CUPRODOXIN

Roswitha PRINZ and Ulrich WESER
Physiologisch-chemisches Institut der Universität Tübingen, 74 Tübingen, Hoppe-Seylerstr. 1, West Germany

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1. Introduction

The metallothioneins belong to a group of actively studied sulphur rich metal proteins containing high concentrations of Zn and Cd, some Hg and occasionally traces of Cu [1–11]. A direct link between the neonatal type mitochondrocuprein and metallothionein was proposed by Porter [12]. Final proof was recently demonstrated in our laboratory [13,14] by the successful conversion of metallothionein into a homogeneous monomeric and polymeric Cu-thionein.

The question whether or not a naturally occurring low mol. wt Cu-thionein can be isolated from biological material prompted us to subject homogenized *Saccharomyces cerevisiae* to ammonium sulphate precipitation followed by ion exchange and gel chromatographic steps. Indeed, we succeeded to prepare a monodisperse microbial Cu-thionein. This thiolate-rich Cu-thionein (24.3% cysteine residues) contained 10 g atoms of Cu per mole of protein. The mol. wt was 9500 ± 500. In contrast to the metallothioneins unusually high concentrations of both aspartate and glutamate residues were present. Thus, it was very attractive to propose some relationship to the protein portion of the group of iron-sulphur proteins.

The copper proved to be non-detectable by EPR. Upon treatment with equimolar concentrations of H_2O_2 a broad absorption band appeared at 565 nm attributable to Cu^{2+} while the ultraviolet spectrum remained unchanged. Added dithionite completely reversed this effect. From X-ray photoelectron spectroscopic measurements it was concluded that copper is coordinated to cysteine sulphur (S 2p = 161.8 eV) while the binding energy of the copper $2p_{3/2}$ levels was 932.8 eV. In the light of both the high content of sulphur and the redox active nature of the protein bound copper it is suggested to call this Cu-thionein 'cuprodoxin'. A cuprodoxin would also fill a long known gap in the evolution of the copper proteins [15].

2. Experimental

All biochemical reagents and inorganic chemicals were purchased and were of analytical grade. Contaminations with metal ions were minimized by employing quartz or polyacrylic ware. Deionized water was additionally distilled in an all quartz unit. The conductivity was 1 μmho.

A homogeneous strain of *Saccharomyces cerevisiae* (Lindenmeyer and Co, Heilbronn) was cultivated at pH 4.5, 30°C. The nitrogen source was Bactotryptone (Difco) and glucose served as the carbon source. The copper concentration was controlled by atomic absorption spectroscopy [16] and was at 0.2 mM which corresponded physiological conditions [17]. To improve the yield of cuprodoxin gassing with air was omitted.

Protein was quantitated by weighing lyophilized aliquots of purified protein solutions of known A_{270} to constant weight. Amino acids were chromatographically separated [18] after hydrolysis of the apoprotein with 6 N HCl for 24, 36 and 65 hr under nitrogen atmosphere. Cysteine residues were separately determined following the oxidation to cysteic acid [19]. Analytical polyacrylamide-gel disc electrophoresis was performed as previously described [1].

Ultraviolet and visible absorption spectroscopy

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was carried out on a Unicam SP 1800 spectrophotometer. The circular dichroism was measured on a JASCO 20 automatic spectrometer. A Varian 4502-II spectrometer was used for EPR measurements. X-ray photoelectron spectroscopic data were collected on a Varian V-IEE-15 spectrometer equipped with an online Varian 620 L,8 K computer.

3. Results

The supernatant of the homogenized yeast cells was subjected to stepwise (10–80%) $(NH_4)_2SO_4$ treatment. After discarding the initial precipitates (10–50% $(NH_4)_2SO_4$) all further fractions were dialysed and chromatographed on DEAE-23 cellulose. The highest Cu concentration was monitored in the fraction eluted with 100 mM phosphate buffer. This fraction was concentrated by ultrafiltration and subjected to Sephadex G-75 gel filtration. Apart from the presence of Cu, Zn-superoxide dismutase a copper protein rich in sulphur was separated. Additional DEAE-23 chromatography yielded a monodispers Cu-thionein which migrated as a single peak during further Sephadex and DEAE-23 chromatography.

The mol. wt was determined using comparative gel filtration on Sephadex G-50 [20] and was in the order of 9500 ± 500. Reference proteins were chymotrypsinogen A (25 000), myoglobin (17 800), ribonuclease A (13 600), cytochrome *c* (13 000), trypsin inhibitor (8400) and kallikrein inhibitor (6500). The electrophoretic behaviour of Cu-thionein was quite intriguing (fig.1). Although the protein appeared chromatographically homogeneous a separation into two distinct components was seen employing polyacrylamide disc electrophoresis. Pretreatment with H_2O_2 in stoichiometric amounts with regard to the copper content resulted in the appearence of only one single band.

The metal determination revealed exclusively copper and 10 ± 0.4 g atoms were quantitated per mole of protein. A striking dissimilarity between the metallothioneins and the present copper protein became apparent. Apart from the high content of cysteine residues glutamate and aspartate were the most abundant residues found in the Cu-thionein (table 1). Attributable to possible side reactions of

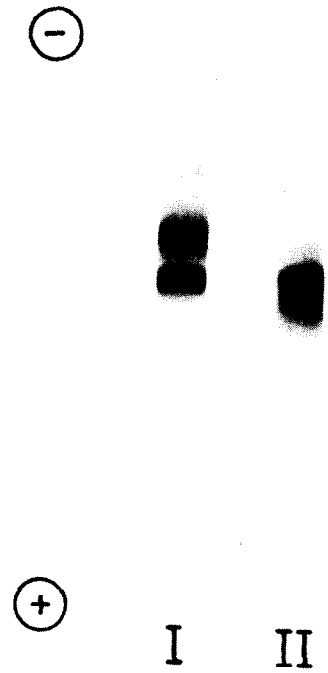

Fig.1. Analytical polyacrylamide disc electrophoresis of yeast-cuprodoxin. The acrylamide concentration was 4% in the concentrating gel and 7.5% in the separating gel. Electrophoresis was performed at pH 8.9 for 1 hr at 130 V and 1.2 mA per gel stick. Tris-glycine buffer pH 8.3. I. 25 μg protein. II. 25 μg protein oxidized with 0.025 μmol H_2O_2.

copper the apoprotein rather than the native Cu-thionein was used.

The electronic spectra in the visible and ultraviolet region are depicted in fig.2. While the freshly prepared Cu-thionein absorbs exclusively in the UV area showing some gradual tailing off into the visible region no further absorption was monitored at longer wavelength. The copper proved completely EPR non-detectable. Upon titrating Cu-thionein with equimolar portions of H_2O_2 (see also fig.1) a braod absorption band appeared at 565 nm. No detectable change was seen in the uv absorption curve. Upon the addition of dithionite this absorption band 565 nm was completely levelled off. Due to the absence of disturbing aromatic amino acid residues the marked circular dichroism properties could be clearly seen. CD-measurements revealed 6 distinct Cotton bands including three positive [245 nm ($[\theta]$ = 183 870),

Table 1
Comparison of the iron sulphur proteins and metallothionein. The values are a percentage of total number of residues, the number of residues are given in parentheses

Amino acid	Cuprodoxin *Sacch. cerev.* (this study)		Ferredoxin *C. pasteur.* [21]		Rubredoxin *C. pasteur.* [22]		Metallothionein Chicken [1]	
	%	(residues)	%	(residues)	%	(residues)	%	(residues)
Lysine	8.9	(7.4)	1.8	(1)	7.4	(4)	10.0	(11.7)
Histidine	1.8	(1.5)	0	(0)	0	(0)	1.7	(1.9)
Arginine	0	(0)	0	(0)	0	(0)	4.5	(5.3)
Tryptophane	0	(0)	0	(0)	1.9	(1)	0	(0)
Aspartic acid	14.6	(12.2)	14.5	(8)	20.4	(11)	9.5	(11.1)
Threonine	4.7	(3.9)	1.8	(1)	5.6	(3)	1.3	(1.5)
Serine	9.6	(8.0)	9.1	(5)	0	(0)	9.0	(10.5)
Glutamic acid	19.2	(16.0)	7.3	(4)	11.1	(6)	3.8	(4.4)
Proline	4.7	(3.9)	5.5	(3)	9.3	(5)	8.5	(10.0)
Glycine	9.6	(8.0)	7.3	(4)	11.1	(6)	6.7	(7.8)
Alanine	0.9	(0.8)	14.5	(8)	0	(0)	9.3	(10.9)
Cysteine	24.3	(20.2)	14.5*	(8)*	7.4	(4)	31.6	(37.1)
Valine	0.7	(0.6)	10.9	(6)	9.3	(5)	1.7	(2.0)
Methionine	0	(0)	0	(0)	1.9	(11)	1.6	(1.8)
Isoleucine	0.4	(0.3)	9.1	(5)	3.7	(2)	0.3	(0.4)
Leucine	0.6	(0.5)	0	(0)	1.9	(1)	0.4	(0.5)
Tyrosine	0	(0)	1.8	(1)	5.6	(3)	0	(0)
Phenylalanine	0	(0)	1.8	(1)	3.7	(2)	0	(0)
			* Labile sulphur excluded					
Stoichiometry metal : sulphur	1 : 2		1 : 2[a]		1 : 4		1 : 4	

[a] Labile sulphur included

328 nm ([θ] = 25 540) and 359 nm ([θ] = 24 520)] and three negative extrema [213 nm ([θ] = −2070), 283 nm ([θ] = −138 000) and a shoulder at 302 nm ([θ] = −91 940)].

An open question was the nature of the sulphur in the protein portion. Freshly prepared species displayed one homogeneous sulphur $2p_{1/2,3/2}$ signal at 161.8 eV indicating the exclusive presence of cysteine sulphur (fig.3). In aged preparations sometimes mixed portions of cystine/cysteine (162.6 eV) were measured. In completely oxidized and deteriorated samples even RSO_3^- sulphur (168 eV) was monitored. A similar oxidative decomposition was already demonstrated using erythrocuprein [23]. The Cu $2p_{3/2}$ level had a binding energy of 932.8 eV (fig.3) and was virtually identical to that of the synthetically prepared Cu-thionein [24]. Since the binding strength of copper to thiolate sulphur is highest [25] it can be concluded that copper is coordinated to cysteine sulphur.

4. Discussion

The rather uncontrolled conditions during polyacrylamide disc electrophoresis with regard to oxidation of the Cu-thionein should be considered. Traces of residual ammonium persulphate or the presence of molecular oxygen could have affected the protein. Therefore, it was not surprising to realize the splitting of freshly prepared Cu-thionein into two components. Whether or not the protein falls into low mol. wt portions needs to be elucidated. Intramolecular disulphide formation certainly will

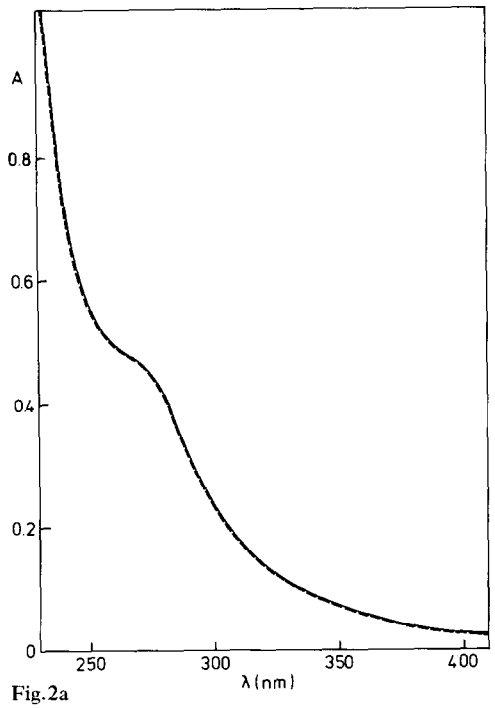

Fig.2a

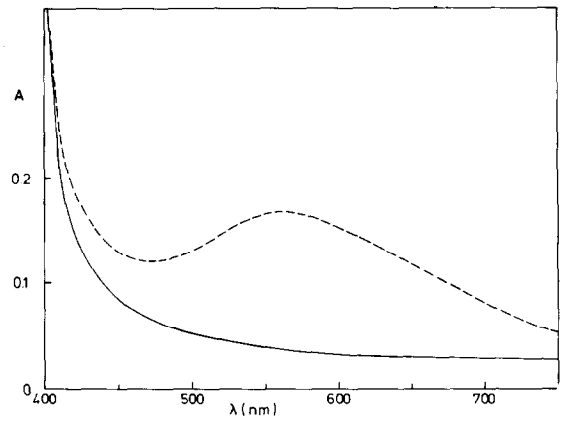

Fig.2b

Fig.2. a) Ultraviolet-spectrum of yeast-cuprodoxin. (——) native and (- - -) oxidized. The protein concentration was 0.46 mg/ml. 10 mM potassium phosphate buffer pH 7.5. Temperature 23°C, 2 mm light path quartz cells. b) Absorption of (——) native and (- - -) oxidized cuprodoxin in the visible region. An elevated protein concentration (0.72 mg/ml) and 40 mm light path quartz cells were used. 10 mM potassium phosphate buffer pH 7.5. Temp. 23°C.

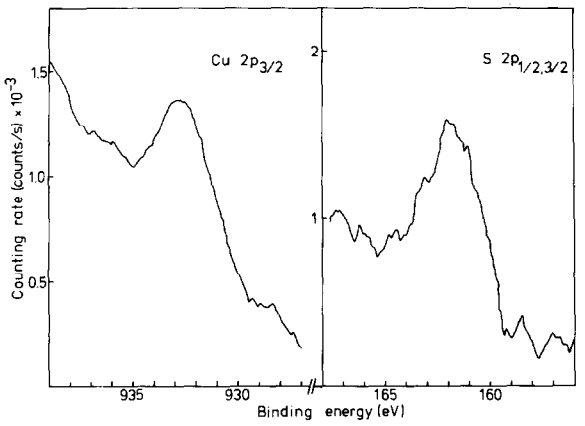

Fig.3. X-ray photoelectron spectra of cuprodoxin. a) Cu $2p_{3/2}$ level. Recording conditions: X-ray source Mg 11 kV, 150 mA. Analyzer energy 50 eV, sweep width 20 eV, sweep time 20 sec. No. of scans 29, no. of channels 200. Smoothing over 19 points. Pressure 1.8×10^{-6} Torr, cooling with liquid nitrogen. b) S $2p_{1/2,3/2}$ levels. Sweep width 20 eV, sweep time 20 sec. No. of scans 50. Smoothing over 15 points. Further conditions as above.

contribute to considerable changes in the overall charge of the molecule and a different migration rate will be the consequence. Indeed, the oxidized protein migrated in one single band. Cu-thionein which will be held under aerobic conditions for a longer period of time will be expected to yield also one single electrophoretic band. In an earlier study we presented evidence of such a monodispers Cu-thionein [26]. A similar splitting into two or more components was occasionally observed using Cd, Zn-metallothioneins of various biological origins [27–29]. The occurrence of such apparently different metallothioneins might be conveniently explained by the above phenomenon including intra or intermolecular disulphide bridge formation (see also [1]).

With the discovery of higher concentrations of copper in the metallothionein like proteins [30–33] an important and beneficial role in metal transport for the metallothioneins was considered. The successful conversion of metallothionein into Cu-thionein [13,14] encouraged to propose even a central function of this low mol. wt copper protein in transportation or intracellular storage of copper. The present experiments allow the conclusion of a completely

new aspect regarding the functional side of Cu-thionein. The reversible oxidation of Cu-thionein by H_2O_2 introduces the usefulness of this metalloprotein in bioenergetic systems. Apart from the redox behaviour of copper the strong polarization of the sulphur ligand should be emphasized. The sulphur chromophore would represent a convenient system in electron transport. The unusual high concentration of glutamate and aspartate as well as the high sulphur content indicate some relationship to the protein portion of the redox active iron sulphur proteins (see table 1). Under anaerobic conditions [26] and in the prenatal stage this copper protein is substantially enriched while the formation of cytochrome c oxidase is rather low. It is attractive to speculate that Cu-thionein may take over some key function in the mitochondrial electron transport. In the light of the redox active nature of the protein bound copper and the relationship to the protein portion of the iron sulphur proteins it is suggested to name this protein 'cuprodoxin'. In any case it should be warned to attribute exclusively the role of metal storage or transportation to this copper protein. The biosynthesis of the metal free thionein or the complexed Zn-thionein rather could be considered as precursors required for 'cuprodoxin' formation.

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