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Does ^{13}C-or ^{15}N-labeling affect Cu(I)-thiolate cluster arrangement in yeast copper-metallothionein ?

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Abstract

It was attempted to examine whether or not isotope labeling may possibly affect an oligonuclear metal-thiolate cluster. Cu-metallothioneins are known to contain strongly distorted Cu-thiolate clusters and seemed appropriate for this study. Thus, yeast ^{13}C-and ^{15}N-Cu-metallothioneins were isolated from *Saccharomyces cerevisiae* cells grown in a minimal synthetic medium and some physicochemical parameters were compared with those of the unlabeled Cu-thionein. Surprisingly, the ^{13}C- and ^{15}N- labeled Cu_7-thioneins are distinctly different in their characteristic spectroscopic properties. The electronic absorption was blue-shifted while both luminescence emission and chiroptic features display a distinct red shift with markedly diminished intensities, respectively. Contrary to common knowledge that isotope labeling does not affect the molecular architecture of a protein the present results support such a phenomenon. Attributable to the fortunate happenstance that there is a strongly distorted structural situation in the oligonuclear Cu-thiolate cluster this isotope effect came to light.

Introduction

Unlike mammalian zinc/cadmium-metallothionein (MT) no exact structure of the one-domain yeast Cu(I)-MT is available. This 53 amino acid peptide contains 12 cysteines ten of which are involved in the binding of seven copper(I) ions (Byrd *et al.* 1988; Narula *et al.* 1991, 1993; Peterson *et al.* 1996). Crystalline forms of exclusively copper-containing thioneins are still unknown. However, a NMR structural model of yeast Cu_7-MT is reported. As ^{63}Cu and ^{65}Cu isotopes are NMR insensitive quadrupolar nuclei this model suffers from the lack of information about Cu(I). Therefore ^{109}Ag was used as a probe in the silver-substituted protein which allowed to propose a solution structure of ^{109}Ag-MT through heteronuclear NMR spectroscopy (Narula *et al.* 1991, 1993; Peterson *et al.* 1996). The problem arises from the consideration that Ag(I) and Cu(I) most certainly do have different coordination properties. Indeed, the coordination radii are different, and Cu(I) tends to favour tricoordination in contrast to Ag(I), which, predominantly is bicoordinated in a linear way (Hathaway 1987). Thus, the silver-cysteine constraints may not apply to copper. Recently, the three-dimensional solution structure of the protein part of Cu_7-MT has been deduced using ^{1}H-two-dimensional NMR spectroscopy (Bertini *et al.* 2000). A peptide structure is obtained which is similar, but not identical, to that from ^{1}H-^{109}Ag heteronuclear multiple quantum coherence spectroscopy. Thus, the structure of the copper polymetallic center may well be different from that proposed for the silver derivative.

During the course of these investigations the question arose whether or not ^{13}C- and ^{15}N-labeled yeast Cu-MT would improve the informations on the NMR structural elucidation of the protein portion. Thus, ^{13}C- and ^{15}N-Cu-MT's were isolated. However, different NMR spectra have been obtained compared to those of unlabeled species. The data were unappropriate for further NMR investigations. It was assumed that a distorted structure might be the reason for this

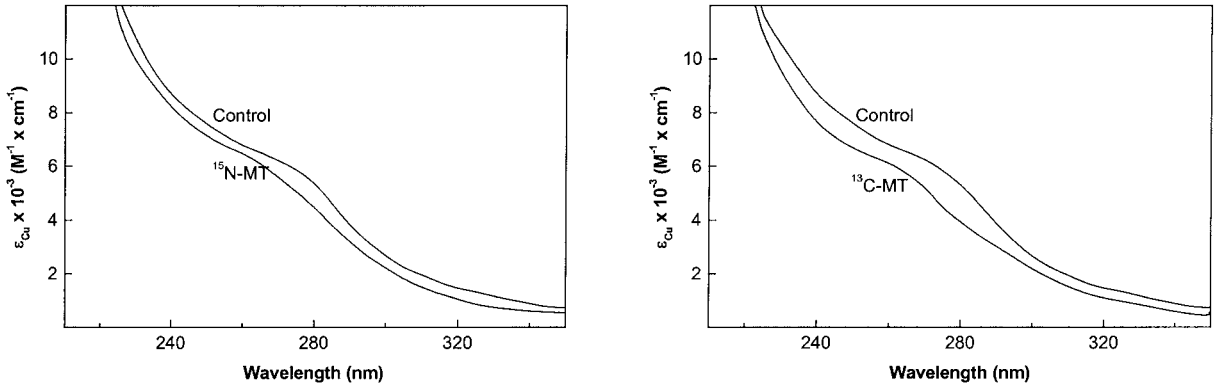

Fig. 1. Electronic absorption of isotope labeled yeast Cu-Metallothioneins. ^{15}N-Cu-MT and ^{13}C-Cu-MT each compared with the control protein. All samples were measured in water at 20 °C immediately after desalting on Sephadex G-25. It has to be emphasized that there was no detectable difference in all the employed spectroscopic properties using either control, Cu_7-thionein grown under minimal conditions or in the fully supplemented growth medium.

intriguing phenomenon. Thus, our interest was especially focused on the Cu(I)-thiolate cluster in either Cu-MT.

Examination of the characteristic physicochemical properties of the Cu(I)-thiolate chromophores including electronic absorption in the ultraviolet region, luminescence emission and circular dichroism seemed suitable and were employed to reveal possible differences in the isotope labeled species compared with the unlabeled protein.

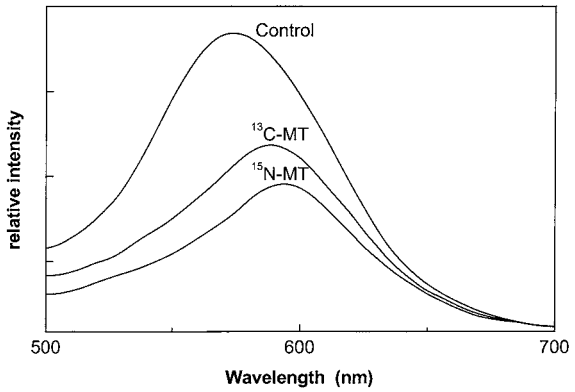

Fig. 2. Luminescence emission of isotope labeled yeast Cu-Metallothioneins. The control protein, ^{13}C-Cu-MT and ^{15}N-Cu-MT. The experimental conditions are the same as in Figure 1. The excitation wavelength was 300 nm.

Experimental

Yeast cell cultivation in minimal growth medium

For the synthesis of ^{13}C- and ^{15}N- Cu-metallothionein (Cu-MT) copper-resistant yeast cells (*Saccharomyces cerevisiae*, strain X 2180-1Aa) were cultivated in a minimal synthetic medium (Yeast Nitrogen Base omitting any amino acids and ammonium sulfate; DIFCO Laboratories, Detroit, USA) (Atlas 1993). The cells were precultured for 48 h under constant shaking at 37 °C in a volume of 500 ml water containing 0.85 g of yeast Nitrogen Base, 4.5 g of either 98% (w/w) ^{15}N-ammonium sulfate and 4.5 g glucose or 99% (w/w) U-$^{13}C_6$-D-glucose and 4.5 g ammonium sulfate in the presence of 1 mM $CuSO_4$, respectively. The main culture was grown under the same conditions in a volume of 5 l using the same concentrations of the above components. The isotope-labeled compounds were purchased from Promochem, Wesel, Germany. Control cells were grown in exactly the same minimal medium using both unlabeled reagent grade D-glucose and ammonium sulfate. Cell growth was monitored at 500 nm to evaluate the cell density.

Protein isolation

Cu-MT was isolated as described earlier (Winge *et al.* 1985; Weser & Hartmann 1991). Cell rupture was successful in the presence of 10 mM Tris/HCl, pH 7.4 using a French press. The progress of cell cleavage was microscopically controlled. After centrifugation of the cell homogenate the supernatant was diluted with the same volume of the above buffer containing 0.2% (v/v) 2-mercaptoethanol, following repeated gel permeation chromatography on Sephadex G-50 previously equilibrated with 10 mM Tris/HCl, pH 7.4, 0.1% (v/v) 2-mercaptoethanol. The proteins were desalted

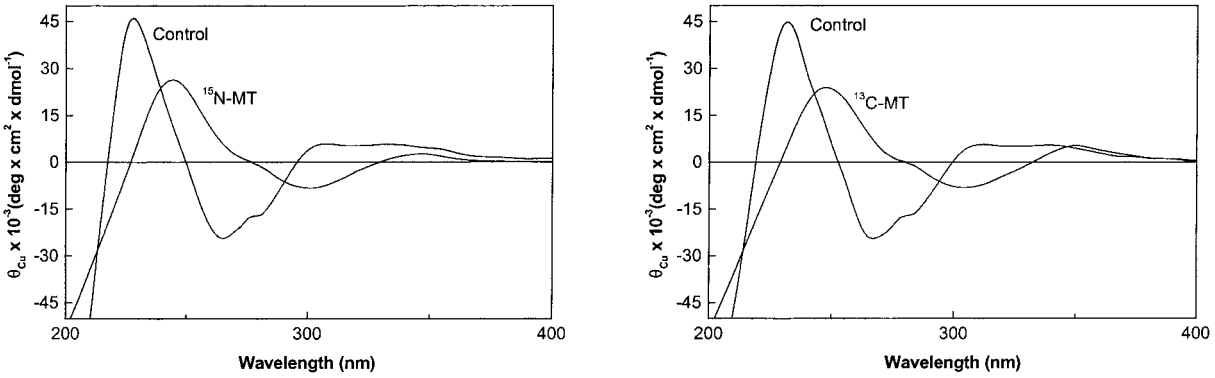

Fig. 3. Circular dichroism of isotope labeled yeast Cu-Metallothioneins. ^{15}N-Cu-MT and ^{13}C-Cu-MT each compared with the control protein. The experimental conditions are the same as in Figure 1.

on Sephadex G-25. After each chromatographic step the Cu-MT containing fraction was freeze-dried.

Spectroscopy

Electronic absorption was recorded on a Beckman 740 spectrophotometer. Luminescence emission was measured on a Perkin-Elmer LS 50 luminescence spectrometer. Excitation was at 300 nm. To suppress the second-order emission of the excitation source an edge filter at 390 nm was used. Circular dichoism was run on a JASCO J-720 spectropolarimeter. The sample chamber was kept under nitrogen to avoid the deleterious reactivity of ozone possibly generated by the Xenon lamp. All spectroscopic measurements were carried out at 20 °C. Copper was quantitated on a Perkin-Elmer 3030 atomic absorption spectrometer.

Results and discussion

In order to isolate the different isotopically labeled Cu-thioneins yeast cells were cultivated in a minimal synthetic medium containing the respective precursors. ^{15}N-ammonium sulfate served as the nitrogen donor while U-$^{13}C_6$-D-Glucose was the carbon source. Exactly the same composition of growth medium was used for the synthesis of unlabeled Cu-MT serving as the control. Using this minimal medium a surprisingly high cell yield of 70% was still obtained compared to that of the usual complete medium. No detectable differences in the course of the isolation procedures of either Cu-MT i.e. control Cu-thionein, ^{13}C- and ^{15}N-Cu-thionein were seen. The three Cu- MT's were isolated independently in duplicate. In all thioneins 6.8 ± 0.2 copper per mol of protein was found. All properties of the respective control proteins isolated from the usual yeast extract containing full medium and from the minimal medium were fully identical.

It is needless to mention that solid structural information on the intact Cu_7-metallothionein could be obtained from X-ray diffractometry only using the crystalline protein. Unfortunately, no successful crystallization has been reported worldwide. Thus, for the time being we have to rely on spectrometric measurements.

The well-known spectroscopic properties of the Cu(I)-thiolate chromophores including UV-electronic absorption, fluorescence and circular dichroism were examined using either the ^{13}C- and ^{15}N-labeled Cu-MT's as well as the unlabeled control. It is very important to note that the spectroscopic features obtained from the above methods are exclusively attributable to the oligonuclear Cu(I)-thiolate cluster (Weser & Hartmann 1991). During all spectroscopic measurements great emphasis was placed to maintain exactly the very same conditions including solution composition, pH-value, temperature and recording time.

Regarding the electronic absorption in the ultraviolet region a clear difference between ^{13}C- and ^{15}N-Cu-MT and the control protein is noticed. The absorption shoulder which is allocated at 275 nm in the case of intact Cu-MT is blue-shifted to 265 nm in the ^{15}N-Cu-thionein amounting to 260 nm when the ^{13}C-labeled protein was used (Figure 1). Throughout the respective intensities remained in the same order of magnitude.

The blue-shifted shoulders allow the assignment of a distincly higher energy level with respect to the charge-transfer copper-sulfur transitions in the isotopically labeled species which may be allocated to a

higher strain in the heptanuclear Cu(I)-thiolate cluster. Much to our surprise and unlike to the ^{15}N-labeled specimen a weak absorption band at 490 nm was additionally seen employing the ^{13}C-derivative. The molar absorptivity ε_{Cu} was 115 $M^{-1}cm^{-1}$. This red-colored absorption is an intriguing phenomenon which cannot be explained at the moment.

It was interesting to notice the characteristic orange-red luminescence emission of the Cu(I)-thiolate cluster at 575 nm which was red-shifted by 15–20 nm in the presence of the isotopically labeled Cu-MT's. The emission maximum of ^{13}C- Cu-MT appeared at 590 and that of the ^{15}N-protein was seen at 595 nm, respectively (Figure 2).

The intensities of the labeled species were, however, distinctively lower compared to that of the control protein sample. On the basis of the Cu(I)-thiolate concentration ^{13}C-Cu-MT displayed roughly half of the control signal whereas the ^{15}N-species was even slightly less intense. The lower intensities might be due to a diminished shielding effect of the Cu(I)-thiolates from water.

A surprisingly strong difference was observed in the circular dichroism spectra (Figure 3).

Both labeled Cu-thioneins exhibited Cotton extrema with similar wavelength positions and intensities. Furthermore, the overall profile with respect to the unlabeled protein was markedly different. All Cotton bands showed marked red-shifts which favor the assignment of altered symmetries in the respective Cu(I)-thiolate clusters.

All these observations are most interesting and intriguing and show that not only the protein mojety of Cu-MT is affected by both ^{13}C- and ^{15}N- labeling as deduced from NMR-spectroscopy. Likewise, the Cu-thiolate cluster may have a different molecular architecture. This proposal is unambigously supported using the above spectroscopic measurements. Although it is commonly thought that isotope labeling cannot change structural properties the present results strongly indicate such an effect. This might be due to the special structural situation in this protein. It was earlier concluded that the secondary structure of yeast Cu-MT is dictated entirely by metal ligation with the absence of regular secondary structural features (Byrd *et al.* 1988). This fact may lead to a very rigid cluster formation where small changes in the peptide backbone are amplified leading to detectable distortions in the spacial structure. Taking into consideration that either 98% of all nitrogen or 99% of the carbon atoms in the protein are exchanged such an effect cannot fully be excluded. At the moment we feel that our proposal appears to be the most attractive one. Of course further studies in this direction will be awaited with great interest. Recent observations that light Fe- and/or- Cu-isotopes are preferentially incorporated in biological systems into Fe- and Cu-proteins support in some way that isotopes must be considered as non innocent. Thus the present study might be another example of distinct reactivities of isotopes (Zhu *et al.* 2002). The sole statistical isotopic distribution of different chemical elements, therefore, must be reconsidered.

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