/2 (61–62 amino acids), that of MT-3 (68 amino acids) shows ~70% se-
sequence identity and contains two inserts as follows: a Thr at position 5 and a Glu-rich hexapeptide in the C-terminal region (Table I). In addition, all known MT-3 sequences contain the conserved 5CPCP9 motif, which is absent in all other members of the MT family (7, 19). Structural data have shown that, similar to MT-1/2 (26, 27), M7-MT-3 possesses two protein domains each encompassing a metal-thiolate cluster as follows: a 3-metal cluster, M5,Cys9, located in the N-terminal 6-domain (residues 1–30), and a 4-metal cluster, M4,Cys11, in the C-terminal 7-domain (residues 31–68) (28, 29). However, markedly increased structural flexibility and cluster dynamics were observed in the biologically active MT-3 compared with MT-1/2 (28, 29). Consequently, whereas the solution structure of the 6-domain of mouse Cd3-MT-3 could be determined from NMR data, the dynamic disorder encountered in the 6-domain prevented its direct structure determination (29). Most interestingly, mutation of the distinct 5CPCP9 motif of MT-3 to 6CSCA9 found in MT-2 was shown to abolish the inhibitory activity of the protein without altering its metal binding affinity but profoundly affecting the dynamics of the 6-domain (9, 30). These findings have led to the proposal that the bioactivity of MT-3 stems from the distinct sequence motif and structure dynamics unique to this isoform (30).

To gain additional insight into the structural features responsible for the marked differences in biological activity between MT-1/2 and MT-3, we have engineered the conserved 5TCPC8 sequence of MT-3 into the inactive and structurally well characterized Zn7–MT-1 isoform, and we examined the biological and structural consequences. The neuronal bioassays with the S6P,SSP and S6P,SSP+T5 mutants of MT-1 revealed that the two conserved Pro residues together with the Thr and Ser residues, 2Cys/6P,S8P and 2Cys/6P,S8P, are necessary and sufficient for biological activity. More-
isoform, *i.e.* S6P,S8P MT-1 mutant. The relative survival of the cultured neurons as a function of increasing concentrations of added proteins is depicted in Fig. 1. As displayed, no biological activity was found with the S6P,S8P MT-1 mutant. In the next step, Thr-5 was inserted into S6P,S8P MT-1 giving rise to a mutant form, S6P,S8P+T5 MT-1, containing the conserved motif of MT-3, *i.e.* 5TCPCP9 (Table I). In the neuronal bioassay, this second mutant showed a biological activity similar to that seen with the MT-3 isoform (Fig. 1). Whereas at high protein concentrations the growth inhibition of MT-3 reached a plateau at about 50% of relative neuron survival, the inhibition by the added proteins is depicted in Fig. 1. As displayed, no biological activity was found with the S6P,S8P MT-1 mutant. In the next step, Thr-5 was inserted into S6P,S8P MT-1 giving rise to a mutant form, S6P,S8P+T5 MT-1, containing the conserved motif of MT-3, *i.e.* 5TCPCP9 (Table I). In the neuronal bioassay, this second mutant showed a biological activity similar to that seen with the MT-3 isoform (Fig. 1). Whereas at high protein concentrations the growth inhibition of MT-3 reached a plateau at about 50% of relative neuron survival, the inhibition by the bioactive MT-1 mutant steadily increases. The necessity of the Thr insert for biological activity of MT-1 is striking. Therefore, its effect on the activity of human MT-3 was also examined.

Corresponding mutational and biological analysis showed that deletion of Thr-5 causes a loss, and its substitution to alanine greatly reduces bioactivity (Fig. 1). Taken together, these studies clearly demonstrate that both the two conserved Pro residues together with the Thr insert of the β-domain of MT-3 are necessary and sufficient for the neuronal growth inhibitory activity exhibited by this MT isoform.

**Electronic Absorption, CD, and MCD Studies**—To examine the structural changes associated with the gain of biological activity, the optical and magneto-optical properties of wild-type MT-1, S6P,S8P MT-1, and S6P,S8P+T5 MT-1, in their zinc and cadmium bound forms, were compared. Because of the increased covalence of the Cd–S bond, the characteristic CysS–Cd(II) ligand-to-metal charge transfer bands of cadmium-substituted MTs are substantially red-shifted from the region of electronic absorption related to the reaction with the electrophilic sulfhydryl reagent 5,5′-dithiobis-(2-nitrobenzoic acid) at 412 nm as a function of time (39). Yet no differences were found (data not shown.)

Although similar spectral changes have also been seen with the zinc-containing MT-1 derivatives. The validity of this approach is supported by the three-dimensional NMR structures of Zn7-MT-1 and Cd7-MT-2 from human liver, showing identical polypeptide fold (35). Moreover, the absence of 113Cd chemical shift changes at resonances from the α-domain of wild-type MT-1 and its mutants (see below) indicates that structural changes in the MT-1 mutants are restricted to the 3-metal thiolate cluster of the β-domain.

The electronic absorption and MCD features of both mutated proteins are similar to those of wild-type Cd7-MT-1. All show characteristic absorption spectra with shoulders at 250 nm (ε ~ 1 × 10^5 M^-1 cm^-1), due to Cys8–Cd(II) ligand-to-metal charge transfer transitions (36), and a biphasic MCD profile, with extrema at ~259 and +235 nm, ascribed to tetrahedrally coordinated Cd(SCys)_4 units (37). In contrast, the corresponding CD spectra, which unlike UV and MCD are highly sensitive to the cluster geometry and conformation of the enfolding polypeptide chain, reveal a small blue shift and slight intensity changes in the derivative CD profile of Cd3S9 upon S6P,S8P mutation (Fig. 2). Interestingly, in the previous CD studies of human Cd7-MT-3, replacement of the two conserved prolines with the amino acids found in MT-2 (P7S,P9A) resulted in the opposite CD effects (30). After insertion of Thr-5 into S6P,S8P MT-1, no additional chiroptical changes have been discerned (Fig. 2). Consequently, the obtained data point to an alteration of the Cd3S9 cluster in the β-domain of MT-1 upon incorporation of the two proline residues found in MT-3. This conclusion is consistent with our previous zinc K-edge extended x-ray absorption fine structure studies on Zn7–MT-3 and Zn7βMT-3 (38). In view of the CD changes between wild-type MT-1 and its mutants, the solvent accessibility/reactivity of their metal-ligating cysteines was assessed by monitoring changes in absorption related to the reaction with the electrophilic sulfhydryl reagent 5,5′-dithiobis-(2-nitrobenzoic acid) at 412 nm as a function of time (39). Yet no differences were found (data not shown).
and non-detection of signal IV in S6P,S8P+T5 MT-1. The corresponding spectra of wild-type and mutated proteins recorded at 323 K revealed marked changes in both the chemical shift and/or line width of the $^{113}$Cd resonances (signals II–IV) of the $\beta$-domain, whereas those of the $\alpha$-domain were virtually unaffected (Fig. 3 and Table II). Apart from the line sharpening due to decreased correlation time, which also affects signals from the $\alpha$-domain, the resonances of the 3-metal cluster experience a temperature-dependent chemical exchange contribution to both their shifts and their line widths. This effect is substantially more pronounced in the spectrum of $^{113}$Cd$_{S6P,S8P}$MT-1 and even more in that of $^{113}$Cd$_{S6P,S8P+T5}$MT-1. In both cases, this temperature-dependent modulation of the $\beta$-domain resonances allowed the three signals (II–IV) to be discerned at 323 K. In addition, a striking temperature effect on the intensity of the $\beta$-domain resonances in the mutants occurred compared with the wild-type protein. Thus, if calculating the total integrated area of the $\beta$ resonances (II–IV) relative to that of the $\alpha$ domain (I and V–VII) and normalizing for the number of cadmium sites, in $^{113}$Cd$_{S6P,S8P}$MT-1 the area of the $\beta$ signals at 323 K accounts for only 60% (47% at 298 K) of the total cadmium present. This value decreases to 45% (38% at 298 K) in the bioactive form $^{113}$Cd$_{S6P,S8P+T5}$MT-1. Conversely, in the wild-type protein, 81% of the total expected $\beta$ signal intensity is detected at 323 K (74% at 298 K). The observed behavior indicates that a substantial part of the signal intensity from the Cd$_{S\alpha}$ cluster of the MT-1 mutants remains undetected under the conditions employed. It may be noted that no additional resonances were detected outside the chemical shift range presented and that the proteins were fully metal-loaded (see above). A similar behavior for $^{113}$Cd NMR resonances of the Cd$_{S\beta}$ cluster in the $\beta$-domain has been observed in our previous studies on the bioactive human $^{113}$Cd$_{MT-3}$ (28). These features have been interpreted in terms of dynamic events involving coupled fast and slow exchange processes, on the $^{113}$Cd chemical shift time scale, between conformational and/or configurational cluster substrates in the N-terminal $\beta$-domain of the protein (28, 30). Thus, minor sub-millisecond changes of the metal coordination geometry would be responsible for line broadening and would place these events in the fast exchange regime. The additional coupled slow exchange processes are connected to major structural alterations which, in turn, lead to the development of more extended configurational cluster fluctuations. The latter can be visualized as the temporary breaking and reforming of the metal-thiolate bonds (28, 40). Accordingly, the detected $^{113}$Cd signals of the $\beta$-domain reflect only a part of the cluster population, whereas the $^{113}$Cd signals of the majority ofconfigurational cluster substrates remain undetected, because of an extensive exchange broadening and/or to their low population. Consequently, the incorporation of two Pro residues into the structure of MT-1 (S6P,S8P) introduces NMR features already observed for the bioactive MT-3 (28). Hence, it appears that the two-conserved Pro in MT-3 introduce a rate-limiting step in the exchange kinetics between the detectable and non-detectable ensemble of interchanging cluster substrates. This conclusion is supported by our previous studies on the biologically inactive double mutant P7S,P9A MT-3, in which a major part of the Cd$_{S\beta}$ cluster signal intensity could be recovered by raising the temperature (30). In addition, the present data reveal the contribution of Thr-5 to the dynamics of the MT-3 $\beta$-domain. Thus, the subsequent mutational insertion of Thr-5 into S6P,S8P MT-1 further contributes to line broadening and intensity reduction of $^{113}$Cd resonances originating from the 3-metal cluster (Fig. 3C).

Despite the large thermodynamic stability of the metal-thiolate bonds in MTs, they are kinetically very labile. The occur-

![Fig. 3. 133-MHz $^{113}$Cd NMR spectra of mouse $^{113}$Cd$_{MT-1}$ (A), $^{113}$Cd$_{S6P,S8P}$MT-1 (B), and $^{113}$Cd$_{S6P,S8P+T5}$MT-1 (C) at 298 and 323 K. 20-Hz line broadening was applied during processing. The arrows indicate the reversibility of the process. The small signals (C) are irreversible and originate from minor sample degradation during the measurement at 323 K.](image-url)
studies on Zn7-MT-1 clearly established that besides the two mutants were found to be inactive (30). The present mutational (Pro-7 and Pro-9) are necessary for the neuronal growth inhibits its mutant forms showed that both conserved Pro residues contribution of the two Pro and Thr residues to the significance of the non-irradiated resonances due to transfer of saturation to other metal sites, providing that metal exchange takes place during irradiation. The presence of such a metal exchange has been interpreted in terms of increased structure flexibility. Therefore, to explore further the dynamic events in the MT-1 structure generated by introducing the MT-3 motif, metal exchange in the Cd3S9 cluster of MT-1 follows the order saturation to other metal sites, providing that metal exchange increases (Fig. 1). However, because the mechanism of the biological assay for MT-3 is not understood, a quantitative comparison of the results is currently not possible. Nevertheless, the bioassay revealed that we have succeeded in engineering a gain-of-function in MT-1. Apart from the three critical residues introduced into MT-1, additional differences exist between the primary structures of MT-1 mutant and MT-3 (Table I). We suggest that both the resulting alterations of three-dimensional structures and of their reactivity may be important for this effect.

The similarity of the UV and MCD spectral features relative to wild-type and mutated MT-1 forms indicates that, in all cases, the nine cysteines of the \( \beta \)-domain are involved in the formation of a 3-metal cluster composed of tetrahedral Cd-(Cys)4 centers. However, the changes observed in the CD spectra reflect an alteration of the wild-type MT-1 Cd3S9 cluster geometry upon the introduction of the two Pro residues into the sequence, with no additional changes occurring upon Thr-5 insertion. Deeper insights into the structure and dynamics of the 3-metal cluster in the modified N-terminal \( \beta \)-domain can be obtained from NMR studies. From the comparison of the 113Cd NMR features of wild-type MT-1 and the mutant S6P,SSP+MT-1, it can be concluded that the presence of the two critical proline residues affects the dynamics of the Cd3S9 cluster in the \( \beta \)-domain in a manner similar to that found in MT-3 (28). Thus,

### TABLE II

|                  | MT-1 | S6P,SSP MT-1 | S6P,SSP+T5 MT-1 |
|------------------|------|--------------|---------------|
|                  | 298 K| 323 K        | 298 K         |
| \( \delta \)     | \( \Delta \nu \) | \( \Delta \nu \) | \( \Delta \nu \) |
| I                | 671  | 87 100 674 84 100 | 671 93 100 674 92 100 |
| V                | 632  | 85 78 635 85 96 | 632 97 93 635 96 89 |
| VI               | 626  | 129 86 630 112 105 | 626 126 111 630 125 105 |
| VII              | 618  | 147 92 618 120 91 | 618 158 91 618 149 90 |
| \( \beta \)      |      |              |               |
| II               | 668  | 112 62 670 105 86 | 659 148 664 133 44 |
| III              | 650  | 168 67 652 115 74 | 655 510 658 133 45 |
| IV               | 648  | 169 62 651 114 79 | 651 251 651 251 40 |

\( ^a \) Apparent line width of the unresolved signals.
\( ^b \) Total intensity of the unresolved signals.
\( ^c \) ND, not detected.

### TABLE III

| Cd saturated | Cd observed | Residual intensity % |
|--------------|-------------|----------------------|
| MT-1         | S6P,SSP     | S6P,SSP+T5           |
| II           | III         | 55 14 6              |
| III          | IV          | 46 27 8              |
| IV           | II          | 47 23 13             |
| III          | IV          | 34 39 15             |
| III          | III         | 45 36 23             |
| III          | III         | 38 36 30             |

\( ^a \) The resonance numbering scheme corresponds to that in Fig. 3.

because the T5A-MT-3 mutant still showed a low level of bioactivity, we currently favor a structural effect for this residue (see below). It should be noted, however, that the inhibitory activity of S6P,SSP+T5 MT-1 differs from that of MT-3 at high protein concentrations. Thus, whereas MT-3 negates the increased neuronal survival caused by AD brain extract (−50%) (13), the inhibitory activity of S6P,SSP+T5 MT-1 further increases (Fig. 1). However, because the mechanism of the biological assay for MT-3 is not understood, a quantitative comparison of the results is currently not possible. Nevertheless, the bioassay revealed that we have succeeded in engineering a gain-of-function in MT-1. Apart from the three critical residues introduced into MT-1, additional differences exist between the primary structures of MT-1 mutant and MT-3 (Table I). We suggest that both the resulting alterations of three-dimensional structures and of their reactivity may be important for this effect.

The previous neuronal cell culture studies on Zn7-MT-3 and its mutant forms showed that both conserved Pro residues (Pro-7 and Pro-9) are necessary for the neuronal growth inhibitory activity exhibited by this MT isoform, as even single mutants were found to be inactive (30). The present mutational studies on Zn7-MT-1 clearly established that besides the two prolines, the Thr-5 insert is also required for generating an MT-3-like inhibitory activity. The lack or substantial reduction of threonine phosphorylation cannot be ruled out. However,
the reduced signal intensities even at 323 K are in line with the generation of a fluctuating ensemble of cluster substrates, which remain non-detectable because of the slow exchange with the NMR-detectable cluster population and to excessive exchange broadening among the intrinsic conformational and configurational cluster substrates. We proposed previously (30) that the kinetic barrier for the exchange between the detectable and non-detectable ensemble of cluster substrates is due to the slow cis/trans interconversion of Cys-Pro amide bonds. By taking into consideration the conformational restraints imposed by the proline residues in a polypeptide chain (42), it is conceivable that the presence of the 6CPCPβ motif in MT-3 and MT-1 mutants perturbs the geometrical requirements from the CysS-metal coordinative bonds within the Cd3S9 cluster, leading to a structure destabilization. Apart from the greater ease of prolyl cis/trans-isomerization, the structure destabilization would also account for the enhanced intesite metal exchange observed in the detectable cluster ensemble. This is in agreement with a recent multidimensional NMR studies (29) of MT-3, which show a restricted conformational flexibility in the 7 first residues of the β-domain together with high internal dynamics and conformational exchange within the rest of the domain.

The mutational insertion of Thr-5 into S6P,S8P MT-1 apparently does not further modify the cluster architecture (Fig. 2) and the solvent accessibility of the metal-ligated cysteines (see above). However, according to the NMR studies the dynamics of the N-terminal β-domain increases substantially. In particular, as judged by the reduced signal intensities at both temperatures (298 and 323 K), the non-detectable cluster population increases. This observation may be explained by the fact that the presence of Thr-5 enhances the kinetic barrier introduced by cis/trans-isomerization of Cys-Pro bond(s), owing either to the increase of the free energy of the transition state associated to this process or to the stabilization of the thereby generated NMR non-detectable cluster ensemble. The latter would be consistent with our preliminary molecular dynamics simulations on MT-1 mutants, which favor the formation of a side-chain-to-backbone hydrogen bond interaction involving Thr-5 only in a specific isomeric state of the prolyl residues.2

The present biological and structural studies reveal that the engineering of the MT-3 conserved motif, 6CPCPβ, into the inactive MT-1 isoform, brings about the neuronal activity and unprecedented cluster dynamics recognized in the β-domain of MT-3. The fact that, in spite of about 70% sequence identity between MT-1 and MT-3, the introduction of the three critical amino acid residues into MT-1 is both necessary and sufficient for the generation of neuronal growth inhibitory activity is striking. MTs belong to a growing class of proteins with a nonregular protein structure under physiological conditions (43). In this context, there is increasing awareness of compact but incompletely folded states of proteins that contain much of the native secondary structure but lack fixed tertiary interactions, playing an important role in many cellular processes (44). Based on our studies it would appear that MT-3 bioactivity resides in the kinetically trapped and intrinsically disordered cluster ensemble induced by the presence of the 6CPCPβ motif. The described dynamic model could be envisaged as being relevant to a biological process requiring recognition of a specific spatial arrangement. The proline-rich motif could provide the scaffolding or the platform for an interaction with another so far unknown component present in the brain extract (45). Taken together, the results provide a basis for understanding the widely different function of this isoform and clearly suggest that the structure/cluster dynamics is required for MT-3 bioactivity. These observations raise intriguing questions as to the role of metal cluster disorder in biological processes.

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