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A luminescence probe for metallothionein in liver tissue: emission intensity measured directly from copper metallothionein induced in rat liver

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We report the first use of an emission probe based on the Cu(I)-thiolate chromophore, for the direct observation of copper metallothionein located in samples of rat liver. Elevated synthesis of Cu-MT in the rat liver was induced by subcutaneous injections of a series of aqueous $CuCl_2$ solutions containing increasing amounts of Cu(II). Luminescence intensity in the 600 nm region, detected from frozen solutions of Cu-MT and from slices of the liver frozen at 77 K, following excitation in the 300 nm region, was dependent on the concentration of the Cu(II) used in the inducing solution. No such luminescence intensity was found for control samples obtained from the livers of rats not exposed to copper salts. It is suggested that this new method will allow direct visualization of Cu-MT in tissue where genetic disorders impare copper metabolism.

Metallothionein; Copper binding; Light emission; Wilson's disease; (Rat liver tissue)

1. INTRODUCTION

The protein metallothionein (MT) is found in all mammalian organs [1,2]. While the specific role of metallothionein has not been defined, elevated concentrations of the protein are detected in the liver and kidneys following exposure to the ions of cadmium, zinc and copper [1]. Metallothionein concentrations are also increased in human livers of subjects suffering from Wilson's or Menke's diseases [3,4]. About 50-80% of all Cu is bound to MT in Wilson's patient's liver [5] and to MT stimulated in liver by the injection of Cu salts [6]. An error in copper metabolism is also considered to be the cause of a debilitating, hereditory disease of Bedlington Terriers; high levels of copper have been found in the livers of these dogs [7]. Very special cluster binding has been demonstrated for cadmium and zinc [8-14], and has been suggested for copper and mercury [12,15,16]. Species with the stoichiometric ratios of 7 and 12, illustrated by Cd_7-MT, Cu_{12}-MT and Hg_7-MT, have been characterized spectroscopically [12,13,16], while X-ray diffraction studies on the Cd_5,Zn_2-MT complex [9] have been completed. It has been shown that Cu-MT, in which copper(I) is chelated by thiolate groups, emits orange light when illuminated with UV light at room temperature and below [12,17-19]. This Cu(I)-MT luminescence is predominantly cluster dependent [12]. Existing methods of detecting excess copper in liver by staining for Cu are unreliable because both Zn and Fe interfere [20].

We now report the first use of this emission probe for the direct observation of copper metallothionein in tissue samples from rat liver and human liver. With excitation of the Cu-MT complexes in the 300 nm region we avoid the interference in tissue from a strong, porphyrin absorption which has a maximum near 400 nm. The very large Stoke's shift places the Cu-MT emission near 600 nm, which is to the red of the natural luminescence from the tissue, which would otherwise completely block the observation of the Cu-MT emission. We find that the intensity of the emission from rat liver depends on the concentration of Cu(II) in the inducing solution.

2. EXPERIMENTAL

Copper-metallothionein synthesis was induced in female rats that were injected with aqueous $CuCl_2$ solution, seven times, every second day, over a two week period, with doses of 1, 2, and 3 mg of Cu per kg body wt. We have found no reports of the specific induction of other copper(I)-containing proteins in liver tissue following a regimen such as this, when the animals are fed a normal diet. The control rat was subcutaneously injected with saline solution instead of the $CuCl_2$ solution. The animals were killed under phenobarbital narcosis, and the kidneys and livers removed and immediately frozen in liquid nitrogen. Copper in the liver was determined using AAS methods, after digestion of the tissue in a mixture of HNO_3 and $HClO_4$. Metallothionein was estimated using the mercury binding method [22]; whereas the concentration of mercury was determined by AAS. The method was calibrated by adding a standard solution of metallothionein which contained 6 Cu ions per molecule of MT. For a 3 mg/kg body wt regimen, the copper and MT levels were significantly enhanced in liver tissue. A value of 58 ± 10 μg of Cu

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and 134 ± 30 μg of MT per g of wet liver tissue was found in the liver of 3 mg-Cu-treated rats, which can be compared to the values of 2.0 ± 0.4 μg of Cu and 41 ± 1 μg of MT per g of wet liver tissue that were determined for the control rat.

Frozen human liver samples were obtained from Dr M.G. Cherian, Department of Pathology, University of Western Ontario. The liver from the Wilson's disease patient had 197 ± 2 μg of Cu and 629 μg of MT per g of wet tissue, while the control liver contained 5.1 ± 0.2 μg of Cu and 536 μg of MT per g of wet tissue.

The distribution of copper and MT in the rat liver samples was analysed by gel chromatography. The analysis of the eluate showed that an estimated 65% of the total copper is bound to metallothionein. The increased copper and MT concentrations are in complete agreement with previous studies on the inducement of Cu-MT in rat livers [6–21]. It has been reported [6] that on average 5–6 Cu and 0.5 Zn are bound to metallothionein located in the livers of rats that have been injected with copper salts.

Control spectra (not shown here) were measured using Cu-MT prepared from Zn_7-MT, which was isolated from rabbit liver following the in vivo induction procedures described previously with Zn^{2+} salts [22]. A 20 nmol/ml solution of Zn_7-MT (isoform 2) in water, was titrated with 6 mol equivalents of Cu^+ ($Cu(CH_3CN)_4$ in 70% water/acetonitrile solution) to form the mixed-metal, Zn,Cu-MT, with increasing fractions of copper(I) bound to the MT. Solutions of the Zn,Cu-MT complexes were frozen and the emission spectra measured at 77 K. The human and rat livers were sliced and transferred into a quartz Dewar filled with liquid nitrogen. Emission spectra and excited state lifetime data were recorded using Perkin-Elmer MPF 4 and Photon Technology Inc. LS1 spectrofuorometers. The spectral data were manipulated and plotted on an HP 7550A plotter using the data management program Spectra Manager [23].

3. RESULTS AND DISCUSSION

Fig.1 shows the uncorrected emission spectra from liver isolated from a rat that had been treated only with saline. The spectra were obtained as a function of excitation wavelength, from a sample of rat liver, frozen at 77 K, after excitation in the range of 280–350 nm. Fig.2 shows the corresponding emission spectra obtained from liver tissue isolated from a rat treated with a solution containing 2 mg/kg body wt. (Emission from a rat treated with 1 mg/kg copper was much less intense, but still significantly greater than for the control). Clearly, new and intense luminescence grows when the rat is treated with copper. This band exhibits a maximum near 605 nm when excited with light of 310 nm wavelength. Significantly, there is minimal luminescence observed from the liver sample isolated from the rat exposed solely to saline when this exciting wavelength is used. The copper-induced emission band closely resembles that reported for Cu-MT formed in vitro [19]. The dips near 550 nm in the emission intensity recorded following excitation at 310 nm, for the '2 mg/kg' tissue, represent reabsorption of the emitted light by heme proteins in the tissue. This is not a problem further to the red (560–700 nm) because protoporphyrin IX does not absorb light in this region, so the intensity of the main emission band is relatively unaffected.

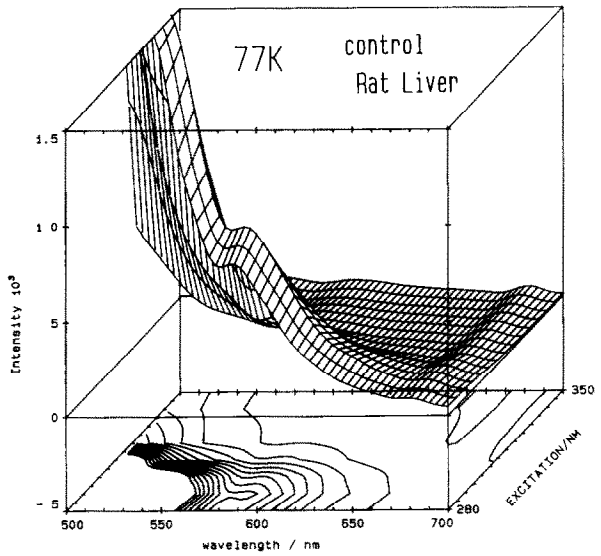

Fig.1. Uncorrected emission spectra from a whole liver sample, isolated from a control rat, after excitation in the range of 280–350 nm at 77 K.

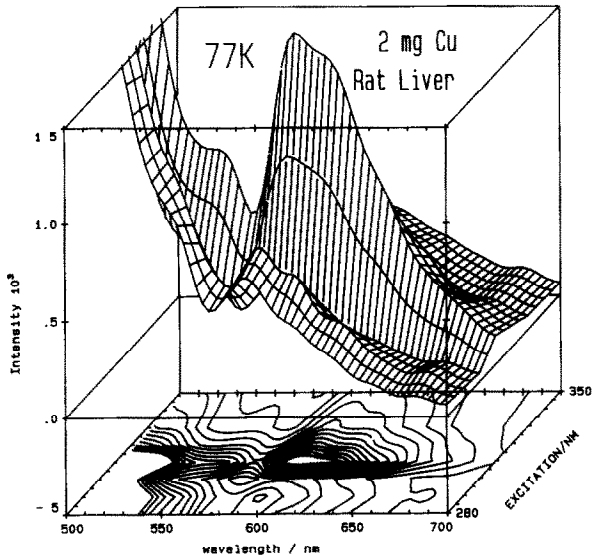

Fig.2. Uncorrected emission spectra from a whole liver sample, isolated from a copper-treated (2 mg/kg body wt) rat, after excitation in the range of 280–350 nm at 77 K.

Enhanced emission intensity was also observed when the emission from liver tissue isolated from a Wilson's disease patient was compared with tissue isolated from a human subject not affected by Wilson's disease. We believe that the additional emission intensity in the 550 to 600 nm region from the Wilson's disease liver also arises from CuMT.

Our previous studies [12,19] of the emission spectra of rat liver Cu-MT 2 formed in vitro, have established that the emission maxima for the Cu(I)-S chromophore lie near 600 nm, and that the lifetime of the emitting state is highly dependent on temperature. Emission spectra recorded at room temperature during titrations of Zn_7-MT 2 with Cu(I) [12] demonstrate a strong

dependence of the emission intensity on the stoichiometric ratio, with a maximum being observed for Cu_{12}-MT in agreement with spectroscopic data on the Cu-S cluster formation in MT [12,15]. In frozen solutions at low temperature, we have found [24] that the emission spectrum is very much dependent on both the stoichiometric ratio of Cu to MT, and on the nature of the MT used at the beginning of the titration, that is whether it was Zn_7-MT or apo-MT. Emission spectra for Cu_6-MT at 77 K have maxima at 550 and 640 nm, reflecting the heterogenity of the copper(I) excited state [19].

The excited state lifetime studies provide an additional assignment criterion. Fig.3 shows a series of traces illustrating the decay of luminescence from the control and Cu-treated rat livers at 77 K. The luminescence decay from Cu-treated liver is bi-phasic. Deconvolution of the luminescence decays for livers isolated from Cu-treated rats at wavelengths of 590–610 nm, when excited in the range 290–310 nm, consistently yielded a contribution of two components to the decay curve. A short-lived component had a lifetime of 11 + 1 μs and the long-lived component showed the lifetime of 98 + 6 μs. The luminescence decay from the control sample at 77 K showed only a fast component with a lifetime of about 9 μs. The luminescence decay from model Cu-MT complexes at 77 K always yields lifetimes in the 100 μs range, but the decays are heterogenous and depend on the Cu content. For example, the luminescence decay from Cu_{10}-MT at 77 K can be deconvoluted into two exponential components with lifetimes of 63 ± 1 μs and 132 ± 30 μs [24].

Thus the luminescence decay in the 600 nm region from the livers of Cu-treated rats can be characterized as a superposition of a nonspecific background luminescence with a lifetime shorter than 10 μs, and a luminescence that is typical of the excited states in Cu-MT with a lifetime of the order of 100 μs.

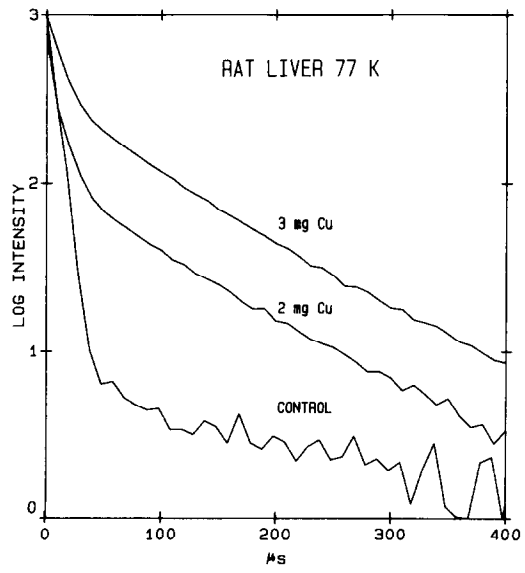

Fig.3. Excited state luminescence decays at 610 nm from a whole liver sample, isolated from a copper-treated rat with 2 mg Cu/kg body wt and 3 mg Cu/kg body wt, and from a whole liver sample, isolated from a control rat, after excitation at 300 nm at 77 K.

The photophysical properties of the Cu-MT emitter in liver can be compared to rare earth chelates used as labels in fluorometric measurements [25]. The combination of these two unusual properties, (i) a large Stokes shift and (ii) a long luminescence decay time, provides the basis for use of the emitting Cu-MT in liver as an excellent internal imaging label. The time resolution method can be readily exploited to distinguish between background luminiscence and the Cu-emitting centers.

Although a variety of different metallated MT complexes have been studied spectroscopically in vitro, no reports of the direct spectroscopic observation of MT in vivo have appeared to date. The commonly accepted methods for detecting MT in vivo include use of immunological probes. Despite their high sensitivity, these probes offer no sensitivity with respect to the metal bound to the protein. The lack of direct observation of metallated metallothioneins by other techniques is due, in part, to the lack of readily available, spectroscopic chromophores with which to observe the protein. There are no aromatic amino acids in MT, and the group 11 and 12 metals (following the new ACS/IUPAC nomenclature) are bound as the nd^{10} ions, that is, with closed shell electronic configurations. Ligand to metal, ^{1}LS $\longrightarrow$ Cd^{2+}, charge transfer transitions at 250 nm [13] allow the detection of Cd-containing MTs in vitro (observable because the protein itself does not exhibit absorption to the red of the 214 nm peptide absorption). However, absorption from similar transitions in vivo are impossible to find amongst all other absorptions of the tissue components. Copper-containing MT exhibits weak and featureless absorption and CD spectra [12,26], quite unlike the spectra of Cd_7-MT [14], so the problem of observing Cu-MT in tissue directly by absorption or CD is even more difficult.

Since the excess copper present in Wilson's patient's liver and stimulated in rat livers by subcutaneous injections of copper salts, is predominantly bound to MT, we interpret the spectroscopic results obtained from the liver tissue in this study, as emission from Cu-MT that is located in the liver tissue. The emission is only observed for copper-enhanced tissue, the emission and excitation maxima are consistent with luminescence from the Cu(I)-S chromophore, and, finally, while emission in the red region of the spectrum is relatively unusual, it has been reported that many complexes of Cu(I) emit at low temperatures.

This experimental measurement offers a new method for determining directly the location of copper(I) bound to MT in liver tissue as a specific staining method for Cu. The procedure will also be able to provide in the future much more detailed information about copper

binding to MT in vivo. Emission spectra have been reported for many metallorganic complexes of Cu(I) [27–29], although not yet in detail for any of the MT-binding site models. Thus this new technique may provide a means of determining for the first time, the extent of copper-sulfur cluster formation and the actual copper-protein stoichiometry in tissue, by comparing the photophysical parameters measured for Cu-MT in vivo, with the parameters obtained for Cu-MT formed in vitro.

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