The ability of metallothionein (MT) to modulate DNA binding by a two-finger peptide of Tramtrack (TTK), a CCHH zinc transcription factor, was investigated using metal-bound and metal-deficient forms of rabbit MT-2 and the TTK peptide. Thionein inhibited DNA binding by zinc-bound TTK, and Zn-MT restored DNA binding by zinc-deficient apo-TTK. “Free” zinc at low concentrations was as effective as Zn-MT in restoring DNA binding by apopeptide but was inhibitory at concentrations equal to zinc bound to 2 mol eq and higher of Zn-MT. Substitution of cadmium for zinc reduced the affinity of the peptide for its DNA binding site. This effect was reversed by incubation with Zn-MT. The circular dichroic spectra of the TTK peptide indicated that zinc removal resulted in loss of α-helical structures, which are sites of DNA contact points. Reconstitution with cadmium resulted in stoichiometric substitution of 2 mol of Cd/mol of peptide but not recovery of α-helical structures. Incubation of Cd-TTK with Zn-MT restored the secondary structure expected for zinc-bound TTK. The ability of Zn-MT and thionein to restore or inhibit DNA binding by TTK was associated with effects on the metallation status of the peptide and related alterations in its secondary structure.

The low molecular weight metal-binding protein metallothionein (MT) is proposed to have functions in metal ion regulation and detoxification (1) and as a scavenger of free radicals (2). Roles for MT in dynamic intermolecular metal exchange reactions are consistent with the high thermodynamic stability (3) and high kinetic lability of its metal binding sites (4, 5). Metal exchange can also be facilitated by cellular redox couples such as GSH and GSSG (6–9). An early focus on enzyme activation by Zn-MT implicated MT as a zinc donor to apometalloenzymes such as aldolase, thermolysin, and carbonic anhydrase (10) and pyridoxal kinase (11). Recent studies on the effect of MTs on interactions between zinc transcription factors and their cognate DNAs implicate MTs in gene regulation. Incubation of metal-free thionein with CCHH zinc finger transcription factors Sp1 (12) and TFIIA (13) inhibits DNA binding, suggesting zinc abstraction by thionein as a mechanism for regulating gene expression. Reciprocal zinc exchange is suggested as a mechanism for regulating gene expression in a study in which Zn-MT and thionein could activate or inhibit DNA binding by the estrogen receptor (14), a CCCC zinc finger protein.

Zinc transcription factors appear uniquely qualified as potential partner molecules in metal exchange reactions with MT. Reported stability constants for zinc binding by zinc finger peptides and proteins are in a similar range, i.e. within 1 or 2 orders of magnitude, as that of MTs (15–17). Additionally, it appears that the kinetics for zinc exchange in these structures are also relatively rapid (18). Direct examination of the metal transfer process has shown that zinc can be transferred from an α-domain peptide of human MT-2 (α-MT-2) to a peptide derived from the third finger of Sp1 (16) and from rabbit Zn-MT-2 to the Zn-transcription factor GAL4 (19). The rate constant for zinc exchange for GAL4 is rapid and similar to that of MTs (18, 19). Although the functional consequences of the transfers were not addressed in these studies; considered together with the preceding examples, it appears that MT can modulate DNA binding of zinc transcription factors by regulating the availability of zinc.

The notion that Zn-MT can modulate intracellular zinc availability can also be extended to interactions of zinc metalloproteins with toxic metals whose toxicity is thought to be due, in part, to their displacement of zinc (20). This condition is considered to be reversible through reactions with Zn-MT, which has the potential to abstract a toxic metal and donate an essential metal. Inhibition of carboxypeptidase activity by cadmium, for example, can be reversed by incubation with Zn-MT (21). This model for participation of MT in metal detoxification has been designated as a “rescue” or “repair” function that confers on Zn-MT an active, as opposed to a passive, role in the protective response to metals (21, 22). It is known that cadmium binding to some zinc finger proteins reduces the affinity of the latter for their DNA-binding sites (23) and that metal-metal exchange can occur between Zn-MT and zinc transcription factors bound to other metals, e.g. between nickel-bound Sp1–3 zinc finger peptide and zinc-bound α-MT-2 peptide (16). These observations raise the possibility that Zn-MT can restore DNA binding activity to zinc transcription factors through metal-metal exchange reactions.

Our study addressed the ability of thionein and Zn-MT to modulate sequence-specific DNA binding by a two-zinc finger peptide (24) of Tramtrack (TTK) (25, 26) prepared in different metallation states (i.e. as zinc-bound, apo-TTK, and cadmium-bound states). TTK is a classical CCHH transcription factor (27) that suppresses genes involved in regulating development and differentiation in Drosophila (28). The zinc atom in a single
zinc finger of TTK is tetrahedrally coordinated in a motif that includes an antiparallel β-sheet containing two cysteine ligands and a short α-helix containing two histidine ligands (29). The amino acid to base contacts reside in the α-helix of the zinc finger motif (29).

Experiments tested the feasibility of 1) reciprocal zinc exchange between TTK and MT in modulating the DNA binding by TTK and 2) cadmium-zinc exchange between Cd-TTK and Zn-MT as a mechanism for cadmium detoxification. Band shift gels were used to assess the effect of various metallation states of MT and the TTK peptide on sequence-specific DNA binding of the latter. Changes in the secondary structure of the TTK peptide and interactions between MT and TTK were monitored by measuring CD. Stepwise substitution of cadmium for zinc in TTK was followed by electronic absorption spectroscopy. The results showed that the peptide structure was dependent on its metallation state and implicated the Zn-MT-thionein conjugate pair in modulation of DNA-binding activity and detoxification of cadmium.

EXPERIMENTAL PROCEDURES

Expression of TTK Peptide and Preparation of Metallated and Non-metallated Forms—The TTK peptide 3911ff was cloned in pET11a (24) and expressed in Escherichia coli strain BL21(DE3) (Novagen, Inc). The peptide was extracted from inclusion bodies in the zinc-reconstituted form basically as described in Fairall et al. (24) and purified by fast protein liquid chromatography (Amersham Pharmacia Biotech) with HiTrap Heparin affinity columns (Amersham Pharmacia Biotech) equilibrated in either 20 mM MES, pH 6.0, 1 mM DTT, 50 μM ZnSO4 or 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 M NaCl, 50 μM ZnSO4. The TTK peptide was eluted with a linear NaCl gradient with 1 mM NaCl in the respective limit buffer. Purification was monitored by SDS-polyacrylamide gel electrophoresis using 4–20% or 10–20% gradient gels (Bio-Rad). A similar procedure was used to reconstitute zinc-bound TTK (previously determined not to alter the CD spectrum of TTK) in another. Cd-TTK was prepared from apo-TTK using a method originally described for the reconstitution of metallated MT from thionein (29) and was purchased from Microsynth (Balghach, Switzerland). To prepare binding site DNA, the top strand was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs) and then annealed with the complementary strand in 10 mM Tris-HCl, pH 7.3, 0.1 M NaCl, 5 mM MgCl2. Unincorporated label and EDTA present in the polymerizer’s end-labelling kit were removed by desalting in a Bio-Gel P10 (Bio-Rad) spin column equilibrated in deionized water, followed by dialysis against water as a further precaution against the presence of EDTA.

To assess the effect of Zn-MT and thionein on DNA binding by zinc-bound or apo-TTK peptide, Zn-MT or thionein was incubated with the peptide for 30 min at 37 °C before the addition of other reagents. The binding site DNA was added last and allowed to react for 15 min before the sample was centrifuged and loaded on the gel. Apo-TTK peptide and thionein, originally dissolved in 0.01 M HCl, were neutralized with Tris-base immediately prior to the addition of other reagents.

Gels were preelectrophoresed for at least 2 h at 200 V to constant current. Dried gels were visualized by autoradiography using a Molecular Dynamics PhosphorImager and the ImageQuant program. Data were further analyzed using the NIH Image program. Apparent dissociation constants (Kd) (34) were estimated for TTK, Cd-TTK, and Cd-TTK + 1x Zn-MT complexes with binding site DNA. Kd values for Cd-TTK and Cd-TTK + 1x Zn-MT were compared against the mean of four estimates for the Kd of TTK in two-tailed t tests.

RESULTS

General Characteristics of Expressed TTK Peptide—The TTK peptide was isolated from inclusion bodies and then renatured and purified in the zinc-bound form. SDS-polyacrylamide gel electrophoresis indicated a single band of the expected molecular weight. The reconstructed ESI-MS spectra obtained under
acidic and neutral conditions indicated the presence of a single major species corresponding to the mass of the metal-free apo-TTK under acidic conditions (observed mass, 8023.5; expected mass, 8025.3) and TTK with two zinc ions (hereafter referred to as TTK) under neutral conditions (observed mass, 8151.7; expected mass, 8152.0). When dialyzed against ammonium acetate prior to ESI-MS, minor peaks indicative of ammonia adducts were also detected under both acidic and neutral conditions and presumed to be artifacts of reactions with ammonia acetate; these peaks were not detected if apo-TTK was prepared by acidification and desalting and analyzed directly without prior dialysis against ammonium acetate.

**Inhibition of TTK-DNA Complex Formation by Thionein and Restoration by Zn-MT**—In titrations of 10 nM binding site DNA with different concentrations of the TTK peptide, band shifts were concentration-dependent with no detectable shift due to TTK-DNA complex formation in the absence of the peptide and a maximal shift at 12 μM peptide, a 1200-fold molar excess over binding site DNA. On subsequent gels, binding site DNA alone and binding site DNA plus 12 μM TTK peptide were used as negative and positive controls, respectively. The addition of unlabeled binding site DNA as a competitor resulted in a concentration-dependent inhibition of the band shift (not shown) and confirmed the previously reported specificity of this peptide for the binding site DNA (24).

Incubation of TTK with thionein resulted in a concentration-dependent inhibition of TTK-DNA complex formation at concentrations of thionein ranging from 0 to 300 μM (Fig. 1). However, a relatively high concentration of 300 μM thionein, corresponding to a 25-fold molar excess of thionein to TTK, was required to achieve complete inhibition of the TTK-DNA complex formation. A concentration between 37.5 and 75 μM thionein, which was equivalent to 3.1- and 6.3-fold molar excess, was needed for 50% inhibition. These findings were in agreement with earlier reports (12, 13) that thionein is capable of inhibiting DNA binding by Sp1 and TFIIIA, zinc transcription factors with similar CCHH motifs.

Incubation of apo-TTK with Zn-MT restored full DNA binding to apo-TTK at concentrations of thionein as low as 3 μM. This corresponded to a 0.25 molar ratio of MT to TTK (Fig. 2) and a ratio of zinc binding sites in MT to those in TTK of 0.9. Because of the high avidity of apo-TTK for Zn$^{2+}$, full inhibition of DNA binding by apo-TTK alone was not observed here, despite efforts to minimize zinc background concentrations. We showed above that complete inhibition could be achieved in the presence of a strong competing ligand such as thionein. Analogies for zinc indicated very low or undetectable concentrations in purified apo-TTK samples and solutions used for the band shift gels; however, it was difficult to control zinc in all phases of running the band shift gels, and this, rather than nonspecific binding, probably accounted for the apparent basal DNA binding activity of the apo-TTK peptide.

In a parallel experiment (Fig. 3), apo-TTK was incubated with Zn$^{2+}$ (as ZnSO$_4$) and sulphydryl (as 2-mercaptoethanol) concentrations equivalent to those in the Zn-MT concentrations above. At the lowest concentrations of 21 and 42 μM Zn$^{2+}$, Zn$^{2+}$ was as effective as Zn-MT in restoring DNA binding activity to apo-TTK. These Zn$^{2+}$ concentrations were equivalent to molar ratios of 0.9 and 1.75 for added Zn$^{2+}$ to the total number of zinc-binding sites on apo-TTK. This corresponded to the amount of Zn$^{2+}$ in 0.25 and 0.5 molar ratios for Zn-MT to apo-TTK in the experiment described above. Thus, roughly equimolar concentrations of Zn$^{2+}$ and zinc-binding sites on TTK were sufficient to fully activate TTK. An increase in Zn$^{2+}$ concentrations to 168 μM and higher resulted in a concentration-dependent inhibition of DNA binding. The presence of mercaptoethanol, a sulphydryl reagent with lower affinity for zinc than MT, was not able to provide protection at concentrations equivalent to the sulphydryl groups in MT.

**Inhibition of DNA Binding by Cd-TTK and Reversal by Zn-MT**—To assess the effect of cadmium substitution on DNA binding and whether incubation of Zn-MT with cadmium-reconstituted Cd-TTK has any effect, the apparent dissociation constant, $K_d$, for complex formation with DNA was estimated by analyzing a range of concentrations of TTK, Cd-TTK, and Cd-TTK + 1× Zn-MT in band shift gels. Four independent measurements were made for the $K_d$ of TTK. Single lanes that showed responses at equivalent concentrations of 6 μM for TTK, Cd-TKK, and Cd-TTK + 1× Zn-MT were selected for presen-

![Fig. 1. Band shift gel showing inhibition of the TTK-DNA complex formation by thionein.](image1)

![Fig. 2. Band shift gel showing the effect of various concentrations Zn-MT on DNA complex formation by apo-TTK.](image2)
tation in Fig. 4. DNA binding by Cd-TTK (K'\textsubscript{d} = 5.3 \mu M) was significantly reduced (p < 0.0001) in comparison with the TTK peptide bound to two Zn\textsuperscript{2+} ions (K'\textsubscript{d} = 0.5 \mu M \pm 0.1 (S.D.; n = 4). This estimate of K'\textsubscript{d} for Cd-TTK agreed with a previously reported value of 0.4 \mu M (24). The K'\textsubscript{d} for Cd-TTK, indicating a 10.6-fold weaker binding to binding site DNA, was considered conservative, due to the possibility that low levels of background Zn\textsuperscript{2+} in the band shifts may have contributed to the formation of a small amount of folded TTK as described for band shifts with apo-TTK. Incubation of Cd-TTK with equimolar Zn-MT reduced the K'\textsubscript{d} to 0.9 \mu M, which was not significantly different from that of TTK (p > 0.05). This restoration of DNA binding activity to the previously inhibited Cd-TTK was probably due to zinc-cadmium exchange and recovery of secondary structure to that expected for the zinc-bound TTK.

**Effect of Zn\textsuperscript{2+} Ions on the Circular Dichroic Spectra of TTK**—The CD spectrum of the native TTK peptide at pH 7.4, with negative maxima at 208 and 222 nm (Fig. 5, solid line), was consistent with a peptide conformation with significant \alpha-helical structure components. A structural composition of 34.5% \alpha-helix, 36.5% \beta-sheet, and 29% random coil obtained by secondary structure analysis (35) of this CD spectrum agreed with the 33% \alpha-helix and 30% \beta-sheet structure of TTK obtained by x-ray crystallography of the TTK-DNA complex (29). The CD spectrum of apo-TTK (Fig. 5, dashed line), with a negative maximum at about 204 nm, showed features mainly of \beta-sheet and random coil structure, with low \alpha-helical content (below 10% in this case). The addition of Zn\textsuperscript{2+} to apo-TTK at neutral pH significantly increased the negative maximum at 222 nm, signifying an increased \alpha-helical structure (not shown). When TTK was prepared by adding Zn\textsuperscript{2+} to apo-TTK at pH 2 and neutralizing to pH 7.3, the CD spectrum of the zinc-reconstituted TTK (Fig. 5, dotted line) resembled that of the native TTK. Thus, the folding of the TTK zinc finger domains to the native structure was coupled to the binding of zinc, a property also reported for other zinc finger domain peptides (36).

The incubation of 11 \mu M TTK with 0.5, 1, and 2 mM Zn\textsuperscript{2+} resulted in little change in the CD spectrum (data not shown). Zinc to TTK ratios in this experiment corresponded to those at the highest Zn\textsuperscript{2+} concentrations in the band shift gel of Fig. 5. Thus, the inhibited band shift at high Zn\textsuperscript{2+} concentrations was probably caused by a polyelectrolyte effect, which prevented formation of TTK-DNA complexes or favored dissociation of TTK-DNA complexes, not by alterations in the secondary structure of TTK.
The electronic absorption spectra of apo-TTK at pH 2 and Cd-TTK reconstituted from this apo-TTK are shown in Fig. 6B. The spectrum of apo-TTK resembled that of native TTK bound to Zn\(^{2+}\) but with the absorption maximum blue shifted about 2 nm to 275 nm. Cd-TTK exhibited characteristic Cys-S-Cd(II) charge-transfer transitions with a maximum at 238 nm (Fig. 6B, inset), similar to that observed above with the cadmium titration of native TTK. The calculated molar extinction coefficient (ε) was about 12,000 M\(^{-1}\) cm\(^{-1}\)/Cd\(^{2+}\). The intensity of the Cys-S-Cd(II) charge transfer band is reported to be proportional to the number of Cys-S-Cd(II) coordinative bonds with ε between 5500 and 6500 M\(^{-1}\) cm\(^{-1}\)/Cys-S-Cd bond (38). The value of about 12,000 M\(^{-1}\) cm\(^{-1}\) was consistent with the presence of two Cys ligands/Cd\(^{2+}\), the expected number of thiolate ligands present in the CCHH finger motif of TTK.

Changes in the CD spectra of TTK titrated with increasing concentrations of Cd\(^{2+}\) were also concentration-dependent (Fig. 7A). Negative and positive maxima at 222 and 193 nm, characteristic of the α-helical structure of the zinc-bound TTK, decreased in intensity with increasing Cd\(^{2+}\) concentrations. This marked alteration of the secondary structure of TTK was clear evidence that the substitution of Cd\(^{2+}\) for Zn\(^{2+}\) was not isostructural and agreed with the previously described inhibition of DNA binding by Cd-TTK. Metal-induced changes in the CD spectra of TTK originated predominantly from alterations in secondary structure elements rather than from Cys-S-Zn(II) and the Cys-S-Cd(II) charge transfer transitions. Although the latter can also give rise to CD bands in the range from 190 to 250 nm as reported for Cd,MT (37), the intensity of CD bands associated with these charge transfer transitions is significantly lower than the CD bands of the amide transitions of the TTK-peptide chromophore, where substantial amounts of well defined secondary structure exist.

The CD spectrum of the Cd-TTK reconstituted from the acidified apo-TTK (Fig. 7B) exhibited features in common with apo-TTK (Fig. 5A), with a secondary structure with only a small contribution from α-helical structure. Incubation of this Cd-TTK with Zn\(^{2+}\) resulted in recovery of the α-helical features characteristic of zinc-bound TTK. Effective Zn\(^{2+}\) concentrations ranged from 38 to 400 μM, which corresponded to a 3.8–40-fold molar excess of Zn-TTK to Cd-TTK (spectrum for 150 μM Zn\(^{2+}\) is shown in Fig. 7B). Thus, the changes in the secondary structure of TTK observed after binding Cd\(^{2+}\) were reversed by the addition of zinc.

Interaction of Cd-TTK with Zn-MT—The effect of Zn-MT on the structure of Cd-TTK is shown in Fig. 8. When Zn-MT was added to Cd-TTK so that concentrations of zinc and cadmium were equimolar, there was a shift in the CD spectrum from that of Cd-TTK (dotted line) to one that showed changes indicative of recovery of the α-helical bands present in zinc-bound TTK, i.e. decrease at 222 nm and increase at 193 nm (dashed line), after 35 min. To isolate the TTK component of the composite spectrum derived from the TTK-Zn-MT mixture, the spectrum of Zn-MT incubated with Cd\(^{2+}\) (thin solid line) was subtracted from that of the mixture (dashed line). The resulting difference CD spectrum (thick solid line) closely resembled the CD spectrum of the native zinc-bound TTK peptide of Fig. 5. These results demonstrated that MT-bound zinc had exchanged for the cadmium bound to Cd-TTK, resulting in full recovery of the secondary structure native to the zinc-bound form of the peptide. This was consistent with the full recovery of DNA binding activity after Cd-TTK was incubated with equimolar Zn-MT as
previously shown in Fig. 4.

The time course of the metal exchange reaction between Cd-TTK and Zn-MT was also monitored over a 30-min period by following the recovery in the ellipticity at 222 nm (data not shown). Exchange was relatively rapid, with 90% of the maximal recovery attained after 10 min and full recovery after 20 min. Thus, the 30-min incubation with MT in the corresponding band shift assays had allowed the cadmium-zinc exchange to go to completion before DNA binding activity was measured.

**DISCUSSION**

The kinetic lability of zinc binding sites (4, 5) confers on MT the potential for intermolecular interactions through metal exchange with other structures, despite the high thermodynamic stability of metal-MT complexes. Early studies (10) demonstrating that Zn-MT is capable of activating Zn-metalloenzymes provided the initial evidence for zinc transfer from MT to potential acceptor molecules. Of the various types of zinc-dependent proteins, those with relatively facile zinc exchange would be suitable candidates to enter into dynamic metal exchange interactions with MT (13, 17). Metalloproteins with zinc atoms bound in cluster or finger motifs, such as MT and certain zinc transcription factors, exhibit the most rapid zinc exchange rates (18, 39). Thus, the reports that thionein was capable of inhibiting sequence-specific DNA binding by the CCHH zinc finger proteins Sp1 and TFIIIA (12, 13) were of interest, since they implicated thionein in a regulatory function involving abstraction of zinc from such proteins. Our observation that removal of zinc abolished the ordered secondary structure of the TTK peptide agreed with results for other zinc finger structures in which zinc-dependent folding of the peptides had been established (36). The inhibitory effect of thionein on the TTK-DNA complex formation was therefore not unexpected and was consistent with these earlier reports. The reverse reaction, i.e., the activation of metal-depleted forms of CCHH zinc finger proteins by Zn-MT, had not been previously reported, however. Zn-MT, for example, was not able to activate apo-Sp1 in *Xenopus laevis* oocytes treated with EDTA (40). This would limit the role of MT to abstracting zinc and inhibiting DNA binding. However, our experiments clearly demonstrated that Zn-MT can activate apo-TTK and, thus, extended observations of Zn-MT-mediated activation of DNA binding to CCHH zinc finger proteins. This would take full advantage of the ability of the Zn-MT-thionein conjugate pair to act as a zinc donor-acceptor mechanism.

Thermodynamic considerations provide some insight into the energetic feasibility of reciprocal Zn$^{2+}$ transfers between Zn transcription factors and MT, both with kinetically labile Zn$^{2+}$. For example, zinc exchange between CCCC zinc transcription factors and Zn-MT is expected to be relatively facile based on the similarity in zinc binding affinities reported to date: e.g., $K_S = 9 \times 10^{11}$ M$^{-1}$ for a model CCCC zinc finger peptide (15) and $2 \times 10^{12}$ M$^{-1}$ for Zn-MT (17). This is consistent with a report describing Zn-MT/thionein-mediated activation-deactivation of the estrogen receptor (14). For CCHH zinc finger peptides and proteins, the apparent stability constants for Zn$^{2+}$ range from lower values of $3.6 \times 10^6$ M$^{-1}$ for the second finger in TFIIIA (41), and $2 \times 10^8$ M$^{-1}$ for the third finger of Sp1 (16) to higher values of $2 \times 10^{11}$ M$^{-1}$ for a designed consensus single finger peptide (Cp-1) (15) and $5 \times 10^{11}$ M$^{-1}$ for intact TFIIIA (17). These considerations provide an explanation for lack of activation of Sp1 by Zn-MT (40) based on the greater affinity of zinc for MT over Sp1. The stability constant for Zn$^{2+}$
in TTK is not known, although our results suggested that the value for TTK should be greater than that for Sp1 and similar or possibly higher than that for the synthetic Cp-1 zinc finger (15). Zn-MT to apo-TTK ratios less than unity were effective in completely restoring DNA binding, attesting to the efficiency of this activation. However, because the levels of thionine required to inhibit DNA binding by TTK were high (3–6-fold molar excess of thionine over TTK for 50% inhibition), it appeared that the reciprocal reactions were not equivalent. TTK appeared to have an unexpectedly high avidity for zinc in comparison with MT.

An alternative hypothesis (7–9) uncouples thermodynamic barriers from the zinc transfer process in proposing a mechanism for coupled zinc exchange between Zn-MT/thionine and apo/Zn-metalloenzymes with much lower Zn\textsuperscript{2+} affinities. It is based on observations that 1) the redox activity of MT thiols and their interaction with other redox couples such as GSH-GSSG can facilitate zinc release from Zn-MT by oxidation of metal-thiolate bonds and stabilization of the liberated thiol group by disulfide bond formation and that 2) zinc transfer from zinc metalloproteins to thionine can be facilitated by the presence of chelators and cellular agents such as Tris, citrate, and GSH. The presence of mercaptoethanol in our TTK samples may have mimicked some of the conditions attributed to GSH during incubations of TTK with thionine (0.5 mM mercaptoethanol during incubation), although effects of the reducing agent on zinc transfer were not apparent.

Both MT-bound Zn\textsuperscript{2+} and “free” Zn\textsuperscript{2+} were equally effective in activating apo-TTK at concentrations equivalent to 1 molar ratio of Zn\textsuperscript{2+} to the number of binding sites on apo-TTK. This observation argues against, but does not preclude, direct molecular interactions between TTK and MT as the basis for the metal exchange and is consistent with a role for MT in regulating the availability of free zinc (Reaction 1).

\[
\text{TTK} \rightleftharpoons \text{apo-TTK} + \text{Zn}^{2+} + \text{thionine} \rightleftharpoons \text{Zn-MT} \\
\text{REACTION 1}
\]

which in cells is reported to occur at extremely low concentrations (100 pm or less) (42, 43). The significance of free Zn as a cellular Zn\textsuperscript{2+} source for metalloproteins is currently a topic of discussion (8). At higher concentrations of Zn\textsuperscript{2+} alone, the effective range for activation of apo-TTK was limited by inhibition of DNA binding at concentrations equal to or greater than 14 mol eq of Zn\textsuperscript{2+}/mol of apo-TTK. Disruption of the protein-DNA complex by high zinc concentrations was also observed with Sp1 (44), although the mechanism could not be inferred from that study. For TTK, CD analysis revealed that inhibition was probably due to effects unrelated to changes in the secondary structure of zinc motifs; the CD spectrum of TTK remained unchanged in the presence of increasing Zn\textsuperscript{2+} concentrations. A polyelectrolyte effect at high Zn\textsuperscript{2+} concentrations can explain the inhibition. When bound as Zn-MT, up to 175 mol eq of Zn\textsuperscript{2+}/mol of apo-TTK, which corresponds to 25 mol eq of Zn-MT/mol of TTK, could be added with no adverse effect on DNA-binding. Thus, binding to MT provided protection at high Zn\textsuperscript{2+} concentrations.

Other metals can also bind zinc finger peptides. Substitution of cobalt for zinc, for example, has been used to probe structural characteristics of the zinc finger motif (15, 36). Binding of Cd\textsuperscript{2+} to CCHH zinc finger proteins has had varied results with regard to effects on structural and functional properties. Cadmium substitutes stoichiometrically for zinc in a three-finger peptide of Sp1 (45), resulting in a form that exhibits both Cys-S-Cd(II) charge transfer bands and full recognition of the cognate DNA (45), although the affinity for the DNA binding site appears reduced. Cobalt, nickel, and manganese are also able to restore DNA binding to Sp1 apoprotein (44), with nickel and manganese less effective than zinc, cobalt, and cadmium.

With a zinc finger peptide of human immunodeficiency virus-1 integrase, titration of the apopeptide with zinc, cobalt, and cadmium results in similar CD spectra with a high degree of ordered structure (32% α-helical content) not seen in the metal-free peptide (46). These findings are not accord with our results with the two-finger TTK peptide, which showed that stoichiometric substitution of cadmium for zinc disrupted the ordered secondary structure normally displayed by the zinc-bound form and inhibited its ability to bind DNA.

Substituting Cd\textsuperscript{2+} for Zn\textsuperscript{2+} by either titrating TTK with Cd\textsuperscript{2+} or reconstituting Cd-TTK from apo-TTK resulted in the appearance of characteristic Cys-S-Cd(II) charge transfer bands and a large decrease in the α-helical content. We suspect that either one or both His ligands of the CCHH motif may not have been involved in Cd\textsuperscript{2+} binding. This would result in an uncharacteristic metal coordination environment and disruption of the TTK-DNA contact points that reside in the α-helix of the zinc finger motif (29). The reverse titration, i.e. titrating Cd-TTK with Zn\textsuperscript{2+}, indicated a preference for Zn\textsuperscript{2+} over Cd\textsuperscript{2+} that was consistent with the rank order of stability constants for the consensus single finger peptide Cp-1 of 2 × 10\textsuperscript{14} M\textsuperscript{-1} for zinc and 5 × 10\textsuperscript{8} M\textsuperscript{-1} for cadmium (calculated from Ref. 15).

Since incubation of TFIIIA with cadmium also inhibited sequence-specific DNA recognition (23), it appears that TTK and TFIIIA have characteristics in common with regard to response to cadmium, which differ from those of Sp1, although the zinc fingers of all three are characterized by the CCHH motif. This may reflect an influence of other aspects of secondary structure on the metal coordination environment of zinc finger motifs of different proteins. Zinc binding to a three-zinc finger peptide of Sp1 is reported to be cooperative (45), while the zinc affinity of a synthetic zinc finger was increased when arranged in tandem with a like motif (47). Given the large numbers of CCHH zinc finger proteins (48) and the specificity attributed to their ability to bind DNA, diversity in their metal binding characteristics probably exists, possibly reflecting differences in zinc-mediated regulation of DNA binding.

Cadmium-induced alterations in the secondary structure of TTK and inhibition of DNA binding were reversed by incubation with stoichiometric amounts of Zn-MT. Measurements of CD indicated a complete and rapid exchange of Cd\textsuperscript{2+} and Zn\textsuperscript{2+} ions between Cd-TTK and Zn-MT. This metal-metal exchange is consistent with the much higher affinity of MT for Cd\textsuperscript{2+} over Zn\textsuperscript{2+} (49) and the preference of TTK for binding Zn\textsuperscript{2+} over Cd\textsuperscript{2+}, indicated by titration of Cd-TTK with Zn\textsuperscript{2+}.

These findings demonstrate the feasibility of an active role for Zn-MT in metal detoxification through metal-metal exchange reactions with adversely affected target structures. The unique properties of high thermodynamic stability and high kinetic lability of metal-binding sites, together with the order of affinity of different metals for MT (e.g. Hg(II) > Cd(II) > Zn(II)) (3, 18), confer on MT a unique ability to “rescue” or “repair” DNA (21, 22) structures that have been compromised by inappropriately binding toxic metals with a greater affinity for MT than zinc. In this way, the Zn-MT-thionine conjugate pair, whose primary role may be to regulate zinc availability, may be diverted to serve detoxification functions when necessary.

Although MT is generally considered to be cytosolic, its localization in the nucleus is known for several cell types and species (50–52). Concentration of MT in the nucleus is reported to be energy-dependent and a means for supplying zinc to this organelle (53). These studies place MT at a site with a high requirement for zinc and also where pathological conditions associated with cadmium-induced genotoxicity or carcinogenesis (54) and interactions with electrophiles such as antitumor.
Metallothionein and Zinc Finger Peptide of Tramtrack

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Drug (55) can occur. Furthermore, thionine is reported to exist in cells in at least one study (56). Our study described mechanisms whereby Zn-MT and thionin may interact with structures within the nucleus for the purpose of zinc regulation and metal detoxification.