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Analogous copper(I) coordination in metallothionein from yeast and the separate domains of the mammalian protein

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The three-dimensional structures of both vertebrate Cu_{12}–metallothionein (class 1) and yeast Cu_8–thionein (class 2) are still unknown. The different copper:protein stoichiometry compared with that of the $(ZnCd)_7$–metallothioneins was expected to alter the metal–thiolate cluster structure considerably. In order to avoid possible domain interactions in the hepatic rat metallothionein, separate chemically synthesized α- and β-domains were used rather than the apoprotein. Apo yeast thionein, and the α- and β-domains of rat liver metallothionein-2 were reconstituted by Cu(I) titration. Reconstitution steps were monitored using spectroscopic methods including luminescence emission and circular dichroism. Upon UV irradiation a linear increase in intensity of the orange–red luminescence was observed near 600 nm up to 6 Cu eq using either compound regardless of the different cysteine sulfur content (yeast thionein 12S, α-domain 11S, β-domain 9S). The characteristic dichroic properties of the yeast copper–protein between 240 and 400 nm were in good agreement with those of the respective class 1 metallothionein domains. All observed Cotton bands were of similar shape and appeared in the same wavelength regions. However, the molar ellipticities were less pronounced in the α- and β-fragments employed. There appears to be a striking similarity between the oligonuclear Cu(I) binding centers in all metallothionein species.

Keywords: circular dichroism, copper–thiolate cluster, fluorescence, metallothionein

Introduction

The ubiquitously occurring metallothioneins are suggested to play an important role in controlling the levels of reactive metals with special emphasis on copper and zinc. Attributable to their biologically prominent functions, these metals require a finely tuned regulation on a cellular level. Due to the pronounced redox function of copper this metal needs special chaperoning in both anaerobic and aerobic metabolism (Felix *et al.* 1989, Weser & Hartmann 1991). Metallothionein represents a good candidate to keep copper in stable Cu(I)–thiolate clusters. Unlike cadmium, zinc–metallothionein, no three-dimensional structure is known for the Cu(I) form of the protein. The different copper:protein stoichiometry compared with that of the zinc and cadmium species is expected to alter the metal–thiolate cluster structure considerably. Nevertheless, it is assumed that in mammalian copper–metallothionein (class 1 metallothionein) a two-domain structure similar to that of the cadmium, zinc protein exists. Twelve copper atoms are deduced to be coordinated to the apoprotein as monitored by means of UV electronic absorption, luminescence and circular dichroism (CD) spectroscopic techniques during Cu(I) titration (Nielson & Winge 1984, Stillman *et al.* 1987, Li *et al.* 1990). Both proteolytically prepared (Nielson & Winge, 1984, 1985, George *et al.* 1986) and chemically synthesized (Li *et al.* 1990) metallothionein domains revealed binding of six copper atoms to either fragment which contains 11 cysteines in the α-domain and nine in the β-domain.

Differently bound Cu(I) species were found in yeast copper–thionein (class 2 metallothionein). Two out of eight cuprous ions coordinated to 12 cysteine sulfur atoms can easily be removed by Cu(I) chelators and do not contribute to the characteristic

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CD spectrum of the protein (Weser & Hartmann 1988).

In addition to the pronounced chiroptical behavior, all copper–metallothioneins exhibit a specific orange–red luminescence emission near 600 nm upon UV irradiation (Beltramini *et al.* 1987, Weser & Hartmann 1991). This luminophore gives proof of an intact Cu(I)–thiolate coordination. Although the primary structure of either class of metallothioneins and/or metallothionein fragments is different it was considered an interesting task to search for some analogous structural properties of each of the oligonuclear Cu(I)–thiolate centers. Due to the lack of X-ray diffraction and two-dimensional NMR data a comparative study using the above spectroscopic techniques was performed. Freshly prepared apothioneins from rat liver and yeast as well as the chemically synthesized α- and β-domains of rat liver metallothionein-2 were employed. The latter two separate fragments were chosen to avoid possible domain interactions in the intact vertebrate protein.

Materials and methods

Yeast copper–thionein was isolated from coper-supplemented *Saccharomyces cerevisiae* (strain X 2180-1Aa). The supernatant of the cell homogenate was subjected to repeated gel filtration using the established method in the author's laboratory (Weser & Hartmann 1991). In the course of the purification of intact rat liver cadmium, zinc–metallothionein both gel filtration and ion-exchange chromatography were employed (Andersen & Weser 1978). The α- and β-domains corresponding to the sequences Lys-30–Ala-61 and acetyl-Met-1–Lys-31, respectively, were chemically synthesized on a MilliGen 9050 continuous flow solid-phase peptide synthesizer (Li *et al.* 1990). Protein and peptide concentrations were assayed spectrophotometrically by thiol titration employing 2,2′-dithiodipyridine (Grasetti & Murray 1967). The apoforms of yeast and rat liver metallothionein were prepared by proton replacement of the coordinated metals (Hartmann & Weser 1985). The apoproteins and the chemically synthesized thionein fragments were dissolved in 50 mM sodium acetate buffer, pH 6.5, in the presence of 25% (v/v) acetonitrile. Cu(I) titration was performed using $(Cu(I)(CH_3CN)_4)ClO_4$ in 50% (v/v) acetonitrile at 22 °C under argon (O_2 impurity less than 0.001%). Anaerobiosis was maintained throughout during the subsequent physicochemical measurements. CD spectra were run on a JASCO J-720 spectropolarimeter at 22 °C. Luminescence emission was recorded on a Perkin-Elmer LS 50 spectrofluorimeter. An edge filter (430 nm) was used to suppress the second-order emission of the excitation source and the background fluorescence of the acetonitrile solvent. Metals were analyzed on a Perkin-Elmer Zeeman/3030 atomic absorption spectrometer.

Results and discussion

The apo forms of yeast copper–thionein and the chemically synthesized α- and β-domains of rat liver metallothionein-2 were converted into the Cu(I) species in the presence of acetonitrile. Anaerobic conditions were maintained throughout. A linear increase of the luminescence emission up to 6 Cu eq was observed with either compound regardless of the different cysteine sulfur content (Figure 1). In the case of the yeast protein a biphasic reactivity was noticed. Upon further copper addition the increase of emission intensity up to 8 Cu eq was less pronounced. Similar behavior with the same protein was also observed by Byrd *et al.* (1988).

It was intriguing to realize a distinct shift of the emission maxima dependent on the cysteine:copper ratio. Correlation of these emission maxima revealed an interesting phenomenon. A low sulfur: copper ratio resulted in a blue shift of the emission band (Figure 2). Indeed, the maximum wavelengths of 590 and 595 nm for yeast copper–thionein and the Cu_6–β-metallothionein domain, respectively (sulfur:copper = 1.5), are fairly consistent with this

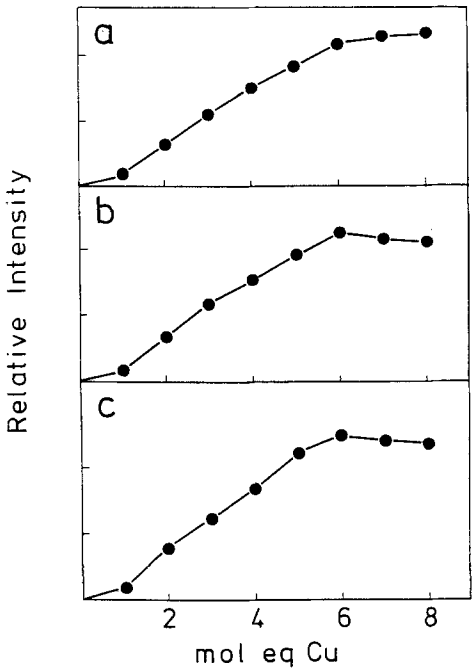

Figure 1. Relative emission intensities of the apoforms of different metallothionein species during Cu(I) titration at maximum emission wavelength: (**a**) yeast metallothionein (590 nm), (**b**) rat liver metallothionein-2 β-domain (595 nm) and (**c**) rat liver metallothionein-2 α-domain (615 nm). Luminescence measurements were performed under anaerobic conditions at 22 °C. Excitation was at 300 nm.

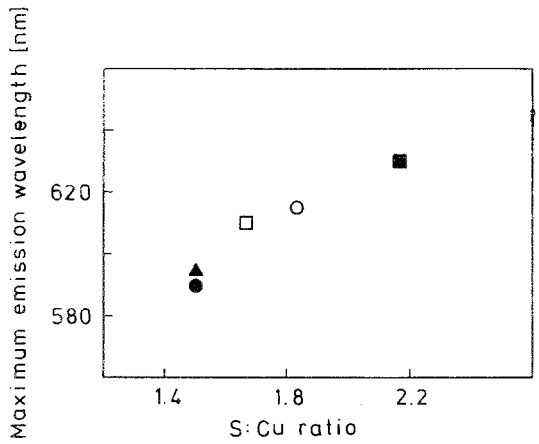

Figure 2. Correlation of the cysteine-sulfur:copper ratio and the maximum emission wavelength of different Cu(I)–metallothioneins. ●, Yeast Cu_8–thionein; ▲, rat liver Cu_6 β-domain; □, rat liver Cu_{12}–metallothionein; ○, rat liver Cu_6 α-domain; ■, Cd_1Cu_3 β-domain in rat liver Cd_5Cu_3–metallothionein.

correlation. This is in accordance with investigations on copper–thiolate model complexes for metallothionein where the average Cu–Cu distances paralleled the Stoke's shift of the corresponding model compound. In clusters with a higher spatial strain an elevated energy difference between the ground state and the emitting state exists with the consequence that the emission band is blue shifted (Beltramini *et al.* 1987).

Due to the lack of aromatic amino acids the dichroic bands of copper–metallothionein between 400 and 230 nm are exclusively attributed to charge-transfer transitions of the metal–thiolate coordination. In the case of yeast Cu_8–thionein a characteristic multibanded CD spectrum with Cotton extrema at 360 (+), 328 (+), 283 (−) and 245 (+) is observed (Prinz & Weser 1975, Weser *et al.* 1977).

Upon Cu(I) titration of the apoprotein a linear increase of all Cotton bands occurs up to six copper atoms per mole of protein. The further addition of two copper atoms had no detectable effect on the dichroic properties. This CD silence is concomitant with the observation that two copper atoms can be removed from the intact Cu_8–thionein without any change in the chiroptical behavior of the protein (Weser & Hartmann 1988).

Mammalian copper–metallothionein prepared from the apoprotein exhibits quite different chiroptical properties compared with that of yeast copper–thionein (Figure 3e & a). However, an intriguing similarity of both the shape and wavelength position of the Cotton bands was observed using the Cu(I) derivative of chemically synthesized rat liver metallothionein-2 β-domain (Figure 3b). Six copper atoms were coordinated to the nine cysteine-residue-containing peptide as shown by electronic absorption and luminescence emission. In this 'class 1 metallothionein' fragment three to four copper atoms were measured to be CD active whereas the specific θ_{Cu} values are lower compared with those of the yeast protein. Unfortunately, the chiroptical properties of the six copper atom α-domain whose cysteine content is closer to that of yeast copper–thionein are less pronounced (Figure 3c). The major CD bands between 320 and 240 nm are readily detectable. The different dichroic behavior during Cu(I) titration of the separate fragments and apometallothionein might be due to domain interactions in the latter species which are also observed with the mixed α- and β-domains. Surprisingly, the specific 'yeast copper–thionein like' CD spectrum was also found in an isolated hepatic bovine copper, zinc–thionein (Figure 3d) (see also Hartmann & Weser 1977, Rupp & Weser 1978).

The analogous CD properties may be attributed to the exclusive copper coordination in one domain which behaves chiroptically like a separate α- or β-fragment.

From both luminescence emission and chiroptical measurements it is concluded that in yeast copper–thionein an oligonuclear Cu(I)–thiolate center is present, similar to that usually observed in either 'class 1 metallothionein' copper domain. The same spectral relationship is noticed with both isolated (Beltramini & Lerch 1983) and synthetically prepared (Kull *et al.* 1990) *Neurospora crassa* copper–metallothionein which can be considered as a partial 'class 1 metallothionein' β-domain.

An attractive structure of the oligonuclear Cu(I)-binding centers of yeast Cu_6–thionein and its Cu_8 species can be proposed taking into account all of the spectroscopic properties (Figure 4). In the Cu_6 species all Cu(I) are coordinated by four sulfur atoms each. The two CD silent Cu(I) and their diminished luminescence could be attributed to one missing sulfur on each of the two Cu(I) of the Cu_8–thionein. In the light of the intriguing spectral similarities of either yeast copper–thionein and the $Cu(I)_6$ complexes obtained using the vertebrate metallothionein fragments a similar cluster arrangement may be assumed for the Cu_6 α- or β-domains.

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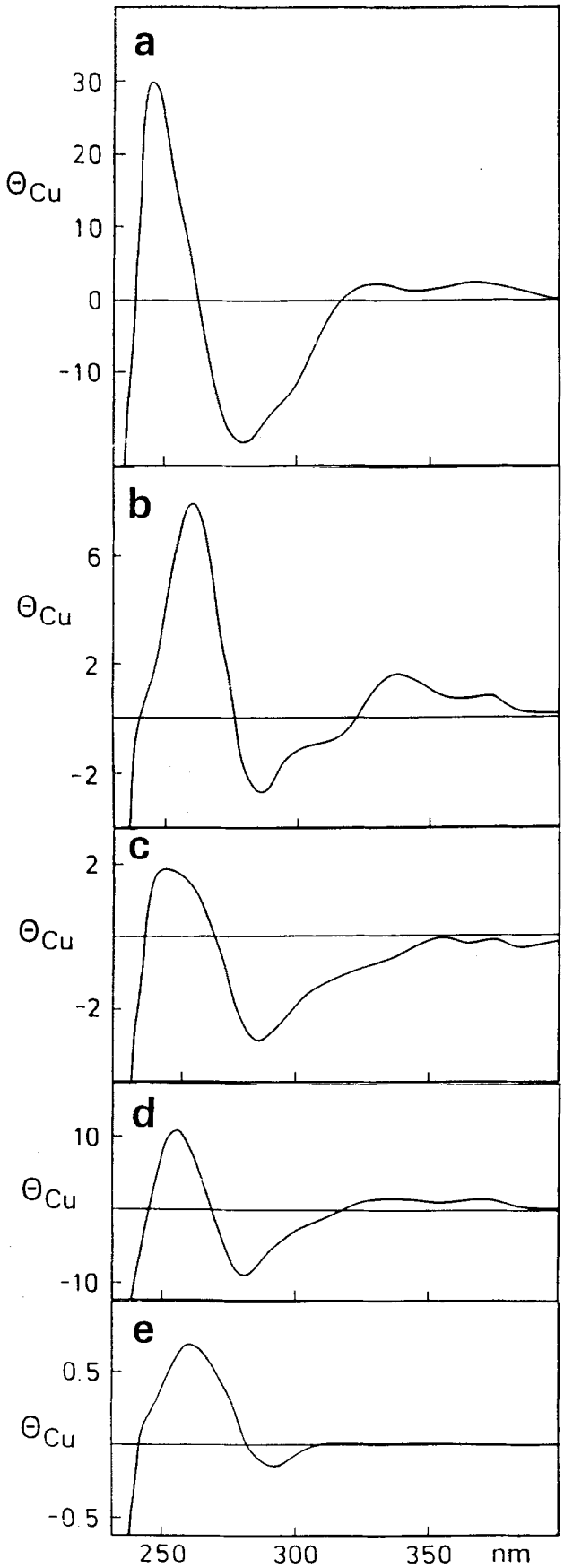

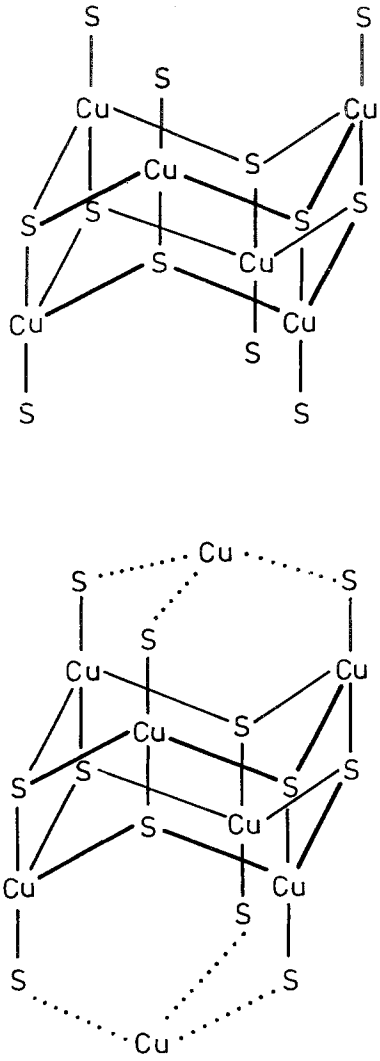

Figure 4. Proposed structures for the metal–thiolate clusters of yeast Cu_6– and Cu_8–thionein.

Figure 3. CD spectra of different Cu(I)–metallothioneins. (**a**) Yeast copper–thionein, (**b**) synthetic rat liver metallothionein-2 copper β-domain, (**c**) copper α-domain, (**d**) isolated hepatic bovine copper, zinc–thionein and (**e**) rat liver copper–metallothionein prepared from the apoprotein. Specific ellipticities calculated on a molar basis of copper are expressed as $\theta_{Cu} \times 10^{-3}$ (deg cm^2 dmol^{-1}).

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