mulation of proteins that severely reduce the digestibility and nutritional quality of the plant leaves to help ward off attacking pests (Ryan, 1983). The isolation and characterization of ATI and the acquisition of specific ATI antibodies are first steps in our efforts to isolate an ATI gene for further studies of wound-regulated expression in alfalfa leaves.

Acknowledgments

We thank Dr. Roger D. Wade for the amino acid analysis of the composite ATI samples and Dr. T. Gurusaddaiah for the amino acid analyses of the isoinhibitors. The help of Sally Combelic for growing the plants is gratefully acknowledged.

Registry No. Trypsin inhibitor, 9035-81-8; trypsin, 9002-07-7.

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Metal Substitution of *Neurospora* Copper Metallothionein†

Mariano Beltramini, Konrad Lerch,* and Milan Vašák

ABSTRACT: The binding of diamagnetic Zn(II), Cd(II), and Hg(II) and paramagnetic Co(II) and Ni(II) ions to the apo form of *Neurospora* metallothionein (MT) was investigated by various spectroscopic techniques. In contrast to native copper MT, which was shown to bind 6 mol of Cu(I)/mol of protein (Lerch, 1980), all substituted forms reveal an overall metal to protein stoichiometry of 3. The charge-transfer (CT) transitions of the complexes containing diamagnetic metal ions as well as the d–d transitions of those with paramagnetic metal ions are indicative of a distorted T_d coordination. Electron paramagnetic resonance and absorption measurements of the Co(II) derivative are in agreement with the presence of a metal–thiolate cluster in this protein. Metal titration studies of the apoprotein reveal characteristic spectral features for the derivatives containing two metal equivalents as compared to those with a full complement of three metal ions. The former features are indicative of an exclusive T_d type of metal–sulfur coordination whereas the latter suggest that the third metal ion is coordinated in a different fashion. This finding is in agreement with the presence of only seven cysteine residues in *Neurospora* MT as opposed to nine cysteine residues in the three-metal cluster of the mammalian MT's [Winge, D. R., & Miklossy, K.-A. (1982) *J. Biol. Chem. 257*, 3471].

Metallothioneins (MT's)[1] are a class of low molecular weight, cysteine-rich proteins binding metal ions like Cd, Zn, and/or Cu (Nordberg & Kojima, 1979). After the first isolation and characterization of a Zn- and Cd-containing MT from equine kidney (Kägi & Vallee, 1961), most of the attention was focused on the proteins isolated from mammalian species. These proteins are characterized by a single polypeptide chain containing 20 cysteines out of a total of 61 amino acids and lacking aromatic residues and histidine. Several physicochemical investigations (Kägi et al., 1974; Otvos & Armitage, 1980; Vašák et al., 1981a,b; Vašák & Kägi, 1981) have shown that the metal binding occurs in the form of a metal–thiolate complex, the metal ions being organized in two clusters with distorted tetrahedral coordination. Metal coordination in the form of a metal–thiolate cluster has been proposed recently also in the case of the Cu-MT isolated from *Neurospora crassa* (Beltramini & Lerch, 1983). *Neurospora* MT consists of only 25 amino acids and binds six Cu(I) ions to seven cysteinyl residues (Lerch, 1980). Although this protein is the smallest MT isolated so far, it shows a striking

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[1] Abbreviations: CD, circular dichroism; CT, charge transfer; EPR, electron paramagnetic resonance; LMCT, ligand to metal charge transfer; MT, metallothionein; apoMT, apometallothionein; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Comparison of Absorption and Chiroptical Maxima of Zn(II)-, Cd(II)-, and Hg(II)-Substituted MT's with Calculated Values Based on Optical Electronegativities for T_d Symmetry Complexes

metal derivative	Zn(II)-MT	Cd(II)-MT	Hg(II)-MT
position of the metal-induced shoulder in the difference spectrum			
λ (nm); $\tilde{\nu}$ (μm^{-1})	205; 4.88	225; 4.44	283; 3.53
$\Delta\epsilon \times 10^{-3}$ (M^{-1} cm^{-1})[a]	16.1	17.0	8.8
position of the first Cotton band			
λ (nm); $\tilde{\nu}$ (μm^{-1})	232; 4.31	243; 4.11	280; 3.57
$[\theta] \times 10^{-3}$ (deg cm^2 $dmol^{-1}$)[a]	5.0	8.4	2.1
calculated position of the LMCT band			
λ (nm); $\tilde{\nu}$ (μm^{-1})	232; 4.31	249; 4.02	312; 3.21
position of the metal-induced shoulder in mammalian MT[b]			
λ (nm); $\tilde{\nu}$ (μm^{-1})	220; 4.55	245; 4.08	308; 3.30

[a] $\Delta\epsilon$ and $[\theta]$ are calculated on a per mole of MT basis. [b] Data from Vašák et al. (1981a).

sequence homology to the NH_2-terminal part of mammalian MT's (Lerch, 1980). However, *Neurospora* MT differs from the proteins from mammalians in that it is induced only by copper and that it binds cuprous ions exclusively. In view of the striking sequence homology with the MT's from mammals, it was of interest to study the binding of Zn(II), Cd(II), and Hg(II) to the apo form of *Neurospora* MT. These studies were extended to the binding of the paramagnetic Co(II) and Ni(II) ions, which have been shown previously to be useful probes in the structural analysis of mammalian MT's (Vašák et al., 1982).

Materials and Methods

Neurospora MT was purified as previously described (Beltramini & Lerch, 1983). The apoprotein was prepared from the air-oxidized derivative (MT_{ox}) (Beltramini & Lerch, 1982). The copper was removed by Sephadex G-25 gel filtration in 50 mM HCl after incubation with 10^{-3} M EDTA for 2 h at 25 °C. The apoprotein contained <2% of the original copper. ApoMT was reduced by overnight incubation at pH 7.5 in the presence of dithioerythritol. The protein was freed from excess reagents with Sephadex G-25 in 50 mM HCl. The concentration of apoMT was determined spectrophotometrically with the extinction coefficient $\epsilon = 19\,500$ M^{-1} cm^{-1} at 220 nm in 50 mM HCl. The content of free sulfhydryl groups per protein molecule was estimated by using *p*-(chloromercuri)benzoate (Boyer, 1954) or 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959). The Cd and Zn derivatives were prepared by adding a 10-fold excess of metal to the apoprotein in 50 mM HCl. After the pH was adjusted to 8 with Tris base (Trizma), excess metal was removed by Chelex 100. The Hg(II) derivative was prepared according to Vašák et al. (1981a) and the Co(II) or Ni(II) one according to Vašák & Kägi (1981). Absorption spectra were recorded on a Hitachi Perkin-Elmer Model 340 spectrophotometer by using a solution of apoMT in 50 mM HCl at the same protein concentration as the reference. Spectrophotometric titrations were carried out in N_2-purged 50 mM Tris-HCl, pH 8.0. CD spectra were recorded on a Cary 61 spectropolarimeter. The ϵ, $\Delta\epsilon$, and $[\theta]$ values were calculated on a per mole of MT basis. Metal analyses of the native protein were performed by atomic absorption (flame mode) using an IL 157 (Instrumentation Laboratory) spectrophotometer. Reagents were of the best grade commercially available and were used without further purification. Buffer solutions were rendered metal free by Chelex 100.

Results

Zn(II), Cd(II), and Hg(II) Substitutions. When *Neurospora* apoMT is incubated with either Zn(II), Cd(II), or Hg(II) ions, derivatives showing a metal to protein stoichiometry of 3 are obtained. The derivatives elute on G-50 with an apparent M_r of 2700, when the carboxymethylated derivative of apoMT is used as a marker. In Figure 1A the absorption spectra of the protonated protein (apoMT) and those of the 2B metal ion substituted proteins are shown. Taking apoMT as a reference, the corresponding difference spectra of Zn-, Cd-, and Hg-MT show the presence of maxima at 205, 225, and 286 nm, respectively (Figure 1B; Table I). Figure 2A shows the CD spectrum of *Neurospora* apoMT. As already shown for the absorption spectra, binding of 2B metal ions to apoMT leads progressively to the appearance of new chiroptical features (Figure 2B–D) with the lowest energy CD band red shifted in the order of Zn < Cd < Hg. The dependency of the spectroscopic properties on the metal to protein ratio has been studied by titrating *Neurospora* apoMT with increasing amounts of either Cd(II) or Hg(II) (Figure 3). In the case of Cd(II) (Figure 3A), the intensity of the absorption when followed at 240 nm levels off at a Cd to MT ratio of about 2 and remains unchanged.

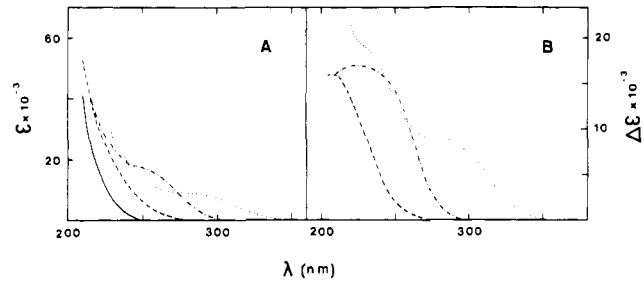


FIGURE 1: (A) Electronic absorption spectra of *Neurospora* apoMT in 0.1 M HCl (—) and derivatives containing Zn(II) (---), Cd(II) (–·–), and Hg(II) (···). (B) Difference absorption spectra obtained by subtracting the contribution of apoMT. Symbols as in (A). The spectra recorded apply to the metal derivatives containing three M(II) per metallothionein molecule. ϵ and $\Delta\epsilon$ values are calculated on a per mole of MT basis.

The Hg(II) titration followed at 280 nm reveals a plateau at a Hg to MT ratio of 2, but above 3 Hg(II) equiv added the absorption markedly decreases (Figure 3B). This change most likely reflects the formation of new species with a different structure for the Hg–S–Cys complex.

In Figure 4A, the absorption spectra for the Hg(II) derivative containing 1, 2, and 3 Hg(II) equiv/thionein are reported. The comparison of the spectra reveals that on going from 1 to 2 Hg(II) equiv bound to the protein, the absorption maximum at 280 nm undergoes a red shift to 283 nm. The main contribution of the third Hg(II) ion bound to thionein in the absorption spectrum occurs below 280 nm with no significant increase at 283 nm. Moreover, the CD spectra of the Hg(II)-containing derivatives show that the binding of the third

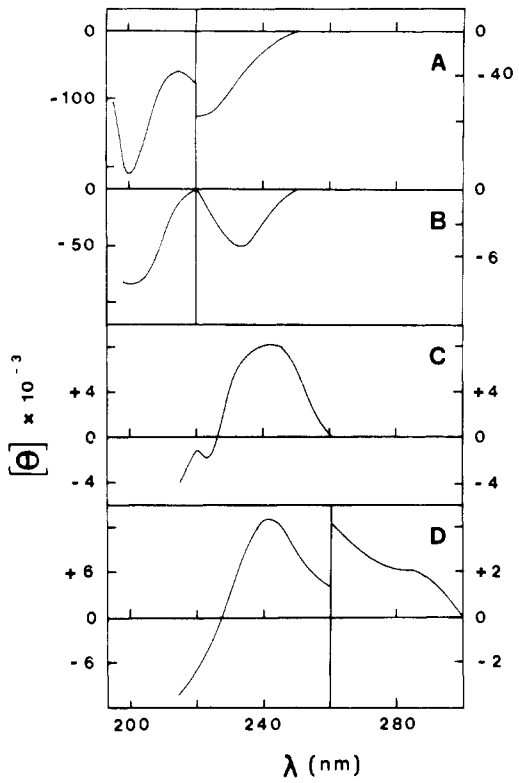


FIGURE 2: CD spectra of *Neurospora* apoMT (A) and derivatives containing Zn(II) (B), Cd(II) (C), and Hg(II) (D). Conditions as in Figure 1.

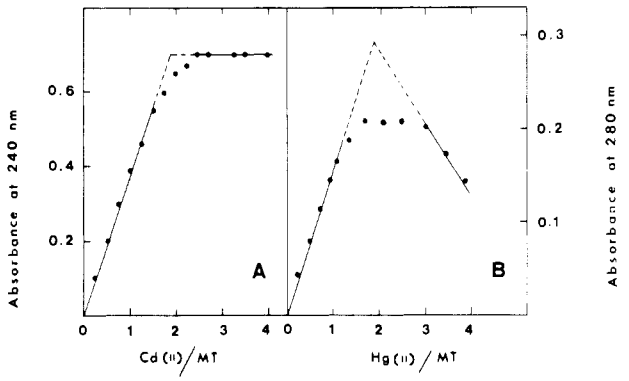


FIGURE 3: Spectrophotometric titration of *Neurospora* apoMT with Cd(II) (A) and Hg(II) (B). Protein concentration is 9 and 18 μM in (A) and (B), respectively. Cd(II)-MT and Hg(II)-MT indicate the Cd(II) or Hg(II) to MT molar ratios.

Hg(II) ion leads to an overall decrease in the chiroptical features of the complex (Figure 4B).

Co(II) and Ni(II) Substitutions. In order to probe for the geometry of the metal-binding sites in the complexes between *Neurospora* apoMT and divalent metal ions, advantage of the spectroscopic features of the paramagnetic Co(II)- and Ni(II)-substituted MT was taken. Similar to the diamagnetic MT derivatives, the paramagnetic ones have a metal to protein stoichiometry of 3. The absorption spectra of both derivatives reveal a number of relatively well-resolved bands and shoulders throughout the spectral region studied.

In Figure 5 is shown the electronic absorption spectrum of *Neurospora* Co(II)-MT. The fully saturated Co(II)-substituted form is characterized by well-resolved spin–orbit coupling components of the d–d transition very similar to those of Co(II)-substituted mammalian MT (Vašák et al., 1981b), which are located at 730, 685, and 610 nm, and by two absorption maxima at 364 and 300 nm in the CT region. The

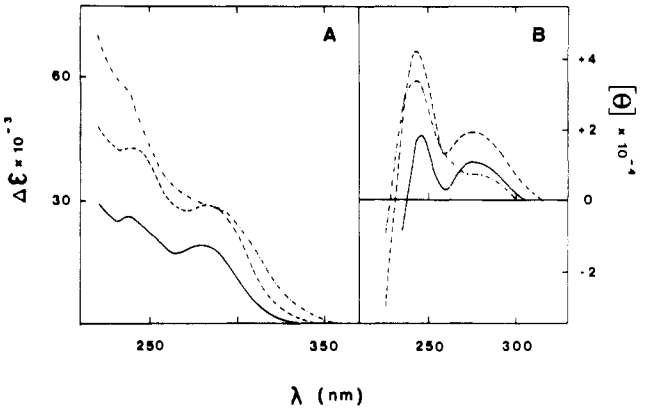


FIGURE 4: Difference electronic absorption (A) and CD (B) spectra of Hg(II)-substituted *Neurospora* apoMT. The spectra refer to the derivatives containing either 1 mol of Hg(II) (—), 2 mol of Hg(II) (---), or 3 mol of Hg(II) (–·–) per mole of MT. Spectra are recorded in 50 mM Tris-HCl, pH 7.8. The difference spectrum is recorded against an equimolar solution of apoMT in 0.05 M HCl as the reference. $\Delta\epsilon$ and [Θ] are calculated on a per mole of MT basis.

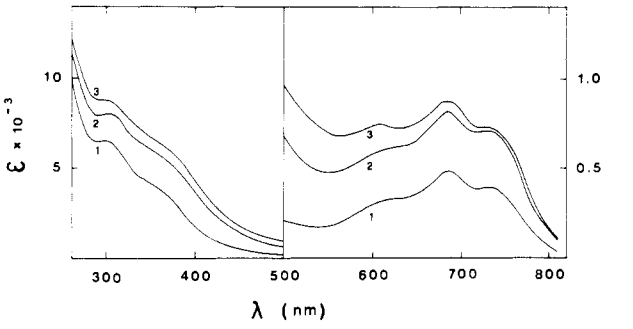


FIGURE 5: Electronic absorption spectra of Co(II)-substituted *Neurospora* MT in 0.05 M Tris-HCl, pH 8.0. The numbers refer to the spectra obtained for the derivatives containing one, two, and three Co(II) per MT molecule. ϵ is calculated on a per mole of MT basis.

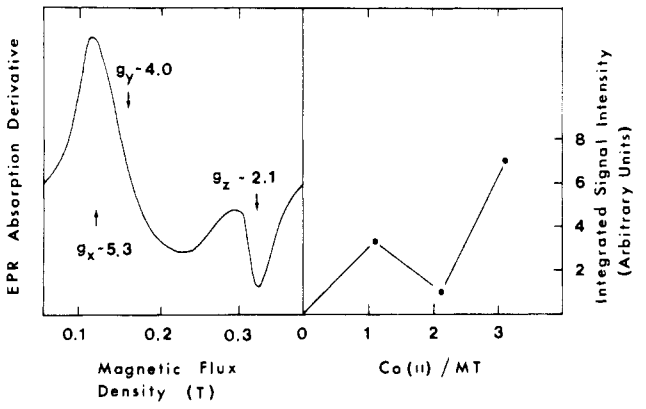


FIGURE 6: (Left) EPR spectrum of Co(II)-substituted *Neurospora* MT containing three Co(II) per MT molecule. Conditions: 0.53 mM Co(II)-MT in 0.05 M Tris-HCl, pH 8.0; microwave power 0.2 mW; microwave frequency 8.98 GHz; temperature 4 K. (Right) Dependency of the EPR signal intensity on the Co(II) to protein ratio. The intensity plotted is calculated by double integration of the observed signal. Co(II)-MT indicates the Co(II) to MT molar ratios.

low-temperature (4 K) EPR[1] spectrum (Figure 6, left) shows the typical features characteristic of a rhombically distorted high-spin Co(II) complex with the g values $g_x \sim 5.3$, $g_y \sim 4.0$, and $g_z \sim 2.1$.

For comparison, the spectral properties of the fully reconstituted *Neurospora* Ni(II)-MT [three Ni(II) per protein] were also studied. The absorption spectrum is shown in Figure 7A, displaying well-resolved shoulders at 570, 420, 315, and 253 nm. The CD spectrum (Figure 7B) shows several negative

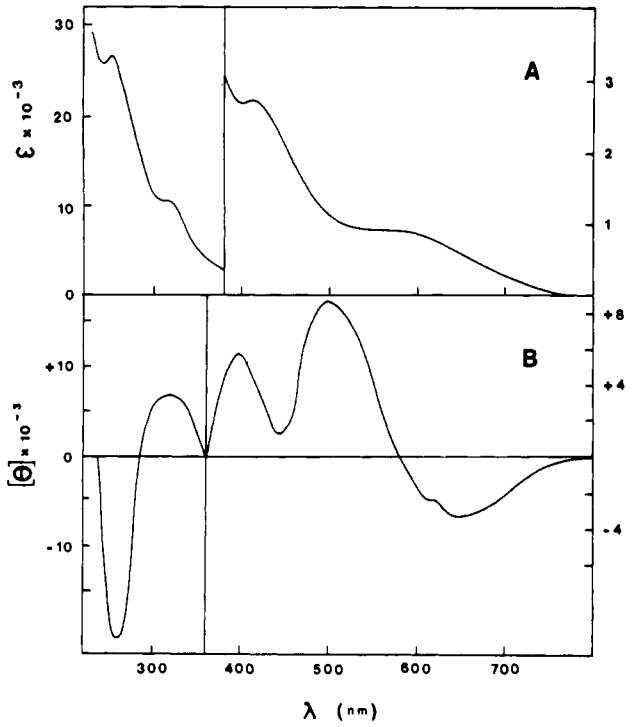


FIGURE 7: Electronic absorption (A) and CD (B) spectra of Ni(II)-substituted *Neurospora* MT in 0.05 M Tris-HCl, pH 8.0. ϵ and [Θ] are calculated on a per mole of MT basis.

Table II: Spectroscopic Properties of Co(II)- and Ni(II)-Substituted *Neurospora* MT

Co(II)-MT absorption[a]		Ni(II)-MT			
		absorption[a]		CD[a]	
nm	$\epsilon \times 10^{-3}$	nm	$\epsilon \times 10^{-3}$	nm	$[\Theta] \times 10^{-3}$
730	0.73				
685	0.87			650	−3.2
610	0.75	570	0.9	610	−2.3
364	5.91	420	2.8	505	+8.7
				405	+5.7
		315	10.7	330	+9.1
300	0.872			300	+5.7
		253	26.4	265	−23.4

[a] ϵ and [Θ] are calculated on a per mole of protein basis. The derivatives bind three metal ions per protein molecule.

and positive Cotton extrema, which are listed in Table II. In order to gain more insight about the process of filling the metal-binding sites as well as about the possible changes in the coordination geometry in the course of this process, the titration of *Neurospora* apoMT with Co(II) ions was investigated. On going from one to two Co(II) per MT, the most pronounced change in the electronic spectrum occurs in the CT region, which undergoes a bathochromic shift of ~4 nm. Moreover, the intensity of the bands in the d–d region is almost additive (Figure 5).

The molar absorptivity in the d–d region at 683 nm calculated per Co(II) is in the order of 450 M^{-1} cm^{-1} in both the derivatives containing either one or two Co(II) ions per protein molecule. However, it decreases to ~250 M^{-1} cm^{-1} upon binding of the third metal ion.

The EPR spectra of Co(II)-MT at different Co(II) to protein ratios display qualitatively the same overall spectral features as the fully saturated form, but they differ remarkably in intensity (Figure 6, right). Thus, the signal intensity of the derivative containing two Co(II) per MT is less than 30% of that measured for the species containing one Co(II) per MT. However, addition of a third Co(II) leads to a strong increase

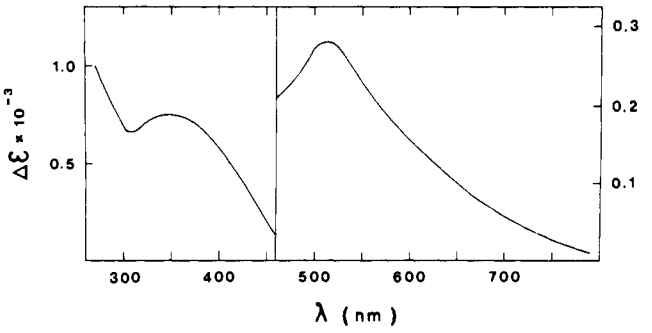


FIGURE 8: Difference absorption spectrum of Co(II)-MT containing three Co(II) per MT vs. an equimolar sample containing two Co(II) per protein. $\Delta\epsilon$ is calculated on a per mole of MT basis.

in the intensity of the overall EPR spectrum. The changes in the absorption spectrum accompanying the binding of the third Co(II) ion are documented in Figure 8. From the difference spectrum of the three Co(II) derivative vs. the two Co(II) derivative, it is seen that the third Co(II) ion bound gives rise to broad bands at ~520 (ϵ ~ 280 M^{-1} cm^{-1}) and ~350 nm (ϵ ~ 750 M^{-1} cm^{-1}).

Discussion

Zn(II), Cd(II), and Hg(II) Substitution. *Neurospora* apoMT is capable of binding group 2B metal ions like Zn(II), Cd(II), and Hg(II) in a complex with a metal to protein stoichiometry of 3:1. This stoichiometry is markedly different from that of the native protein, which was shown to bind six Cu(I) ions (Lerch, 1980). The elution position indicates that the group 2B metal-substituted derivatives behave as monomers.

In agreement with the lack of aromatic residues, apoMT is devoid of spectral features above 250 nm. The absorption band in the far-UV observed in apoMT can be attributed to π–π^* and n–π^* amide transitions (Gratzer, 1967). Similar to the binding of Cu(I) (Beltramini & Lerch, 1983) also the binding of group 2B metal ions to *Neurospora* MT intensifies the far-UV absorption. In addition, typical shoulders at the red edge of apoprotein band develop, whose positions change as a function of the metal bound. Similar Zn-, Cd-, and Hg-induced absorption shoulders are also observed in the corresponding thiolate model complexes and in mammalian MT's (Kägi & Vallee, 1961; Vašák et al., 1981a; Willner et al., 1983).

The similarity of the position of the metal-induced absorption shoulders with those of the corresponding derivatives of mammalian MT and complexes with 2-mercaptoethanol (Vašák et al., 1981a) documents their common origin in metal–thiolate coordination and is indicative of LMCT[1] transitions. As documented for mammalian MT's (Vašák et al., 1981a), the red shift of the band maxima observed on going from Zn and Cd to Hg *Neurospora* MT can be explained on the basis of the Jørgensen concept of optical electronegativity of the ions involved in the complex (Jørgensen, 1970). According to this theory, the predicted wavenumber σ (in μm^{-1}) of the first allowed LMCT transition of posttransition group complexes can be calculated as

$$\sigma = 3.0[\chi_{opt}(x) - \chi_{opt}(m)] \ (\mu m^{-1}) \qquad (1)$$

where $\chi_{opt}(x)$ and $\chi_{opt}(m)$ are the optical electronegativities of ligand x and of the metal m, respectively. Thus the position of the absorption maximum is expected to change for a set of complexes with the same ligand x and different metal ions differing in their optical electronegativities. The increase of

$\chi_{opt}(m)$ is a measure of the electron-withdrawing capability of the metal ion involved in the complex. As a consequence, the red shift of the maximum position is directly correlated with the increase in covalency of the ligand–metal bond. The positions of both absorption and chiroptical maxima of substituted *Neurospora* MT can be compared with the corresponding values calculated according to Jørgensen's theory. Thus, assuming $\chi_{opt}(S^-) = 2.6$ (McMillin, 1978) and $\chi_{opt}[Zn(II)] = 1.15$, $\chi_{opt}[Cd(II)] = 1.27$, and $\chi_{opt}[Hg(II)] = 1.50$ reported for tetrahedral metal–thiolate complexes (Vašák et al., 1981a), the corresponding LMCT bands in Zn, Cd, and Hg *Neurospora* MT are expected at 232, 249, and 312 nm, respectively. However, the difference maxima of Zn(II), Cd(II), and Hg(II) *Neurospora* MT vs. apoMT appear to be blue shifted (see Table I) with respect to the expected values and to those observed earlier for the mammalian MT's (Vašák et al., 1981a). In the case of mammalian MT's, the spectral envelopes in the far-UV have been resolved into the contributions of at least three Gaussian components. The corresponding first LMCT transitions were attributed to the type $t_2 \rightarrow a_1$ in T_d symmetry. Although the absorption maxima of Zn-, Cd-, and Hg-MT appear to the blue shifted, the better resolution of transitions at energies corresponding to the expected values comes from the CD spectra of these derivatives (Table I).

As documented for the absorption properties, the chiroptical features of apoMT can be attributed to the amide transitions of the polypeptide backbone. The comparison of the spectra of apo- and metal-substituted *Neurospora* MT indicates large contributions arising from metal–protein interactions. The CD features of the polypeptide backbone in *Neurospora* apoMT are similar to the one observed for the CD spectra of random coil structures, indicating the lack of substantial secondary structure. However, as shown in the case of the native *Neurospora* MT (Beltramini & Lerch, 1983), the lack of a regular polypeptide structure in the apoprotein does not preclude the formation of a well-defined tertiary structure in the metal-containing derivatives. Binding of 2B metal ions to *Neurospora* MT introduces CD features throughout the spectral region under study. Hence, an evaluation of the metal-induced conformational change is excluded.

The lowest energy Cotton extrema of the Zn, Cd, and Hg *Neurospora* MT derivatives undergo a progressive bathochromic shift on going from the Zn to the Hg derivative. The molecular origin of the metal-induced chiroptical features of substituted *Neurospora* MT as well as those of the native form (Beltramini & Lerch, 1983) can be attributed to asymmetric structures of the multidentate metal–mercaptide complexes. Moreover, the better resolution in CD of the metal-induced transitions allows the identification of the lowest energy bands as the corresponding transitions calculated on the basis of Jørgensen's optical electronegativity theory. The observed maxima are in fairly good agreement with the positions calculated by using $\chi_{opt}(m)$ values for complex with tetrahedral symmetry (Table I).

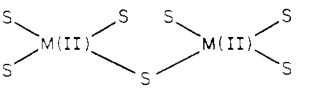
On these grounds, a tetrahedral geometry and a metal–thiolate coordination are suggested for the binding of divalent metals by *Neurospora* MT. Although the protein is able to bind three metal ions per molecule, titration data show that the complexes formed with two or three metal ions are spectroscopically different, indicating two different coordination modes. This idea is supported not only by spectroscopic titrations with both Cd(II) and Hg(II) showing a break point at a metal to protein ratio of 2 but also from the spectral comparison of the Hg(II) derivatives containing one, two, or three Hg(II) per MT molecule. Both the electronic absorption and CD spectra of the complexes containing either 1 or 2 equiv of Hg(II) have the same shape, and their intensities are almost additive. However, the spectra recorded differ markedly. Thus, the change of the absorption spectrum is accompanied by a sharp decrease of the chiroptical features (Figure 4).

Co(II) and Ni(II) Substitution. Binding of Co(II) to *Neurospora* apoMT leads to the appearance of well-resolved absorption bands in the d–d region of the spectrum. The features observed in the case of either one or two Co(II) bound are typical for a tetrahedral Co(II)–thiolate coordination and closely resemble those reported for inorganic tetrathiolate–Co(II) complexes (McMillin et al., 1974; Anglin & Davison, 1975; Dance, 1979) and for Co(II)-substituted metalloproteins with four cysteine ligands (Drum & Vallee, 1970; Maret et al., 1979) and rabbit Co(II)-MT (Vašák & Kägi, 1981; Vašák et al., 1981b).

The resolved band pattern can be assigned to the spin-allowed $\nu_3[^4A_4(F) - {}^4T_1(P)]$ transition. The large splitting of this transition with an energy separation of the components of 1800 and 900 cm^{-1} suggests distortion from T_d symmetry. Moreover, the molar absorptivity of the main d–d component at 685 nm is also compatible with tetracoordination of the Co(II) ion (Bertini et al., 1982). In the Ni(II) derivative of *Neurospora* MT (Figure 7A) the persistence of a substantial absorption at 750 nm ($\epsilon \sim 150$ M^{-1} cm^{-1}) suggests that Ni(II) ions, too, are bound in a nonplanar geometry (Lever, 1968), reflecting a situation similar to that found in rabbit MT (Vašák et al., 1981b).

In addition to the d–d bands discussed above, the remaining absorption bands of *Neurospora* Co(II)- and Ni(II)-MT can be attributed to CT transitions (Vašák et al., 1981b). The band at 300 nm seen in Co(II)-MT can be attributed to a S $\rightarrow$ Co(II) CT transition (McMillin et al., 1974). The position of the positive bands at 330 and 405 nm observed in the CD spectrum of *Neurospora* Ni(II)-MT (Figure 7B) closely corresponds to the one reported for the CD profile of nickel(II) azurin (Tennent & McMillin, 1979), whose tetrahedral metal-binding site consists of two nitrogen and two sulfur ligands. The origin of the high-energy CD bands at 300 and 265 nm, observed also in rabbit Ni(II)-MT, remains to be established.

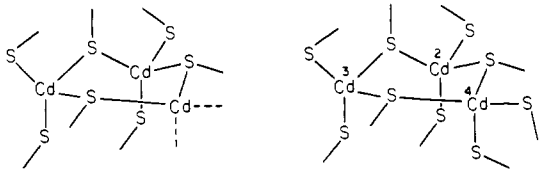
Independent evidence for the distorted tetrahedral coordination geometry is given by the EPR spectra of *Neurospora* Co(II)-MT, strongly resembling that of a rhombically distorted high-spin Co(II) complex (Aasa et al., 1976). The remarkable loss of signal strength on going from one to two Co(II) per MT is compatible with an antiferromagnetic spin coupling of the metal ions mediated by a bridging thiolate ligand (Vašák & Kägi, 1981). This idea is supported also by the red shift ($\sim$3–5 nm) observed in the CT maxima of the absorption spectrum (Vašák & Kägi, 1981). A similar shift has been observed also in the case of the Hg titration experiments on going from the one to two Hg(II) derivative (see above). From the data presented herein, a tentative model describing the structure of the binuclear metal cluster in *Neurospora* MT can be depicted as follows:



The third metal ion is bound in a different fashion and/or by different ligands, as shown by the increase of the EPR signal and by new absorption features documented by the difference absorption spectrum of the three Co(II) minus two Co(II) derivatives. This view is also supported by the results of the

Cd(II) and Hg(II) titrations of apoMT (see above). In this case the marked spectral changes observed on going from two to three metal ions per protein molecule occur predominantly in the far-UV region of the absorption spectra, thus leaving the region of the first CT S → M(II) transitions (Table I) almost unchanged. Although the ligation and the geometry of the third Co(II) ion in Co(II) MT are not yet clear, the presence of the difference absorption maximum at 350 nm, a position consistent with a S → Co(II) CT transition (McMillin et al., 1974), suggests the involvement of at least one thiolate group in the complex.

Mammalian Cd-MT's have been shown recently to be composed of two distinct metal clusters: cluster B containing three metal ions involves the first 9 cysteinyl residues of the NH_2-terminal part; cluster A involves the remaining 11 cysteines binding four metal ions (Otvos & Armitage, 1980; Winge & Miklossy, 1982). In view of the striking sequence homology of *Neurospora* Cu-MT with the NH_2-terminal part of the mammalian MT's, it is tempting to compare the structure of the three metal ion cluster (Boulanger et al., 1983) with the one of Cd-substituted *Neurospora* MT. As is shown in structures a and b, the major difference between the two



clusters resides in the total number of sulfur ligands. This difference most likely accounts for the observed difference in the coordination mode of the third metal ion bound in *Neurospora* MT.

In conclusion, *Neurospora* apoMT is capable of binding divalent metal ions in a fashion analogous to that of the mammalian MT's. The first two metal ions are bound exclusively to the thiol residues as a coupled binuclear complex with distorted tetrahedral geometry. The third metal ion, however, is bound in a different environment, reflecting the smaller number of thiol ligands in *Neurospora* MT as compared to the three-metal cluster of the mammalian MT's.

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Registry No. Zn, 7440-66-6; Cd, 7440-43-9; Hg, 7439-97-6; Co, 7440-48-4; Ni, 7440-02-0.

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