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# Spectroscopic characterization of rat kidney Hg,Cu-metallothionein

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Absorption, circular dichroism (CD), magnetic circular dichroism (MCD) and emission spectra are reported for rat kidney Hg,Cu-metallothionein isoform 3 isolated following induction of the metallothionein with $HgCl_2$. While the absorption spectrum is featureless, both the CD and MCD spectra show resolved bands that arise from the Cu-thiolate and Hg-thiolate groups. The emission spectrum at 77 K is much more complicated than would be expected for a copper(I)-containing metallothionein. It is suggested the emission only arises from the copper-thiolate groups but that the presence of the mercury results in copper ions in several different environments depending on the nature of the nearest neighbour.

While the binding of cadmium and zinc to metallothioneins (MT) isolated from the livers and kidneys of animals has been well documented by a variety of spectroscopic techniques using MT metallated both in vivo and in vitro (1-10), there are far less data available for MT containing other metals. Metallothionein containing copper, cobalt, mercury, lead and silver can be prepared in vitro by substitution using Cd,Zn-MT at neutral pH, or by remetallation of the metal-free protein prepared at low pH (1,2,6,7,11,13-16). $^{113}Cd$ nmr results (3,5) suggest that metal binding in MT takes place in two clusters, A and B, in which bridging thiolate groups are used to increase the maximum number of metal ions that can be bound. In rabbit liver MT the two clusters appear to be able to bind 4 and 3 metal ions, respectively. However, it is not clear yet whether both clusters are always fully populated, some spectroscopic evidence obtained from cobalt loading experiments (13,17) suggests that metal binding does take place in which bridging is not used when the metal loading is less than the maximum

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possible in the cluster. It is also unclear whether the distribution of two or more different metals between the two clusters is always same when metallation takes place in vivo. Finally, it is not known whether metallation in vitro by different experimental techniques, ie. metal replacement near pH 7 or metallation of metal-free MT at a low pH followed by neutralization, result in metal binding that is the same as for an MT in which metallation took place in vivo. Comparison of the data from metal titration studies carried out in vitro with the data obtained from native protein samples that contain two different metals can give valuable information about the distribution of metal ions between the two clusters.

In this paper we report the first optical study of a native mercury- and copper-containing metallothionein isolated from rat kidneys following induction of the protein with mercuric chloride.

## Experimental

A low molecular weight, mercury-binding protein (Hg,Cu-MT) was isolated from the kidneys of rats exposed to $HgCl_2$ (1 mg Hg/kg). Mercury was applied subcutaneously every second day over 14 days; the protein was purified according to Zelazowski et al. (18). Polyacrylamide gel electrophoresis of the protein was performed according to Jovin et al. (19); the protein was separated into three isoforms of $R_m$: 0.35, 0.57 and 0.80 (20). Each of these isoforms contained considerable amounts of mercury and copper. Isoform 1 contained: 2.0 g at Hg/mol, 2.2 g at Cu/mol and 0.5 g at Zn/mol; isoform 2 contained: 2.0 g at Hg/mol, 3.6 g at Cu/mol and 0.4 g at Zn/mol; and isoform 3 contained: 1.8 g at Hg/mol, 4.4 g at Cu/mol and 0.2 g at Zn/mol (20). The protein isoform with $R_m$ = 0.80 ,Hg,Cu-MT 3, was used for the spectroscopic studies described in this paper.

Absorption spectra were recorded on a Cary 219, circular dichroism (CD) and magnetic circular dichroism (MCD) spectra were recorded using a highly modified Jasco J5 which used a Morvue optical modulator, an Ithaco 391A Lock-In amplifier and an Oxford Instruments (UK) SM2 superconducting magnet operating at 5.5T. The CD intensity was calibrated with d-10-camphorsulfonic acid [$\Theta_{289}/A_{291}$=+226 (21)], while the MCD intensity was calibrated using an aqueous solution of $CoSO_4$[at 505nm $[\theta]_M$ = -61.6 deg $cm^2$ $mol^{-1}{}_T{}^{-1}$ or $\Delta\epsilon_M$ = 1.87 x $10^{-2}{}_L$ $mol^{-1}cm^{-1}{}_T{}^{-1}$]. All data were digitized by computer. Emission spectra were recorded at 77 K using a Perkin Elmer MPF4 fluorimeter operating in the ratio mode. The spectrometer was equipped with a Hamamatsu R928 phototube; this tube has a very good red response and an almost flat quantum efficiency between 230 nm and 500 nm.

## Results and Discussion

The absorption, CD and MCD spectra for Hg,Cu-MT 3 are shown in Fig. 1. Clearly there is little detail in the absorption spectrum. The MCD and CD spectra indicate the presence of several transitions, with the major

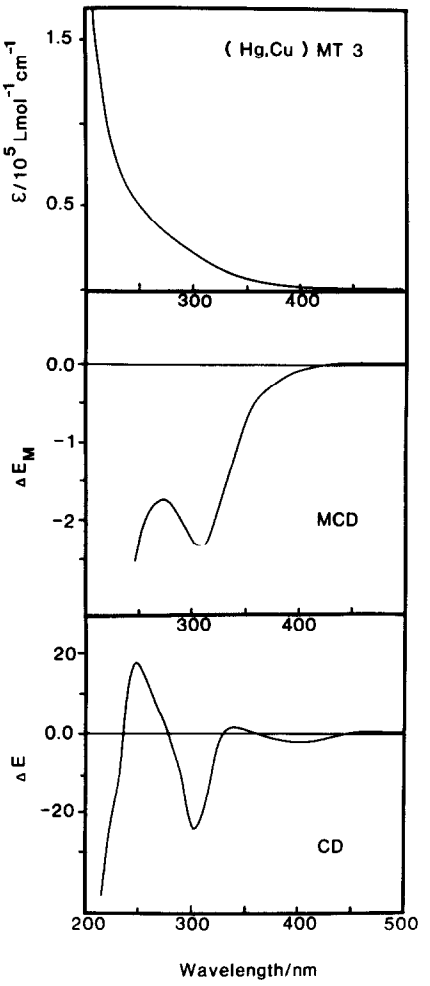


Figure 1. Absorption, MCD and CD spectra of Hg,Cu-MT 3. The units of $\Delta\varepsilon_M$ are L mol$^{-1}$ cm$^{-1}$T$^{-1}$, and the units of $\Delta\varepsilon$ are L mol$^{-1}$ cm$^{-1}$.

bands at 305 nm (negative CD and MCD signals) and 255 nm (a positive CD signal). The absorption spectrum is reminiscent of spectra from other Hg- and Cu-containing metallothioneins (1,6,11,12). We can suggest a tentative identification of the chromophores responsible for the CD and MCD features in the Hg,Cu-MT spectra by examining the reported spectra of other Hg- and Cu-containing species.

The CD spectra of Cu-thionein (2), copper-saturated Cd,Zn-MT (12,22) and Cu-MT from Neurospora crassa (8), each exhibit the following general features: there is a positive band between 240 nm and 280 nm, a negative band near 290 nm and a very weak positive band above 300 nm. The CD

spectra which have been reported for metallothioneins containing $Hg^{2+}$ bound in vitro (8,11,13,15) are considerably less intense than the data recorded for the Hg,Cu-MT, this is especially apparent in the titration of Cd,Zn-MT with $Hg^{2+}$ (11). We assign, therefore, the major CD bands in Fig. 1 as arising from the copper binding sites.

No MCD spectra have been reported previously for either a native Cu- or Hg-MT. The MCD spectrum of copper-saturated rat liver Cd,Zn-MT (22) exhibits a rather weak, broad band at 270 nm, while in native rat kidney Cd,Cu-MT the MCD spectrum closely resembles that of the cadmium-thiolate group in Cd,Zn-MT (23). This suggests that the MCD envelope of the copper-thiolate group may exhibit little intensity, which may not be clearly resolved when other metals are bound to the MT. MCD spectra are also available for Hg-MT prepared in vitro by titration of rat liver and horse kidney Cd,Zn-MT, and bismuth-induced Zn-MT with $HgCl_2$ (11,13,23), for complexes between $Hg^{2+}$ and BAL (22) and for some tetrahalomercurates (24), in each of these spectra we observe a distinct derivative-like signal in the 240 nm - 350 nm region. We do not see any evidence for a band of this shape in the data for the Hg,Cu-MT. Indeed, the MCD band at 310 nm most closely resembles a Faraday B term (25); in this feature the band centres of the MCD and absorption transition are coincident, which implies, in the absence of a degenerate ground state, that the transition is nondegenerate. This leads us to the suggestion that the MCD spectrum is dominated by mercury-sulfide charge transfer transitions, and that the mercury may occupy a site that is not tetrahedral. In titrations of bismuth-induced Zn-MT with $HgCl_2$ (23), a spectrum can be recorded when both $Zn^{2+}$ and $Hg^{2+}$ are bound to the metallothionein, under these loading conditions the MCD spectrum does not exhibit the derivative-shaped signal. It is possible that the geometry of the binding sites in this Hg,Zn-MT resembles those in the Hg,Cu-MT.

Fig. 2 shows the emission spectrum of Hg,Cu-MT in an aqueous glycerol solvent recorded at 77 K. Unlike the single-banded spectrum of Cd,Cu-MT (10), of Cu-MT from Neurospora crassa (26) or of copper complexes

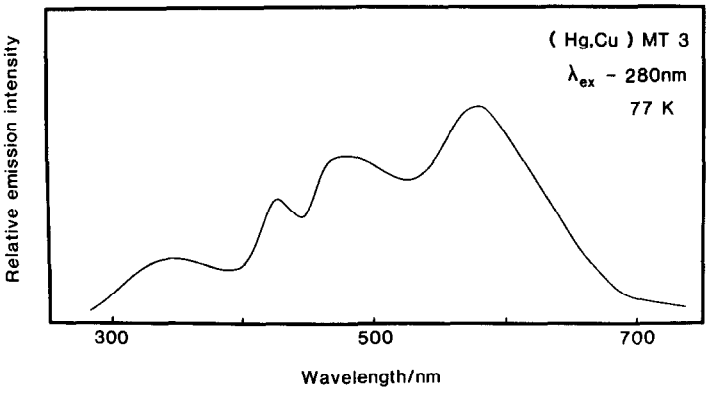


Figure 2. Emission spectrum of Hg,Cu-MT 3 at 77 K. The exciting wavelength was 280 nm.

with thiol-containing ligands (27), we see a complex series of bands between 400 nm and 700 nm. The prominent band near 580 nm is assigned as arising from the $Cu^+$-thiolate groups in the protein as its energy is almost identical to the red band in the Cd,Cu-MT (10) and Cu-MT (26). The weak 340 nm and 430 nm bands are found in the 77 K emission spectra of all the metallothioneins that we have studied and their intensities appear to be independent of the metal bound to the MT. The new feature observed in the emission spectrum of the Hg,Cu-MT is the broad band near 480 nm. We do not believe that this band arises from Hg emission as the intensity diminishes if $HgCl_2$ is added to the sample. At present we suggest that the intensity above 450 nm arises solely from the copper thiolate-groups in the MT. Because the Cu/Hg ratio is approximately 2:1 there will be a distribution of environments for the $Cu^+$ within the two clusters. Thus some $Cu^+$ ions may be in tetrahedral sites with no adjacent $Hg^{2+}$, while other $Cu^+$ ions are in sites which have bridging sulfides bound to both $Cu^+$ and $Hg^{2+}$ ions. There is some evidence that the energy of the red band in $Cu^+$ emission does depend quite significantly on the nature of the chelating ligands (27). We suggest that the different environments for the $Cu^+$ results in two main bands, one at 580 nm and the other near 500 nm.

The emission spectrum of isoform 1 at 77 K is very weak and only exhibits a band at 500 nm, in this isoform the Cu/Hg ratio is close to 1. This suggests that although the overall effect of $Hg^{2+}$ as a nearest-

neighbour to a $Cu^+$ ion is to quench the emission intensity, as in the $HgCl_2$ titration of Cu-MT (8), the energy of the emitting state is also perturbed quite considerably and the band centre shifts to 500 nm. Coupling between adjacent $Co^{2+}$ ions bound through bridging thiolates in MT has been suggested as the explanation for the loss of EPR intensity at high metal loading concentrations (13), and it does not appear to be unreasonable that there should be large differences in the energies of the $Cu^+$ emitting states between $Cu^+$ linked through a thiolate bridge to a $Hg^{2+}$ ion rather than another $Cu^+$ ion.

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