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COBALT-(CYSTEINYL)$_4$TETRAHEDRA IN YEAST COBALT(II)-THIONEIN

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The conversion of yeast Cu(I)-thionein into the Co(II) derivative was successful. 2.6 Co atoms were incorporated per mole of protein yielding a Co : S ratio of 1 : 3. The electronic absorption of this highly air sensitive Co(II)-thionein is virtually identical to those of the Co(II) derivatives of other metallothioneins originating from vertebrates and N. crassa. Weaker Cotton extrema are noticed and the two doublet splittings of Cu-thionein disappeared. Throughout the molar ellipticities of the cobalt protein were markedly lower compared to those of the Cu-thionein. Owing to the characteristic charge transfer bands and d-d transitions a tetrahedral Co-thiolate coordination was deduced. The best fit proposal maintaining the above Co : S ratio of 1 : 3 was a six-membered ring with three bridging cysteine sulphurs.

The tetrahedral arrangement of four thiolate sulphurs around a d^{10} metal centre belongs to the established phenomenon of all known metallothioneins (1-6). In vertebrate metallothioneins many different metals are coordinated including Zn, Cd and Cu (1,7,8). By way of contrast, in yeast and Neurospora crassa only copper is capable to induce a metallothionein exclusively loaded with copper (9,10). In the Neurospora protein there is a striking sequence homology to the NH_2-terminal part of mammalian metallothioneins (10). The sequence of amino acid residues of the yeast type proteins is markedly different to that of the other reported species (11,12). Nevertheless, there is a close relationship to Cd and Cu binding thiolate rich proteins isolated from plants (13,14).

Four coordinate Cu(I)-thiolate centres were deduced from EXAFS measurements (4,5). These four Cu(I)-thiolate tetrahedra of yeast Cu-thionein were proposed to be arranged in a cubane type cluster. Only three sulphur to copper bondings were suggested using pig liver Cu-thionein (15). Thus, the close relationship of the yeast type Cu-thiolate cluster to that of the vertebrate Cu-thioneins became debatable. Additional convincing proof of tetrahedral metal to sulphur coordination was obtained from Co(II) replacement studies of both vertebrate and

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Neurospora metallothioneins (3,16,17). The pseudo tetrahedral Co(II)-(thiolate)$_4$ centres of rubredoxin (18) and alcohol dehydrogenase (19) served as prominent reference proteins.

It was very promising to examine whether or not the yeast Cu-thionein can be converted into a protein containing the same tetrahedral Co(II)-(thiolate)$_4$ centres. Special emphasis was placed on the preparation of the Co(II)-protein avoiding mercaptides as reducing compounds. The correct insertion of Co(II) was followed by electronic absorption spectrometry and circular dichroism measurements. These data are expected to lend further support for our earlier conclusion to assign the yeast type protein regardless of its different amino acid sequence to a genuine metallothionein analogue.

MATERIALS AND METHODS

Yeast Cu-thionein was isolated as previously described (9). The copper content was 4 Cu per M_r=4800. Removal of copper was successful after dialyses of one ml containing 5-10 mg Cu-thionein against two changes of a 200-fold volume each of 600 mM HCl for 2h and 1h against 100 mM HCl at 20°C. The dialysis tubing used had a relative molecular mass cutoff of 3500 (Spectrum Medical Industries, Los Angeles). The apoprotein contained less than 1% of the original copper. Quantification of dissolved apothionein in 100 mM HCl was performed spectrophotometrically using the absorption coefficient ϵ_{220}=25000.

The conversion into the Co(II)-thionein was carried out under nitrogen. To 500 µl of apothionein dissolved in 100 mM HCl in the presence of variable concentrations of $CoCl_2$ the same volume of 1 M Tris/HCl buffer, pH 8,6 was added to yield a final pH of 7.5-8.0. Due to partial oxidation of sulphydryl groups of the apoprotein at elevated pH values the addition of $NaBH_4$ was advisable to ascertain fully reduced metalthiolate chromophores.

Metal analyses were performed on a Perkin-Elmer 400S atomic absorption spectrometer furnished with a HGA 76B unit. Electronic absorption spectra were run on a Beckman Model 25 spectrophotometer. Circular dichroism was measured employing a JASCO 20A spectropolarimeter. Anaerobiosis was maintained using a nitrogen-vacuum line flushed with N_2 of 99.996 % purity.

RESULTS AND DISCUSSION

In order to minimize possible mixed disulphide formation between apo Cu-thionein and mercaptide chemicals $NaBH_4$ was employed as a powerful reducing agent. No measureable deterioration of the protein was noticed after this treatment. Unlike the 4Cu-thionein, stepwise titration of the apoprotein using 0.5 Mol of $CoCl_2$ each resulted in the binding of 2.6 Co per mol of protein. No further specific cobalt coordination was measured. It was intriguing to realize that these 2/3 of cobalt compared to the original copper content corresponded exactly to a

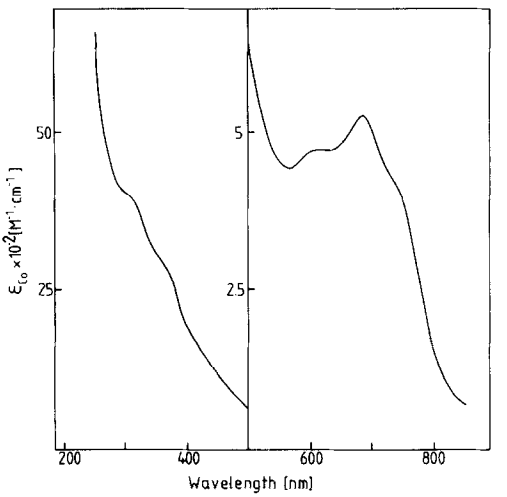

Figure 1. Molar visible and ultraviolet absorption of cobalt bound in Co(II)-yeast-thionein. Measurements were performed under nitrogen at 20°C.

ratio of 1 cobalt : 3 mercaptide sulphurs. This 1:3 ratio is usually seen in vertebrate metallothioneins containing Zn, Cd or Co (1,3,16). Each metal is coordinated to four sulphurs, one sulphur acts as a bridging ligand to the next metal thiolate centre. The distorted tetrahedral arrangement of these metal-thiolate centres have been generally accepted. For example, in yeast Cu-thionein two bridging sulphurs are required to form copper-sulphur tetrahedra at a ratio of 1 Cu: 2 cysteine residues (4,5). The above low cobalt content of the converted yeast Cu-thionein is essential for a 1:3 stoichiometry and, thus, is in perfect agreement with the situation of the former vertebrate metallothioneins.

All chemical data are supported by the observed spectrometric properties. The electronic absorption of yeast Co-thionein is remarkably similar to that of the other reported Co-thioneins (Figure 1, Table 1). All absorption bands are positioned at the very same wavelength regions. Two bands are detectable at 300 and 365 nm with the highest molar absorptivity between 3000 and 4000 $M^{-1} \cdot cm^{-1}$. A three-banded absorption is seen at 610, 685 and 740 nm, respectively, with a molar absorptivity close to 500 $M^{-1} \cdot cm^{-1}$. In fact, the molar absorptivity in the 300 nm region of the microbial Co-thionein is roughly one third higher compared to that of the vertebrate species. This higher absorption might be attributed to a somewhat stronger absorption of the Co-thiolate cluster.

Table 1. Cobalt(II)-thionein prepared from metallothionein of different origin

band position [nm]	molar absorption, ε_{Co} [$M^{-1} \cdot cm^{-1}$]			
	yeast	N. crassa[a]	horse kidney[b] MT 1A	rabbit liver[c] MT 1
740	410	370	310	450
685	520	440	320	440
610	480	380	300	290
365	2900	3000	2100	2160
300	4000	4360	2860	2900

Calculated from a) Table 2 of (17) using the spectrometrical active cobalt atoms, b) the molar absorptivity of Ref. (3) and c) the data of Fig. 1 and 2 of Ref. (16).

The d-d bands in the visible region are as expected consistent with a tetrahedral high-spin Co(II). Both position and magnitude of each band are attributable to a distorted tetrahedral high spin Co(II)core (3,16-20). As in 2Co(II)-rubredoxin from Pseudomonas oleovorans the absorption at 350 nm ($\epsilon_{Co,\ 350}$ = 4700) can be assigned to a $S^- \rightarrow$ Co(II) charge transfer transition. The same situation is observeable by looking at the absorption properties of the Co(II)-$(SR)_4$ unit of cobalt substituted liver alcohol dehydrogenase ($\epsilon_{Co,\ 340}$ = 4000 $M^{-1} \cdot cm^{-1}$) (19).

The rather strong absorption band at 610 nm is very interesting. Isolated Co-$(SR)_4$ units do not show a considerable electronic absorption at this wavelength. For example in the spectra of $[Co(SPh)_4]^{2-}$ and the non catalytic $Co(SR)_4$ centre of alcohol dehydrogenase this 610 nm band is absent (20,19). Furthermore substoichiometric addition of Co(II) to vertebrate cysteine-thionein resulted in a less pronounced 610 nm absorption.

The chiroptical properties of Co(II)-thionein were compared with those of Cu-thionein (Figure 2). Six Cotton extrema [Θ_{Co}-values ($\frac{deg \cdot cm^2}{dmol}$) are given in parenthesis] are seen at 260 nm (-1380), 290 (-400), 335 (2160), 385 (-200), 470 (-200) and 590 (400). The numeric Θ_{Co}-values are much lower compared to Θ_{Cu} of native 4Cu-thionein [245 (23000), 283 (-14300), 302 (-10300), 330 (2300) and

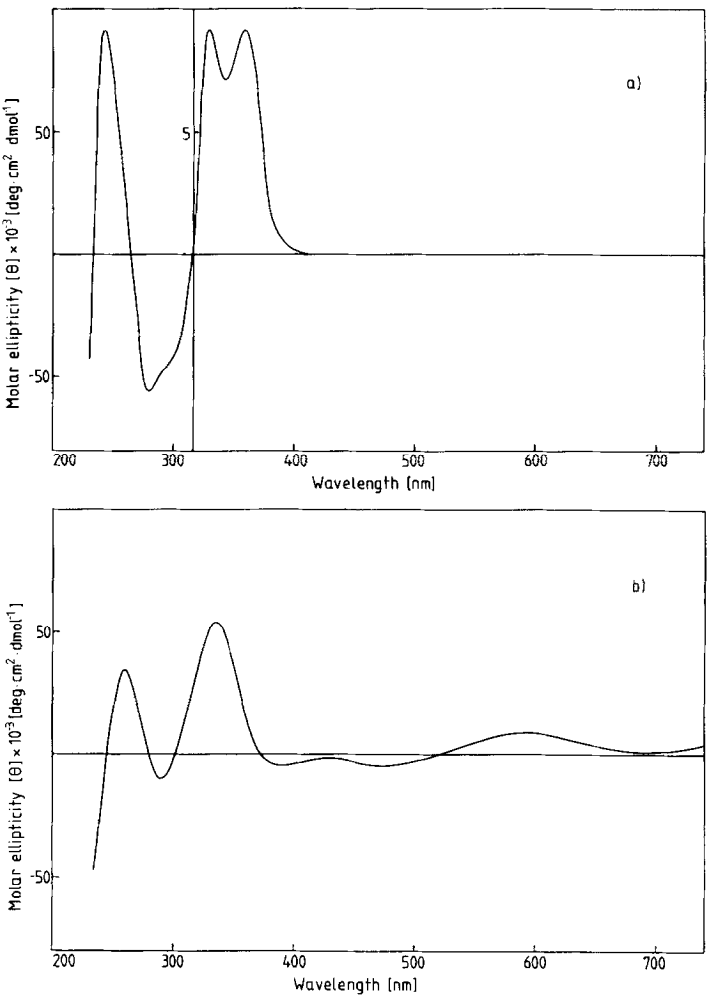

Figure 2. Circular dichroism of a) yeast Cu-thionein (4 Cu/M_r = 4800), b) cobalt substituted yeast Cu-thionein (2.6 Co/Mol). The spectrum of the cobalt derivative was run under anaerobiosis at 20°C. Protein concentrations were 0.9 mg/ml.

360 (2300)]. The splitted negative Cotton bands at 283 and 302 nm and another positive doublet at 330 and 360 nm are smoothed to give one negative [$\Theta_{Co,\ 290}$ = $-400\ \frac{deg \cdot cm^2}{dmol}$] and one positive [$\Theta_{Co,\ 335} = 2160\ \frac{deg \cdot cm^2}{dmol}$] Cotton extremum each. There is also a striking similarity with the circular dichroism spectrum of 2Co-rubredoxin (18). Owing to the diminished magnitude of the Θ_{Co}-values and the leveled off splitting of Cotton bands the Co-thiolate tetrahedra are expected to be differently arranged. The best fit proposal to arrange 2.6 Co and 8 cysteine sulphurs maintaining a Co : S ratio of 1:3 could be a six membered ring including three bridging sulphurs in a way similar to the known 3Fe-3S-binding centre of

aconitase (21). Each cobalt has two additional cysteine sulphurs to ascertain the former suggested tetrahedral coordination. Nevertheless, the identity of the yeast cobalt-thiolate tetrahedra with all other reported cobalt-metallothionein binding centres is supporting proof to include the yeast Cu-thionein to the general class of metallothioneins.

The redox properties of Co-thionein appear to be much different from those of the 2Co-rubredoxin. The metallothionein derivative is extremely air sensitive and strict anaerobiosis had to be maintained throughout all measurements. The cobalt substitute of rubredoxin resists exposure to air for days, and, is in fact more stable than the native 2Fe(II)-rubredoxin. The different redox properties of either microbial cobalt protein can certainly be attributed to the markedly different sequence of amino acid residues. The redox potential of all Co-thioneins including those of vertebrates, however, they all lie in the negative region.

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REFERENCES

1. Rupp, H. and Weser, U. (1978) Biochim. Biophys. Acta 533, 209-226.
2. Kägi, J.H.R. and Nordberg, M. (1979) Metallothionein, Experientia Supplementum 34, Birkhäuser Verlag, Basel.
3. Vašák, M. (1980) J. Am. Chem. Soc. 102, 3953-3955.
4. Bordas, J., Koch, M.H.J., Hartmann, H.-J. and Weser U. (1982) FEBS Lett. 140, 19-21.
5. Bordas, J., Koch, M.H.J., Hartmann, H.-J. and Weser U. (1983) Inorg. Chim. Acta 78, 113-120.
6. Garner, C.D., Hasnain, S.S., Bremner, I. and Bordas J. (1982) J. Inorg. Biochem. 16, 253-256.
7. Hartmann, H.-J. and Weser, U. (1977) Biochim. Biophys. Acta 491, 211-222.
8. Winge, D.R., Geller, B.L. and Garvey, J. (1981) Arch. Biochem. Biophys. 208, 160-166.
9. Weser, U., Hartmann, H.-J., Fretzdorff, A. and Strobel, G.-J. (1977) Biochim. Biophys. Acta 493, 465-477.
10. Lerch, K. (1980) Nature 284, 368-370.
11. Kimura, M., Otaki, N., Hartmann, H.-J. and Weser, U. (1981) Regard sur la Biochimie 3, 101.
12. Butt, T.R., Sternberg, E.J., Gorman, J.A., Clark, P., Hamer, D., Rosenberg, M. and Crooke, S.T. (1984) Proc. Natl. Acad. Sci. USA 81, 3332-3336.
13. Rauser, W.E., Hartmann, H.-J. and Weser, U. (1983) FEBS Lett. 164, 102-104.
14. Rauser, W.E. and Curvetto, N.R. (1980) Nature 287, 563-564.

15. Ross, I., Binstead, N., Blackburn, N.J., Bremner, I., Diakun, G.P., Hasnain, S.S., Knowles, P.F. Vašák, M. and Garner, C.D. (1983) in EXAFS and Near Edge Structure (Bianconi, A., Incoccia and Stipcich, S., eds.) pp.337, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.
16. Vašák, M. and Kägi, J.H.R. (1981) Proc. Natl. Acad. Sci. USA 78, 6709-6713.
17. Beltramini, M., Lerch, K. and Vašák, M. (1984) Biochemistry 23, 3422-3427.
18. May, S.W. and Kuo, J.-Y. (1978) Biochemistry 17, 3333-3338.
19. Maret, W., Andersson, I., Dietrich, H., Schneider-Bernlöhr, H., Einarsson, R. and Zeppezauer, M. (1979) Eur. J. Biochem. 98, 501-512.
20. Dance, I.G. (1979) J. Am. Chem. Soc. 101, 6264-6273.
21. Ghosh, D., Furey, W., O'Donnell, S. and Stout, C.D. (1981) J. Biol. Chem. 256, 4185-4192.