CONVERSION OF METALLOTHIONEIN INTO Cu-THIONEIN, THE POSSIBLE LOW MOLECULAR WEIGHT FORM OF NEONATAL HEPATIC MITOCHONDROCUPREIN

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1. Introduction

Our knowledge on the preparation and physicochemical properties of metallothionein has improved in recent years [1–15]. On the other hand, a highly polymeric sulphur and copper rich protein called neonatal hepatic mitochondrocuprein was described by Porter [16–22]. Although this protein bears the name cuprein, it proved completely different from those intracellular cupreins containing 2 g-atoms of each of copper and zinc and which display superoxide dismutase and singlet oxygen decontaminating activities (for a review see [23]). The neonatal type cuprein normally being present under physiological conditions in newborn species was also found in mitochondria of the livers of adults suffering from Wilson's disease. Apart from these polymeric sulphur rich copper proteins some low molecular weight species of similar compositions were found [22,24,25]. It was assumed that the copper is in the cuprous state [24,25] and bound with the cysteine sulphur. Due to the similar amino acid composition of both the metallothionein and neonatal hepatic mitochondrocuprein Porter suggested some relationship to metallothionein [22].

In this context we were highly interested in the possibility of a direct conversion of metallothionein into Cu-thionein. The partially loaded Cu-thionein was expected to polymerize via disulphide bridges in the presence of oxygen to form the 'neonatal type mitochondrocuprein'. Chicken metallothionein was titrated using the stable $Cu(CH_3CN)_4ClO_4$ which is known to react strongly with thiolate sulphur [26–30]. Marked changes of the UV absorption properties of metallothionein were detected. Fifteen g-atoms of Cu^+ were bound by the protein. Displacement of remaining Zn^{2+} and Cd^{2+} by H^+ using partially loaded Cu-thionein yielded highly polymeric species of surprising similarity to neonatal hepatic metochondrocuprein. The binding energy of the sulphur core electrons of this polymer (162.5 eV) was inbetween the respective sulphur binding energies of Cu-thionein and the fully oxidized cystine-thionein.

2. Materials and methods

$Cu(CH_3CN)_4ClO_4$ was obtained using the method given in [26]. Acetonitrile of spectroscopic purity (Uvasol) was from Merck, Darmstadt. Chicken liver metallothionein and the metal free cystine-thionein were prepared as previously reported [9,10]. UV-spectroscopy was performed in a Unicam SP 1800 spectrometer. The X-ray photoelectron spectra (XPS) were recorded with a Varian IEE-15 electrostatic spectrometer. All binding energies were corrected using the C1s line at E = 284.0 eV as an internal standard. In order to minimize possible oxidation of the samples due to the deleterious action of X-ray irradiation the sample holder was cooled to approximately −100°C using liquid nitrogen. Circular dichroic spectra were recorded by means of a Roussel-Jouan Dichrograph CD 185 instrument at 5°C.

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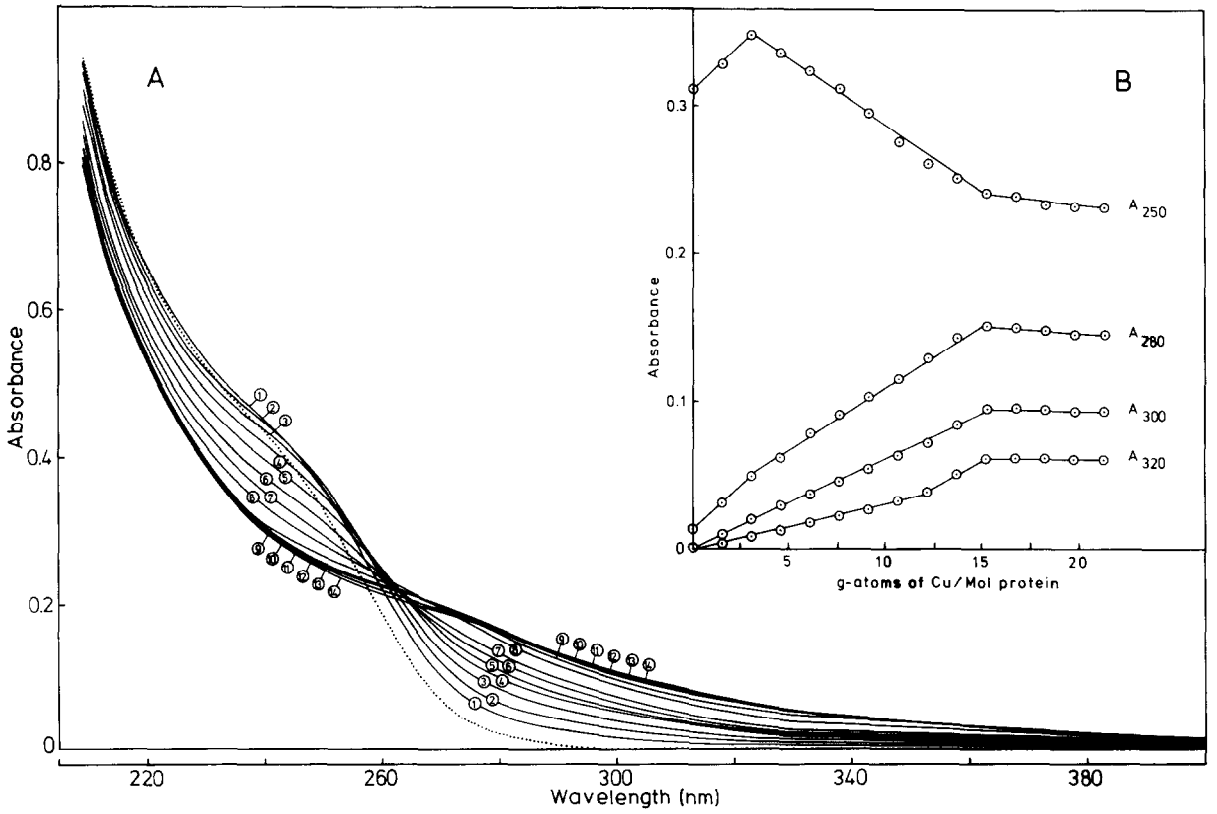

Fig. 1. Titration of metallothionein with $Cu(CH_3CN)_4ClO_4$. A 1 cm light path quartz cell contained in a volume of 3 ml 8.3 mM sodium phosphate buffer, pH 7.0 and 0.158 mg metallothionein. The reference cuvette contained 3 ml 8.3 mM sodium phosphate buffer, pH 7.0 and the corresponding Cu^+ concentrations of the Cu^+ titration. To the metallothionein-containing cuvette the calculated amounts of copper dissolved in CH_3CN-H_2O (1:2, v/v) were added employing a 5 µl pipette. The titration was carried out at 5°C by adding to both cuvettes 5 µl of a 4.0 mM Cu^+ solution to a final concentration of 0.28 µmol (17.8 µg Cu^+). The end point of the titration, 0.2 µmol Cu^+ (12.7 µg Cu^+), corresponds to a copper concentration of about 8.0% of the protein or 15.2 g-atoms/12 000 g of protein. All measurements were carried out in a Unicam SP 1800. A) To 13.2 nMol (158 µg) metallothionein the following concentrations of Cu^+/Mol of protein were added: ① 1.5; ② 3.0; ③ 4.6; ④ 6.1; ⑤ 7.6; ⑥ 9.1; ⑦ 10.6; ⑧ 12.2; ⑨ 13.7; ⑩ 15.2; ⑪ 16.7; ⑫ 18.2; ⑬ 19.8; ⑭ 21.3. The dotted spectrum was obtained using the original metallothionein. B) Plotting the absorbance at 4 constant wavelength against g-atoms of Cu^+/mol of protein. The values were taken from the 14 different spectra of fig. 1A.

3. Results

The total number of metal ions of freshly prepared metallothionein was 8.2 g-atoms/12 000 g of protein and was in accordance with earlier published data [9,10]. The UV spectrum of metallothionein is seen in fig. 1A. Upon the addition of increasing concentrations of $Cu(CH_3CN)_4ClO_4$ the character of the UV absorption spectrum started to change progressively. No absorbance at all was seen in the visible region. The elucidation of the stoichiometry of bound cuprous ions was possible after plotting the concentration of added Cu^+ versus the absorbance at constant wavelengths. At 250 nm the straight line was descending while at 280, 300 and 320 nm all three straight lines were ascending. All four curves showed a surprisingly sharp bending at exactly the same point. This point corresponded to 15.2 g-atoms of cuprous ions per 12 000 g of protein. The Cu-thionein was separated by gel filtration and virtually no metal ions other than copper could be

measured in the protein employing atomic absorption spectroscopy.

The conversion of metallothionein into cuprous-thionein was even better expressed using CD measurements. The presence of elevated Cu^+ concentrations caused the appearance of two, new Cotton effects. One positive extremum at 259 nm ($[\theta]$ = 88 000) and one negative species at 302 nm ($[\theta]$ = −24 000). The negative Cotton effect at 238 nm ($[\theta]$ = −108 000) of the original metallothionein was levelled off. The two new Cotton effects may be assigned to electronic transitions of copper chelates in which the free functional groups of Lys, His [31], or as in the present case, most likely Cys participate as ligands. As in the case of native metallothionein it was of interest to examine the question whether or not the sulphur-containing amino acid residues of Cu-thionein are attributable to cysteine or cystine. It was demonstrated by XPS spectroscopy that in the colourless Cu-thionein the sulphur was exclusively assigned to cysteine-sulphur (161.9 eV) i.e. the replacement of cadmium and zinc by copper caused no oxidation of the RS^- residues (fig. 2). For comparative reasons the fully oxidized metal-free cystine-thionein was recorded. A significant shift of the S 2 $p_{1/2,3/2}$ binding energy by 1.5 eV to 163.4 eV was seen which was attributed to disulphide bridged species.

In solutions of freshly prepared cystine-thionein, the metal-free metallothionein showed after exposure to air an increasing turbidity followed by occasional precipitation of flakes. This phenomenon was attributed to intra- and/or intermolecular disulphide formation of the cysteine residues. It was attractive to consider the possibility that this highly polymeric cystine-thionein could be regarded the metal-free form of the 'neonatal type mitochondrocuprein' [16–22]. Using homogeneous metallothionein the incomplete displacement of cadmium and zinc was successful by adding substoichiometric concentrations of Cu^+ (about 10 g-atoms of Cu^+ per mole of protein). Most of the remaining portions of Cd^{2+} and Zn^{2+} were removed by dialysis versus 50 mM HCl–NaCl buffer, pH 2.5 for 12 hr while all the added Cu^+ was tightly bound with the protein portion throughout. This result was consistent with the low pH resistant binding of copper in the half cystine rich copper protein ('neonatal-type copper protein' [22]). The pH was adjusted to 7.8 under aerobic conditions to allow disulphide formation of free RS^- residues.

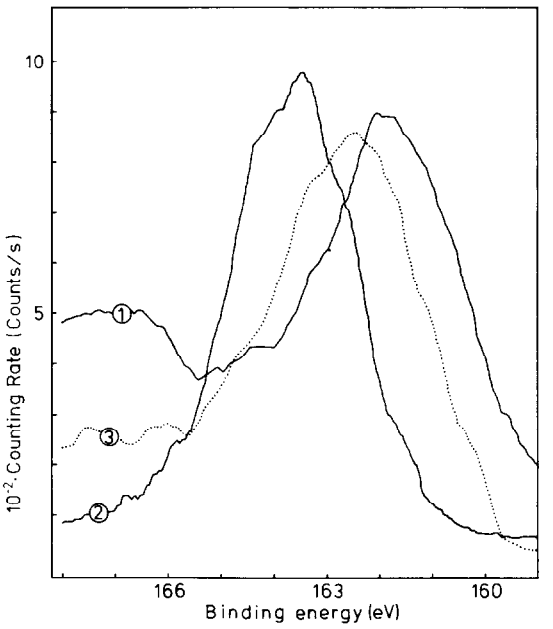

Fig. 2. X-Ray photoelectron spectra of the sulphur $2p_{1/2,3/2}$ levels of ① Cu-thionein, ② cystine-thionein, ③ partially oxidized Cu-thionein. Recording conditions: X-ray course Mg-$K_{\alpha 1,2}$ 1253.6 eV, 100 mA; analyzer energy 50 eV; pressure $2 \cdot 10^{-6}$ Torr; sweep width 10 eV; sweep time 20 sec; no. of scans 150 ①, 50 ②, 91 ③; no. of channels 200; work function 4.6 eV (① and ③), 6.6 eV ②; smoothing over 13 points; recording under constant cooling using liquid nitrogen; the temperature of the applied sample was approximately −100°C.

Indeed, turbidity and precipitation was detectable after these processes. Surprisingly, all the added Cu^+ was still present. This highly polymeric Cu-thionein seemed very similar to the 'neonatal type polymeric mitochondrocuprein'. From the corresponding XPS-spectrum of the sulphur 2 $p_{1/2,3/2}$ level a numerical value of 162.5 eV was obtained. The observed binding energy is roughly half between that of R–S^- (monomeric Cu-thionein 161.9 eV) and R–S–S–R (cystine-thionein 163.4 eV). Considering both the averaged binding energy of 162.5 eV and the marked line broadening of the signal it can be concluded that polymeric Cu-thionein is composed of either sulphur-containing amino acid residues.

4. Discussion

The occurence of a copper rich protein other than the Cu-, Zn-cupreins is known since 1961 [16–22,24, 25]. Porter found it very attractive to correlate this copper and half-Cys-rich polypeptide with metallothionein and other Zn-binding Cys-rich proteins [32, 33]. His conclusions were supported by the successful preparation of a low molecular weight half-cystine-rich polypeptide (molecular weight 5000–10 000) by subjecting the polymeric mitochondrocuprein to sulfitolysis. However, a final decision was not possible due to many unsolved questions, for example, the binding status of sulphur and copper. In the present study the relationship between neonatal hepatic mitochondrocuprein and metallothionein is much more clear. It is possible that the 'neonatal type mitochondrocuprein' is present under biological conditions in the monomeric form. Since the copper concentration of the naturally occurring protein is low (4%) compared to our Cu-thionein (8%) preparation it is very likely that during the course of the preparation of mitochondrocuprein substantial portions of cysteine residues being loosely bound to zinc, other metal ions or even protons, respectively, were oxidized to cystine. Thus polymeric species are expected to be isolated. The polymerization should have occurred in a way similar to our present polymeric Cu-thionein preparation, i.e. oxidative processes in the respective cellular compartments.

The existence of low molecular weight forms of sulphur rich-copper binding proteins was demonstrated in patients suffering from Wilson's disease [24,25]. As in the cadmium-induced stimulation of metallothionein synthesis [6,8–10] the corresponding synthesis of the half-cystine-rich copper protein is also stimulated at elevated copper concentrations normally being found during the course of the illness. It has to be emphasized that throughout the conversion of metallothionein into the 15 g-atoms of copper containing Cu-thionein all copper remained in the cuprous state [34]. This conclusion and the proof of the exclusive presence of RS^- residues was drawn from X-ray photoelectron spectroscopic data.

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