COPPER TRANSFER BETWEEN *NEUROSPORA* COPPER METALLOTHIONEIN AND TYPE 3 COPPER APOPROTEINS

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1. Introduction

Metallothioneins are a class of low-M_r, cysteine-rich proteins which bind unusually high amounts of Zn, Cd and/or Cu. These proteins occur ubiquitously in eukaryotic organisms where they are believed to play an important role in metal metabolism [1]. In contrast to the detailed knowledge on the molecular structure and the physical–chemical properties of the metal-binding sites of metallothioneins, their biological function is still a matter of controversy. Besides a metal detoxification and storage function [2], metallothioneins were also proposed to be involved in metal transfer to apometalloproteins. In particular, the zinc ions of metallothioneins were reported to reactivate a number of zinc-dependent enzymes [2].

Here, we have investigated the copper transfer between *Neurospora* copper metallothionein [3] and the apo-forms of *Neurospora* tyrosinase and *Carcinus* hemocyanin. The reconstitution efficiency was found to be strongly dependent on the oxidation state of copper metallothionein.

2. Materials and methods

Neurospora copper metallothionein and tyrosinase were purified according to [3] and [4], respectively. Hemocyanin from *Carcinus maenas* was provided by Dr B. Salvato. Protein concentrations were measured spectrophotometrically using the coefficient $A_{280}^{1\%,\,1\text{ cm}} = 22.0$ for tyrosinase [4] and $A_{278}^{1\%,\,1\text{ cm}} = 12.4$ for hemocyanin [5] in 0.1 M sodium phosphate (pH 7.5). The concentration of copper metallothionein was expressed on a per mole copper basis. Tyrosinase activity was measured using L-DOPA as substrate [4]. The concentrations of oxytyrosinase and oxyhemocyanin were determined spectrophotometrically. Fully oxygenated tyrosinase and hemocyanin showed absorption ratios of $A_{345}/A_{280} = 0.17$ [4] and $A_{337}/A_{278} = 0.18$ [5], respectively. Apotyrosinase and apohemocyanin were prepared by KCN treatment according to [6] and [7], respectively. X-band EPR measurements were performed at 90 K using a Varian E-112 spectrometer.

The pentapeptide Gly–Cys(–S–S–Cys)–Ser–Ser was prepared by solid-phase synthesis techniques [8] and its purity assessed by HPLC [9]. Apotyrosinase (12 μM in 0.15 M potassium phosphate, pH 6.0) was reconstituted using copper metallothionein (100 μM in 10 mM Tris–HCl (pH 8.0), 5 mM sodium citrate) pre-incubated for 5 min at 10°C with an equimolar amount of sodium dithionite.

The reconstitution was followed by measuring enzymatic activity and formation of oxytyrosinase. Reconstitution of apohemocyanin (12 μM in 0.1 M potassium phosphate, pH 6.0) was monitored by following the increase of absorption at 336 nm. Copper metallothionein (100 μM) and sodium dithionite (200 μM) were added directly into a 3 ml cuvette containing the apohemocyanin solution at 10°C. The reconstituted proteins were fre from excess metallothionein by gel filtration.

Abbreviations: MT, freshly isolated metallothionein; MT_{ox}, air-oxidized metallothionein; MT_r, air-oxidized metallothionein after dithionite reduction; HPLC, high-performance liquid chromatography; L-DOPA, L-3-(3,4-dihydroxyphenyl)-alanine

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3. Results

Freshly isolated *Neurospora* copper metallothionein (MT) is characterized by a broad absorption spectrum in the ultraviolet region displaying a typical shoulder at ~240 nm [10] and is completely EPR-silent (fig.1 right, a). On exposure to air for a few days at 4°C, the protein undergoes spontaneous oxidation leading to distinctly different physical–chemical properties. In particular the absorption spectrum of oxidized metallothionein (MT_{ox}) shows a new absorption band responsible for the blue color of the solution in the visible region (λ_{max} = 610 nm, fig.1 left, ——). Concomitantly an EPR spectrum typical of Cu(II) ions emerges (fig.1 right, b) with the following parameters: $g_{II} = 2.23, g_{I} = 2.05, A_{II} = 19$ mT.

Upon treatment of MT_{ox} with a stoichiometric amount of dithionite the blue color (fig.1 left, ——) as well as the EPR signal (fig.1 right, c) vanish. As shown in fig.2a the dithionite-reduced metallothionein (MT_r) is able to reconstitute *Neurospora* apotyrosinase very efficiently. The reconstitution is complete within 10 min as monitored by measurements of both activity and oxytyrosinase absorption.

The final product is a reconstituted enzyme whose properties, after removal of excess metallothionein, are identical with those of the native form (table 1). The reconstitution reaction with $CuSO_4$, after dithionite treatment as described for MT_r, proceeds rather slowly (fig.2b) resulting in an enzyme with only

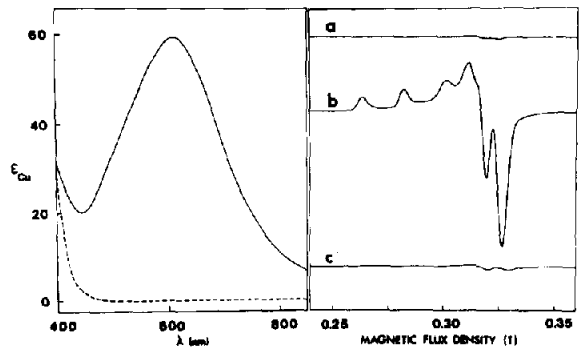

Fig.1. Electronic absorption and EPR spectra of different forms of *Neurospora* metallothionein: (left) absorption spectrum in the visible region of MT_{ox} (——) and MT or MT_r (– – –) in 10 mM Tris–HCl (pH 8.0); (right) EPR spectra of MT (a), MT_{ox} (b), MT_r (c) (1.5 mM in copper). Conditions: temp., 90 K; microwave frequency, 9.11 GHz; microwave power, 10.0 mM; modulation amplitude, 0.1 mT.

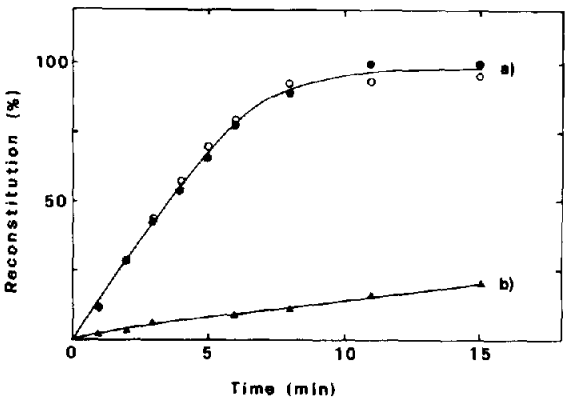

Fig.2. (a) Reconstitution of apotyrosinase with MT_r. The reaction was followed by enzymatic activity (•) and oxytyrosinase formation (○). (b) Reconstitution of apotyrosinase with dithionite treated $CuSO_4$ measuring enzymatic activity (▲). The data are expressed in percent of specific activity and oxytyrosinase formation. For experimental details see section 2.

80–85% of final specific activity. The same results are obtained when MT_{ox} or $CuSO_4$ alone are used for reconstitution. The kinetic of reconstitution observed with MT_r is complicated and cannot be expressed with a single apparent rate constant. Nevertheless, from the initial slope of the plots presented in fig.2, it is seen that the reaction with MT proceeds ⩾10-times faster than that with copper sulfate. Very remarkably, no metal transfers occurs at all from MT to apotyrosinase.

The metal transfer from MT_r to *Carcinus* apohemocyanin is shown in fig.3. In this case too, the reconsti-

Table 1
Physicochemical properties of native-, apo- and reconstituted tyrosinase and hemocyanin

	Native	Apo	Reconstituted
Tyrosinase			
Activity (U/mg)	1200 ± 100	10–20	1200 ± 100
A_{345}/A_{280}	0.17	0	0.16
Cu/protein (g atoms/mol)	2.0 ± 0.1	0.1	2.2 ± 0.1
Hemocyanin			
A_{337}/A_{278}	0.18	0	0.19
Cu/protein (g atoms/mol)	2.0 ± 0.1	0.05	2.2 ± 0.1

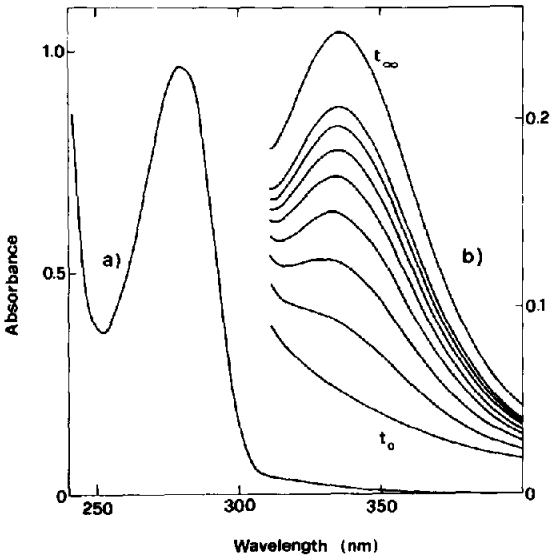

Fig.3. Reconstitution of apohemocyanin with MT_r: (a) absorption spectrum of apohemocyanin before metallothionein addition; (b) increase of the 337 nm band recorded in 5 min intervals; 5 min t_0 and t_∞ indicate the absorption spectra recorded before dithionite addition and at the end of reaction, respectively. For experimental details see section 2.

tution is complete as shown by metal stoichiometry and oxyhemocyanin formation (table 1). The time dependency of the reconstitution reaction (fig.3) can be described by a first order kinetic process with $k = 9.4 \times 10^{-4}$ s^{-1}. In contrast to apotyrosinase, no reconstitution is observed when apohemocyanin is incubated in the presence of MT_{ox}. As found with apotyrosinase, MT is also incompetent of metal transfer to apohemocyanin.

Reconstitution experiments were also carried out with the oxidized form of the synthetic pentapeptide Gly–Cys–Ser–Cys–Ser (representing part of the amino acid sequence of *Neurospora* metallothionein (res. 16–20 in [3])). Essentially the same results were obtained with the Cu(II)–pentapeptide complex reduced with dithionite before reconstitution.

4. Discussion

Metal transfer between metallothionein and apometalloproteins has been studied recently by different groups [2]: reconstitution reactions were shown to proceed almost with the same efficiency either in the presence of metallothionein or of inorganic metal complexes. These data show that metallothionein is required for obtaining a complete and efficient reconstitution of the 2 apoproteins investigated. Remarkably, the reconstitution was found to be strongly dependent on the oxidation state of both the metal ions and the metal ligands of copper metallothionein. Thus, freshly isolated *Neurospora* copper metallothionein proved to be completely incompetent in transferring the metal ions to apotyrosinase and apohemocyanin. This finding is accounted for by the strong binding of Cu(I) to the cysteinyl residues of metallothionein in the form of Cu(I)–thiolate complexes [10,11]. For the Cu(I)–cysteine complex, a binding constant of 1.15×10^{19} M^{-1} was measured [12] which is >4 orders of magnitude larger than that reported for apotyrosinase and Cu(I) [13].

The sensitivity of copper metallothioneins towards oxidation in the presence of air was shown in [11,14]. This property is also typical for *Neurospora* metallothionein which becomes oxidized both with respect to the metal ions and the metal ligands. This is borne out by the emergence of an absorption band in the visible region, an EPR signal, both characteristic for tetragonal Cu(II) (fig.1), and the lack of free sulfhydryl groups, as well as the complete disappearance of its luminescence [10].

The bleaching of the 600 nm band and disappearance of the EPR signal, together with the lack of free sulfhydryl groups in MT_r suggest that dithionite leads to a reduction of the Cu(II) ions leaving the disulfide bridges intact. This metallothionein derivative (MT_r) transfers copper ions very efficiently to apotyrosinase and apohemocyanin (table 1). Both the metal stoichiometry of 2 g copper atoms/functional unit and the specific enzymatic activity of tyrosinase were shown to be indistinguishable for the native and the reconstituted forms.

Hemocyanin and tyrosinase are members of a group of copper proteins containing a binuclear copper center [15]. In the reduced state, the copper is capable of binding molecular oxygen reversibly giving rise to pronounced spectral features in the near ultraviolet and the visible region. Therefore, the fact that apotyrosinase and apohemocyanin were always reconstituted to the oxygenated forms in the presence of MT_r (fig.2,3) lends direct support for a metal ion transfer in the form of Cu(I). Moreover, the reconstitution of hemocyanin has been shown to depend strictly on Cu(I) [16].

In agreement with the presence of Cu(II) ions in

MT_{ox} no metal transfer to *Carcinus* apohemocyanin is observed at all. Under the same conditions, however, apotyrosinase regains enzymatic activity slowly. This partial reconstitution of apotyrosinase is consistent with the fact the Cu(II)-ions (in contrast to apohemocyanin) are capable to enter the apotyrosinase active site, where they are autocatalytically reduced to Cu(I) by endogenous groups of the protein [17].

The results obtained with the Cu–pentapeptide complex suggest the –S–S– groups of MT_r to be involved in the stabilization of the Cu(I) ions. As was pointed out in [18], disulfides represent highly specific ligands for Cu(I) and hence, are expected to greatly stabilize the lower oxidation state of the copper in MT_r. Furthermore, the stability of the Cu(I)–disulfide complex in MT_r is distinctly lower than the one of the Cu(I)–thiolate complex occurring in MT. These stability differences most likely account for the observed differences in reconstitution efficiency of the two metallothionein derivatives.

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