Articles

Spectroscopic Studies on *Neurospora* Copper Metallothionein†

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ABSTRACT: The spectral properties of *Neurospora* copper metallothionein were investigated and compared with those of the Cu(I)–2-mercaptoethanesulfonic acid complex. In both cases, the absorption spectra are rather similar, showing a characteristic shoulder at ~250 nm. However, marked differences were observed in their emissive properties. Thus, only metallothionein emits detectable luminescence in solution, but both the copper protein and the Cu(I) complex are luminescent at 77 K. The circular dichroism spectrum of *Neurospora* copper metallothionein shows several Cotton extrema attributable to asymmetry in metal coordination. The influence of $HgCl_2$ and *p*-(chloromercuri)benzoate on the spectral properties of metallothionein was also investigated. The two mercurials exerted a pronounced effect on the electronic absorption, chiroptical, and emissive properties of the protein. Spectroscopic titrations followed by gel filtration experiments indicate that two mercurials can be bound per metallothionein molecule without loss of copper. This binding is responsible for the disappearance of the emissive properties of metallothionein and for the distinct changes in its electronic absorption and circular dichroism spectra. From these data, it is suggested that the Cu(I) ions are coordinated to the cysteinyl residues in the form of a single metal cluster.

Metallothioneins are a class of low molecular weight, cysteine-rich proteins binding metal ions like Cd, Zn, and/or Cu. These proteins have been obtained from a large variety of organisms including invertebrates and microorganisms (Nordberg & Kojima, 1979). Their wide occurrence in nature suggests that they are involved in important biological functions. Besides a detoxification (Piscator, 1964; Nordberg, 1972) and storage function (Bremner & Davies, 1975; Rydēn & Deutsch, 1978), metallothioneins were also proposed to be involved in metal transfer to apometalloproteins (Udom & Brady, 1980; Li et al., 1980; Beltramini & Lerch, 1982; Geller & Winge, 1982). After the first isolation and characterization of a Zn- and Cd-containing metallothionein from equine kidney (Kägi & Vallee, 1961), most of the attention was focused on the proteins isolated from mammalian species, and comparative studies have stressed some structural features shared by all metallothioneins (Nordberg & Kojima, 1979). 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. Mammalian metallothioneins typically bind 7 mol of Zn and/or Cd per mol of protein, and all cysteinyl residues were shown to be involved in metal binding (Kägi & Vallee, 1961; Weser et al., 1973). Comparative sequence studies have demonstrated that the positions of the cysteines are strongly conserved during the evolution, supporting the idea that they are essential in determining a very specific metal binding mode. On the basis of recent spectroscopic and chemical investigations, mammalian metallothioneins are proposed to bind the metal ions in two separate metal clusters in which each ion is tetrahedrally coordinated to four cysteinyl residues (Otvos & Armitage, 1980; Vašāk et al., 1981a,b; Vašāk & Kägi, 1981; Winge & Miklossy, 1982; Briggs & Armitage, 1982; Otvos et al., 1982). In contrast to the metallothioneins of higher eucaryotic organisms, binding different metal ions, fungal metallothioneins contain exclusively copper. Weser et al. (1977) isolated from yeast a copper metallothionein containing 10 mol of metal per mol of protein (M_r 10000). X-ray photoelectron spectrometry has indicated the copper to be bound as Cu(I)–thiolate complexes, and on the basis of recent extended X-ray absorption fine structure (EXAFS)[1] studies (Bordas et al., 1982), a tetrahedral geometry was proposed for the metal binding sites. Although the yeast protein shares with other metallothioneins several physicochemical properties, no sequence homology to the vertebrate metallothionein was detected (Kimura et al., 1981).

From the ascomycete *Neurospora crassa*, a small copper metallothionein has been recently isolated (Lerch, 1980). The protein contains 7 cysteinyl residues out of a total of 25 amino acids and binds 6 mol of copper per M_r 2200. All the cysteines are involved in metal binding. In contrast to yeast metallothionein, this protein showed a striking sequence homology to the mammalian Cd/Zn binding metallothioneins. In particular, the positions of the seven cysteinyl residues are identical with those of the seven cysteines in the N-terminal region of the mammalian metallothioneins (Lerch, 1980). The binding of copper was shown to be responsible for a typical red-shifted luminescence which can be attributed to the presence of Cu(I)–thiolate complexes (Beltramini & Lerch, 1981). *Neurospora* metallothionein differs markedly from the other proteins, including yeast, as its copper to sulfur ratio approaches 1 in contrast to values of $^1/_3$ and $^1/_2$ reported for mammalian and yeast metallothioneins, respectively (Nordberg & Kojima, 1979; Bordas et al., 1982). In the present paper, we report spectroscopic data obtained on *Neurospora* copper metallothionein in the absence and in the presence of spectroscopic perturbants.

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[1] Abbreviations: PCMB, *p*-(chloromercuri)benzoate; CD, circular dichroism; MESNA, 2-mercaptoethanesulfonic acid; Hg^{2+}/MT, molar ratio of $HgCl_2$ to metallothionein; PCMB/MT, molar ratio of *p*-(chloromercuri)benzoate to metallothionein; EXAFS, extended X-ray absorption fine structure; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Materials and Methods

Neurospora copper metallothionein was isolated from copper-exposed cells grown as described by Lerch (1980). The following steps of the purification procedure were all carried out at 4 °C. The lyophilized mycelium (20 g) was disrupted with a Waring blender and the powder extracted with 0.1 M sodium phosphate, pH 7.2 (200 mL). The suspension was centrifuged for 30 min at 20000*g*. The supernatant was then applied to a Sephadex G-50 column (5 × 150 cm) equilibrated in nitrogen-purged 10 mM Tris-HCl, pH 8.0. The copper-containing fractions eluting at an apparent molecular weight of 2500 were pooled and immediately applied to a DE-32 column (2.5 × 8 cm) equilibrated in 10 mM Tris-HCl, pH 8.0. Copper metallothionein was eluted with a linear gradient by using 250 mL each of 10 mM Tris-HCl, pH 8.0, and 10 mM Tris-HCl, pH 8.0, plus 0.1 M NaCl. The protein was stored at −80 °C under argon. Protein concentrations were determined by metal analysis using a metal content of 6 mol of copper per mol of protein (Lerch, 1980). For spectroscopic measurements, the protein was rechromatographed on a Sephadex G-25 column in 20 mM potassium phosphate, pH 7.5. Buffer solutions were rendered metal free by passage through Chelex-100. Chemicals were of the best grade commercially available and used without further purification. The Cu(I) complex with 2-mercaptoethanesulfonic acid (MESNA) was prepared according to Vortisch et al. (1976).

Spectroscopic Measurements. Absorption spectra were recorded in a Hitachi Perkin-Elmer Model 340 recording spectrophotometer. Circular dichroism (CD) spectra were recorded with a Cary 61 spectropolarimeter using 1-cm cells. The ϵ, $\Delta\epsilon$, and $[\theta]$ values were calculated on a per mole of metallothionein basis in the case of the protein and on a per mole of copper basis in the case of Cu(I)–MESNA. Fluorescence measurements were made with a Perkin-Elmer MPF-2A spectrofluorometer operating in the ratio mode. If not otherwise indicated, excitation at 305 nm was used. The emission intensities were corrected for the inner filter effect by using the coefficient X (Chignell, 1972):

$$X = \text{antilog}\ \frac{A_{exc}}{2} \qquad (1)$$

where A_{exc} is the absorbance at the excitation wavelength. Emission spectra at 77 K were recorded in a 1 to 1 buffer–glycerol mixture. All the spectroscopic measurements were carried out at 10 °C. Metal analyses were performed on an IL 157 atomic absorption spectrophotometer (Instrumentation Laboratory) using acid-washed glassware.

Titration with Spectroscopic Perturbants. The changes in absorption, emissive, and chiroptical properties of metallothionein upon addition of $HgCl_2$ and *p*-(chloromercuri)-benzoate (PCMB) were followed by adding stepwise increasing amounts of ligand. Absorption spectra in the presence of perturbants were recorded by using a solution of an equal concentration of native metallothionein as a reference. As PCMB shows some spectral features in the region where metallothionein absorbs, titration was performed by using a pair of double-chamber quartz cuvettes as described by Herskovits & Laskowski (1962).

Results

Spectroscopic Properties of the Native Protein. Neurospora copper metallothionein (MT) shows an absorption spectrum characterized by a very broad band displaying a shoulder at 250 nm (Figure 1A solid line). No absorption features are observed in the visible region. In Figure 1A (dashed line) is

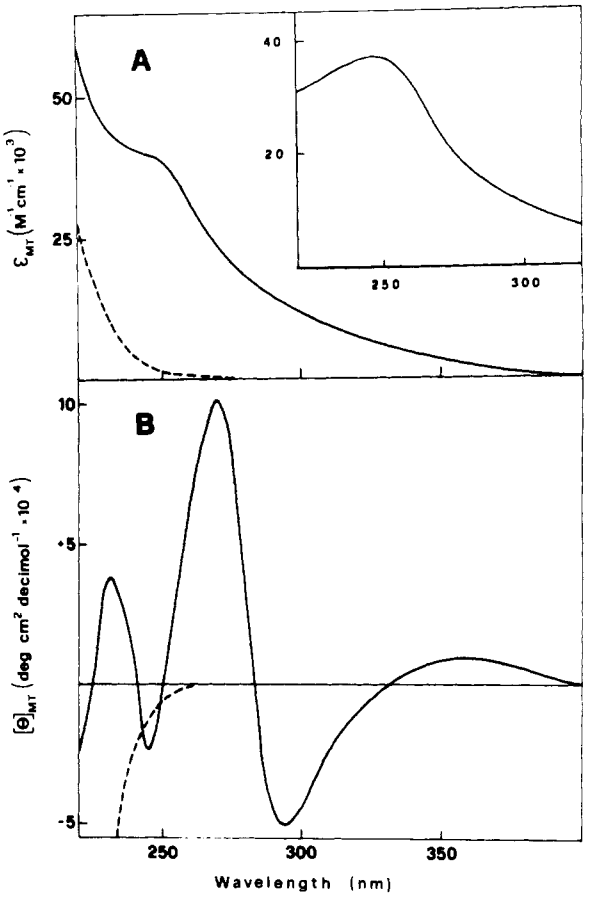

FIGURE 1: Electronic absorption (A) and CD (B) spectra of *Neurospora* copper metallothionein. Solid lines, native protein; dashed lines, apometallothionein; inset, difference absorption spectrum of metallothionein vs. apometallothionein. For details, see Materials and Methods.

reported the absorption spectrum of apometallothionein (apo-MT), obtained by metal displacement with HCl (pH ~0.5). It shows no absorption above 260 nm and lacks the shoulder at 250 nm. The difference spectrum obtained for MT vs. apo-MT is shown in the inset of Figure 1A. In contrast to the rather featureless absorption spectrum, the circular dichroism spectrum of the native protein reveals several positive and negative Cotton extrema located at 230(+), 245(−), 270(+), 295(−), and 355(+) nm (Figure 1B, solid line). As shown above for the absorption features, the chiroptical properties of the protein vanish upon removal of the metal (Figure 1B, dashed line). *Neurospora* metallothionein was reported to emit, upon excitation at 305 nm, an unusual orange-yellow luminescence centered at 565 nm with a quantum yield of 0.013 at 10 °C (Beltramini & Lerch, 1981). The protein also strongly luminesces when irradiated in a buffer–glycerol glass at 77 K, displaying the same spectral features as in solution. At room temperature, the emission intensity is sensitive to the presence of oxygen and to the medium viscosity. In fact, the quantum yield is 2.5 times larger in solutions equilibrated with argon or in a 1 to 1 glycerol–buffer mixture. The effect of oxygen is reversible, and no change in the absorption spectrum is observed. The luminescence intensity depends on the nature of the solvent. Substitution of H_2O with D_2O in solution brings about an increase of emission intensity of ~50%. These results are summarized in Table I.

Spectroscopic Properties of the Cu(I)–MESNA Complex. To get more information about the electronic and geometric structure of the Cu(I)–thiolate complex of copper metallo-

Table I: Quantum Yields (Q) of *Neurospora* Copper Metallothionein under Different Conditions

condition[a]	Q
air, H_2O	0.013[b]
air, D_2O	0.026
argon, H_2O	0.033
50% glycerol, in air	0.033

[a] 20 mM potassium phosphate buffer, pH 7.5, 283 K. [b] Data from Beltramini & Lerch (1981).

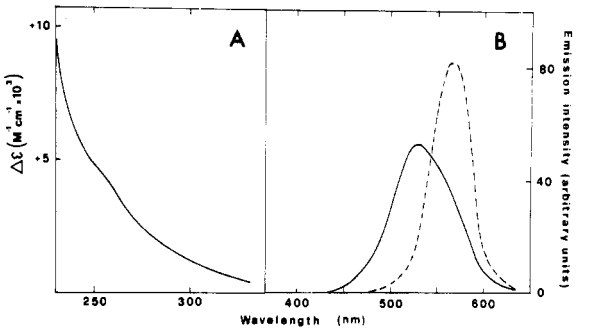

FIGURE 2: Spectroscopic properties of the Cu(I)–MESNA complex. (A) Difference absorption spectrum of Cu(I)–MESNA vs. MESNA. (B) (Solid line) Emission spectrum (λ_{exc} = 305 nm, 77 K); (dashed line) emission spectrum of *Neurospora* copper metallothionein (λ_{exc} = 305 nm, 77 K). The metal concentration in MESNA is 4.4 mM and in metallothionein 0.9 mM.

thionein, we compared the spectroscopic properties of *Neurospora* metallothionein with those of Cu(I)–MESNA. This complex has been characterized in great detail (Vortisch et al., 1976) and is soluble in aqueous solutions. In Figure 2 are reported the difference absorption (A) and luminescence (B) spectra of the Cu(I)–MESNA complex. As shown for *Neurospora* copper metallothionein, the difference absorption spectrum Cu^+–MESNA vs. MESNA shows a broad band displaying a shoulder at ~250 nm. The Cu^+–MESNA complex does not luminesce appreciably in solution; however, in a glass at 77 K, a strong band at 520 nm with a shoulder at 570 nm becomes apparent (λ_{exc} = 305 nm) (Figure 2B, solid line). For comparative purposes, the emission spectrum of copper metallothionein at 77 K is also reported (Figure 2B, dahsed line).

Spectral Perturbations of Copper Metallothionein. Addition of $HgCl_2$ to a solution of native MT brings about several perturbations in its absorption, chiroptical, and emissive properties. As the changes in the absorption spectrum are rather small, it is convenient to measure the spectra of native MT in the presence of Hg^{2+} vs. a sample of native MT at the same concentration. The difference spectra show signals whose shape and intensity change for the different Hg^{2+} to MT ratios (Hg^{2+}/MT) used (Figure 3A,B). At $0 < Hg^{2+}/MT \leq 2$, the difference spectra obtained show a negative ($\lambda_{max} \sim 255$ nm) and a positive band ($\lambda_{max} \sim 300$ nm), the former decreasing and the latter increasing in intensity. Two isosbestic points are evident (λ = 272 and 244 nm; Figure 3A, solid and dashed lines). With higher Hg^{2+} concentrations, the difference spectrum drastically changes. At $2 < Hg^{2+}/MT \leq 4$, both the positive and the negative bands decrease in intensity with a concomitant loss of the isosbestic points (Figure 3A, dotted line). Finally at Hg^{2+} concentrations equal to or larger than the copper content in the native protein, a completely positive difference spectrum is observed with a maximum progressively shifted toward 280 nm (Figure 3B). The results of the spectrophotometric titration are also shown in Figure 4A where the $\Delta\epsilon$ values, calculated at 300 nm, are plotted against the Hg^{2+}/MT ratios. In this plot, the intersection of the two straight lines indicates a value of 2 as a critical Hg^{2+}/MT ratio. As expected, the circular dichroism spectrum of native MT is also affected by the presence of $HgCl_2$. During the titration at $0 < Hg^{2+}/MT \leq 2$, the main spectral changes observed are not only a decrease in intensity but also a progressive shift of the two major CD bands at 270 and 295 nm toward 255(+) and 280(−) nm, respectively, with the appearance of an isosbestic point at 295 nm (Figure 5). After addition of more than 2 mol of Hg per mol of native metallothionein, these bands decrease in intensity until the spectrum shown in the inset of Figure 5 (solid line) is reached at a mercury concentration equal to the copper content in the native MT. The dashed line of the inset of Figure 5 describes the spectrum observed at a Hg^{2+}/MT ratio of 3. The luminescence properties of *Neurospora* metallothionein are very sensitive to $HgCl_2$. The emission spectrum of the native protein is progressively quenched by addition of $HgCl_2$. This quenching does not involve a shift in the maximum position and is linearly correlated to the Hg^{2+}/MT ratio. After addition of 1 mol of $HgCl_2$ per mol of protein, 98% of the luminescence intensity is quenched (Figure 6A). This effect is independent of the protein concentration.

Spectral Perturbations of Copper Metallothionein: PCMB Titration. The difference absorption spectra of *Neurospora* copper metallothionein obtained in the presence of PCMB show changes in shape and intensity at the different PCMB/MT ratios analogous to those documented above for

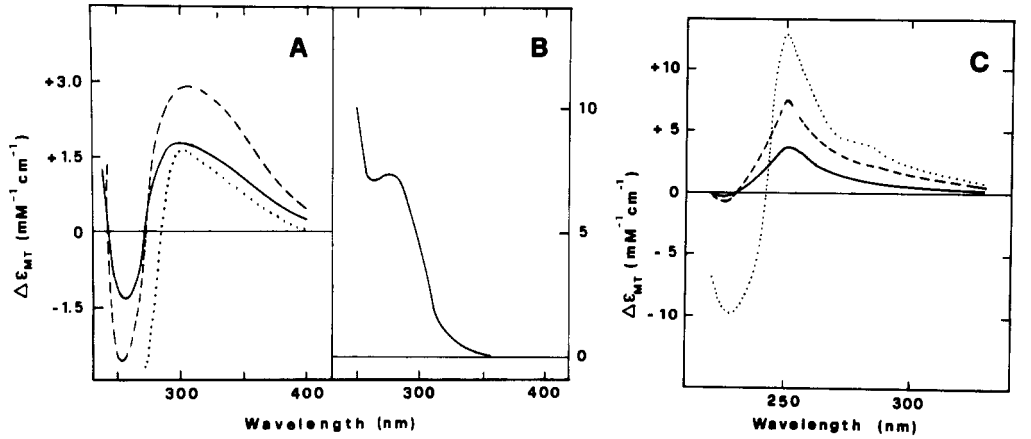

FIGURE 3: Difference absorption spectra of *Neurospora* copper metallothionein in the presence of $HgCl_2$ (A and B) and PCMB (C). For details, see Materials and Methods. (A) Hg^{2+}/MT = 1.0 (solid line), Hg^{2+}/MT = 2.0 (dashed line), Hg^{2+}/MT = 4.0 (dotted line); (B) Hg^{2+}/MT = 6.0; (C) PCMB/MT = 1.0 (solid line), PCMB/MT = 2.0 (dashed line), PCMB/MT = 6.0 (dotted line).

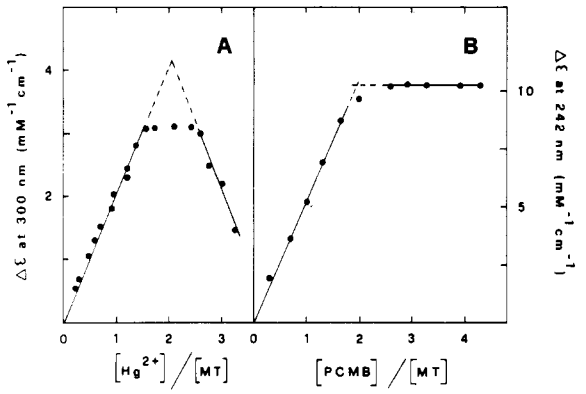

FIGURE 4: Absorption titration of *Neurospora* copper metallothionein with $HgCl_2$ (A) and PCMB (B). For details, see Materials and Methods.

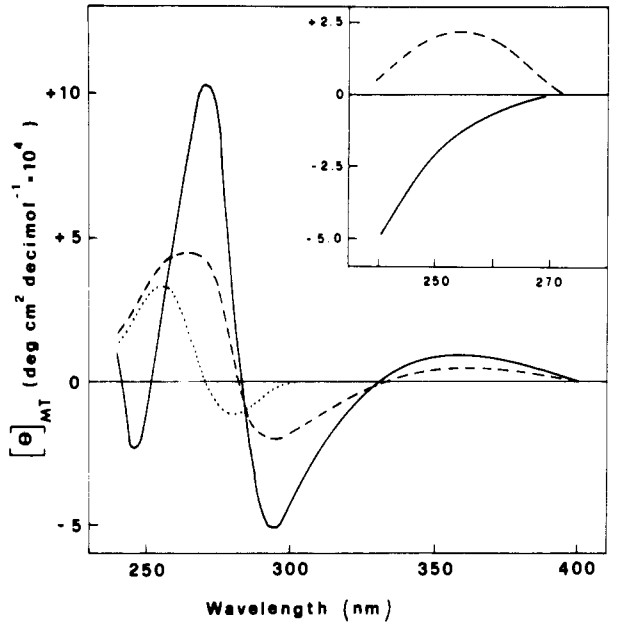

FIGURE 5: Circular dichroism spectra of *Neurospora* copper metallothionein in the absence (solid line) and in the presence of Hg/MT = 1.0 (dashed line), Hg/MT = 2.0 (dotted line), Hg/MT = 3.0 (dashed line, inset), and Hg/MT = 6.0 (solid line, inset). For details, see Materials and Methods.

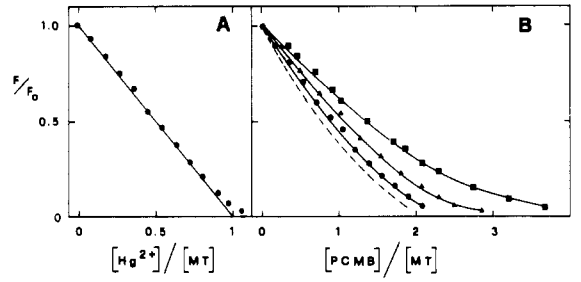

FIGURE 6: Luminescence quenching of *Neurospora* copper metallothionein with $HgCl_2$ (A) and PCMB (B). The solid line in (A) represents the plot expected in the case of proportionality between Hg^{2+}/MT and the quenching effect; the closed circles represent the experimental points. In (B), the symbols are relative to metallothionein concentrations of 8.0 (■), 19.33 (▲), and 38.67 μM (●). The dashed line corresponds to the calculated plot when the protein concentration is extrapolated to infinity. F_0 and F are the emission intensities in the absence or in the presence of quencher, respectively, measured at the emission maximum (λ = 565 nm).

$HgCl_2$. At $0 < PCMB/MT \leq 2$, the difference spectra are characterized by a broad positive (λ_{max} = 250 nm) and a small negative band (λ_{max} = 225 nm). Increasing concentrations

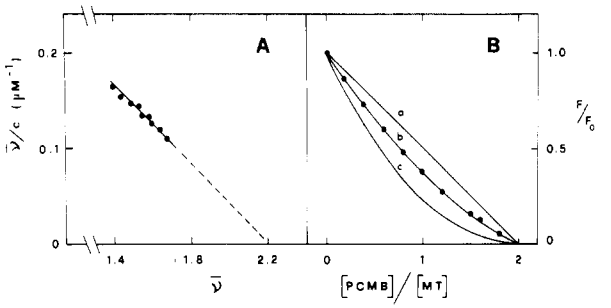

FIGURE 7: Binding of PCMB to *Neurospora* copper metallothionein. (A) Scatchard plot obtained from the data of Figure 6B according to the method of Halfman & Nishida (1972); $\bar{\nu}$ represents the moles of PCMB bound per mole of metallothionein; c is the concentration of free PCMB. (B) Comparison of the experimental binding data with the "random binding model" of Lehrer (1969). The different plots are calculated by assuming (a) $K_1 = {}^1/_2K_2$, (b) $K_1 = {}^3/_4K_3$, and (c) $K_1 = K_2$ (see text). The closed circles represent the experimental points calculated from the experiment of Figure 6B, extrapolating the protein concentrations to infinity. F and F_0 are the emission intensities in the presence and in the absence of quencher, respectively, measured at the maximum of emission (λ = 565 nm).

of PCMB lead to an increase of the positive band and a decrease of the negative band with the appearance of two isosbestic points (λ = 230, 220 nm) (Figure 3C, solid and dashed lines). When the PCMB/MT ratio is >2, the negative 225-nm difference band increases drastically with a concomitant shift of its maximum toward higher wavelengths and the loss of the isosbestic points (Figure 3C, dotted line). Following the titration at a convenient wavelength (242 nm), it is possible to define the value PCMB/MT = 2 as the critical point where further additions of PCMB do not increase the absorbance (Figure 4B). As observed for $HgCl_2$, PCMB leads to a quenching of the emissive properties of native MT. In this case, however, the decrease in luminescence intensity is not linearly correlated with the PCMB/MT ratio (Figure 6B), and the quenching effect depends on the protein concentration used (Figure 6B). The dashed line in Figure 6B describes the quenching process calculated for PCMB at a protein concentration extrapolated to infinity. Note that in this case too the PCMB titration cannot be fitted by a straight line and the value required for a complete quenching is 2 mol of PCMB per mol of protein.

The binding phenomenon can be described by means of a Scatchard plot where the parameters involved are $\bar{\nu}$ (molar ratio of PMCB bound to protein) and c (molar concentration of free PCMB). These parameters can be calculated from experiments at different MT concentrations (Figure 6B) by using the method of Halfman & Nishida (1972).

The Scatchard plot describes the dependency of $\bar{\nu}/c$ against $\bar{\nu}$ according to

$$\frac{\bar{\nu}}{c} = -\frac{\bar{\nu}}{K_d} + \frac{n}{K_d} \qquad (2)$$

where $\bar{\nu}$ and c are as defined above. K_d is the dissociation constant of the PCMB–MT complex, and n is the number of binding sites in MT. In a Scatchard plot, the intercept on the abscissa gives n and that on the ordinate the value n/K_d from which K_d can be easily calculated. From the Scatchard plot calculated for PCMB binding to *Neurospora* copper metallothionein (Figure 7A), the values obtained are n = 2.2 and $K_d = 4.86 \times 10^{-6}$ M.

The luminescence quenching process was further treated according to the binding models proposed by Lehrer (1969). The MT molecule can be considered to have two metal binding sites, a and b, and the binding of one molecule of PCMB to

one site leads to a certain extent of quenching.

In general, for any addition of a mercurial, the luminescence emitted by the solution is

$$F = F_0P(0) + F_{1a}P(1)/2 + F_{1b}P(1)/2 + F_2P(2) \quad (3)$$

where $P(0)$, $P(1)$, and $P(2)$ represent the fractions of molecules with 0, 1, and 2 PCMB molecules bound, respectively, and F_0, F_{1a}, F_{1b}, and F_2 are the corresponding values of luminescence intensity. F_{1a} and F_{1b} are treated separately because the quenching related to the binding to one site is not necessarily the same for the second one. According to Lehrer's model (1969), in the case of random binding the fractional fluorescence emitted is

$$F/F_0 = (1 - K_1)/\phi + (2K_1 - K_2)\phi^2/4 \quad (4)$$

where $K_1 = (F_0 - F_1)/F_0$ and $K_2 = (F_0 - F_2)/F_0$ with $F_1 = (F_{1a} + F_{1b})/2$ and ϕ is the PCMB/MT molar ratio. From eq 4, it is possible to generate a number of curves by assuming different values of K_1 and K_2. The curves shown in Figure 7B are calculated by assuming (a) $K_1 = {}^1/_2K_2$ in the case where the binding to each site contributes equally to the quenching, (b) $K_1 = {}^3/_4K_2$ is an intermediate case, and (c) $K_1 = K_2$ in the case where the total quenching is attributable to the binding to the first site.

Discussion

Comparison of the absorption spectra of native and fully protonated *Neurospora* copper metallothioneins (apometallothionein) shows that binding of copper increases the absorption in the near- and far-UV and introduces the characteristic feature at 250 nm. The position of this shoulder is, however, different in yeast and fetal liver copper metallothioneins occurring in these proteins at ~270 nm (Weser et al., 1977; Hartmann & Weser, 1977). A similar shoulder is also observed in the absorption spectrum of the Cu(I)–MESNA complex attributable to copper–thiolate complexation. In contrast to the poorly resolved absorption spectrum of *Neurospora* copper metallothioneins, the circular dichroism measurements reveal a high degree of asymmetry in the protein chromophore. As pointed out above for the absorption features, the five characteristic Cotton extrema can again be attributed to metal complexation because the chiroptical properties are absent in the apoprotein.

Both copper metallothionein and Cu(I)–MESNA are luminescent in a glass at 77 K, showing an emission maximum in the region of 500–600 nm. Luminescence emission from Cu(I) complexes involving nitrogen or thiol ligands has been observed either at room temperature in dried samples (Anglin et al., 1971) or at 77 K in a glass (Buchner & Mc Millin, 1978). As these metal complexes generally interact with the solvent, the luminescence emission is expected to be strongly quenched in solution. On grounds of such a solvent collisional effect, it is possible to explain the lack of detectable emission in Cu(I)–MESNA solutions at room temperature. Furthermore, the considerable emission intensity of *Neurospora* copper metallothionein in aqueous solutions at 10 °C could be explained on the basis of partial shielding of the luminescent copper complex from solvent vibrations. A shielding effect could be elicited by the polypeptide backbone which would decrease the rate of the solvent collisons. This rate is also expected to be influenced by the medium viscosity, resulting in a higher quantum yield in glycerol–buffer mixtures. Finally, the increased quantum yield in D_2O–buffer is also consistent with a reduced frequency of collision between solvent and protein, due to the higher molecular weight of D_2O. In addition, as shown by the oxygen quenching of the luminescence, not only the solvent but also dissolved oxygen can be involved in the deactivation process. A similar effect was also reported for the emission of the Cu(I)–CO complex of hemocyanins (Finazzi-Agrõ et al., 1982).

The peptide backbone in *Neurospora* metallothionein is rather short and simple: it consists of only 25 residues lacking completely the bulky and hydrophobic aromatic residues. Therefore, it is rather surprising to observe an extended shielding effect on the luminophore. This shielding effect could be, however, brought about by a compact tertiary structure of the protein elicited by metal complexation.

The spectral properties of metallothionein are strongly affected by the presence of mercurials; however, the changes are qualitatively different in the case of $HgCl_2$ or PCMB. Hg^{2+} is able to quench completely the emission of metallothionein at a $HgCl_2$/MT molar ratio of 1, namely, at a concentration one-sixth of the copper concentration in the protein. The linear dependency of the quenching vs. the Hg^{2+}/MT ratios and the spectral changes observed in absorption and circular dichroism spectra indicate binding of the Hg^{2+} ions to the protein. A strong binding of $HgCl_2$ to metallothionein is expected on the basis of the high affinity of the metal for sulfhydryl groups.

However, as shown by gel filtration experiments, no copper is lost upon binding of two mercurials. At a Hg^{2+}/MT ratio >2, the loss of copper correlates with the appearance of a difference signal with a maximum around 280 nm, typical for tetrahedral mercury thiolate coordination (Jörgensen, 1980). The results of the absorption and CD titrations show an inflection point at an Hg^{2+}/MT ratio of 2, indicating the presence of two mercury binding sites. Marked differences are observed for PCMB quenching of metallothionein luminescence. The decrease of emission intensity is not a linear function of the PCMB/MT ratio. A Scatchard plot of the binding data shows the presence of two binding sites for PCMB characterized by a rather weak binding constant. Moreover, the binding model indicates that the two sites are independent of each other with respect to binding probability (Lehrer, 1969). The fit of the experimental data with the curve $K_1 = {}^3/_4K_2$ suggests that binding of PCMB to each site does not contribute equally to the quenching. The difference absorption spectrum is dominated by an intense positive band with a maximum at 250 nm typical of PCMB–thiol complexation (Boyer, 1954). The results of the spectrophotometric titration, showing an inflection point at a PCMB/MT ratio of 2, are in agreement with the quenching data.

The apparent discrepancy between the luminescence quenching behavior of the two mercurials investigated can be explained by assuming in both cases that there are two binding sites but that the binding of Hg^{2+} to one site affects the emissive properties of both sites. The difference in the quenching behavior reflects the difference in the coordination number between Hg^{2+} (2) and PCMB (1). Moreover, from the absorption and CD data, two binding sites for $HgCl_2$ are evident.

Taken together, the spectral perturbation data lead us to suggest that the six Cu(I) ions coordinated to the seven cysteine residues in *Neurospora* copper metallothionein are behaving as a single metal cluster. In the case of the mammalian Zn- and Cd-containing metallothioneins, the metal ions were shown to be organized in clusters where the thiolate groups are bridging two tetrahedrally coordinated metal ions (Otvos & Armitage, 1980; Vašák et al., 1981a,b; Vašák Kägi, 1981; Otvos et al., 1982; Briggs & Armitage, 1982). In the case of *Neurospora* copper metallothionein, the experimental data are in agreement with a polymeric copper (I) μ-thiolate structure

earlier proposed on the basis of the metal to sulfur stoichiometry (Lerch, 1980). In this structure, the six copper ions are complexed to five cysteinyl groups, bridging two Cu(I) ions each, and to two terminal cysteinyl residues, coordinating a single Cu(I) ion. The two monocoordinated terminal cysteines are thought to provide additional binding sites for the mercurials $HgCl_2$ and PCMB. This copper binding mode is also in line with the spectroscopic data of the Cu(I)–MESNA complex, which is known to form polymeric μ-thiolate chains (Vortisch et al., 1976). However, the observed differences in the emission behavior between Cu(I)–MESNA and metallothionein and the presence of distinct chiroptical features in the protein strongly suggest that the protein backbone has a marked influence on the emission intensity and the asymmetry of the metal binding sites in *Neurospora* copper metallothionein.

Registry No. Cu, 7440-50-8; Cu(I)–MESNA, 84849-51-4; $HgCl_2$, 7487-94-7; PCMB, 59-85-8.

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