Kinetics of the H_2O_2 Dependent Cleavage of Cu–Thiolate Centres in Yeast Cu_8–Thionein

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Abstract

The usefulness of Cu(I)–penicillamine as a Cu–thionein model was examined by circular dichroism and fluorescence spectrometry. The chiroptical properties were of striking similarity compared to the native yeast protein. There was an emission at 655 nm which was quite similar to the 610 nm emission of Cu_8–thionein. It should be emphasized that the emissions of both Cu-complexes were detectable at 20 °C. The H_2O_2 dependent cleavage of the Cu–thiolate centres in yeast Cu_8–thionein was reexamined. From both circular dichroism and fluorescence measurements a pseudo-first-order decay was deduced. No detectable signs of interconversion reactions and/or metastable intermediates were noticed.

Introduction

Copper(I)–thionein belongs to a class of ubiquitously present metalloproteins. Their role is discussed in the homoeostasis of copper metabolism [1–3]. Unfortunately, the molecular mechanism by which copper is released is unknown. Several possibilities leading to the cleavage of the stable Cu–thiolate centres in Cu–thionein have been proposed [3]. Among these is the controlled Cu(I) transfer into the vacant Cu-binding sites of apo-copper proteins [4, 5]. Proteolysis is only successful after prior damage of the Cu-binding centres [3]. Great emphasis was placed on an oxidative mechanism induced by activated leukocytes [2, 6, 7] and/or H_2O_2-generating enzymes [8].

There remains an important question as to whether or not there are cooperative effects during the release of copper. Furthermore, kinetic data are not available over the course of the copper liberation. An approach was made by titrating native Cu_8–thionein with H_2O_2. Cleavage of the Cu–thiolate centres was monitored by comparing both circular dichroism and emission properties of the native and oxidized copper binding centres.

In relation to the emission properties, excitation by ultraviolet light of *Neurospora crassa* metallothionein emits luminescence at 565 nm attributable to the Cu–thiolate bonding [9]. It was expected to measure essentially the same luminescence employing yeast Cu–thionein. In order to gain further support for the possible structural analogy of Cu(I)–penicillamine and Cu_8–thionein, the fluorescence of the former Cu(I)–thiolate complex was also examined.

Experimental

Chemicals

All reagents used were of analytical grade purity. D-Penicillamine was purchased from Serva, Heidelberg. Yeast Cu_8–thionein was isolated as earlier described [10]. Cu(I)–penicillamine was prepared and stored under argon following the procedure of Gergely and Sovago [11]. *Anal.* Calc.: C, 28.3; H, 4.7; N, 6.6; S, 15.1; Cu, 30. Found: C, 29.0; N, 6.8; S, 15.5; Cu, 27%.

Spectrometry

Copper was quantitated on a Perkin-Elmer atomic absorption spectrometer Model 400 S equipped with an HGA 76 B graphite furnace.

Circular dichroism measurements were run on a JASCO 20A spectropolarimeter.

Fluorescence excitation and emission spectra were recorded on a Spex fluorolog 222 double-beam unit for real-time comparison of samples with a digital data registration (computer controlled) equipped with a 450 W xenon high-pressure lamp (XBO, Osram), double-grating 1680 B monochromators (Spex) and a peltier-cooled R 928 photocathode (Hamamatsu) as photomultiplier. Using this spectrometer the direct recording of corrected spectra was possible. A double-beam module served as the sample module (S) which allowed an on-line recording of the radiation intensity as reference (R) (a totally absorbing Rhodamine B solution connected to a detector). Each emission signal was the result of

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the directly plotted sample over the reference quotient (S/R), thus allowing the collection of corrected data.

The release of Cu from Cu_8–thionein in the presence of up to a 5 molar excess of H_2O_2 was followed by the decline of characteristic Cotton extrema at 354, 329 and 283 nm. Alternatively, the levelling off of the luminescence emission band near 610 nm was monitored. All measurements were carried out at 25 °C in aqueous solutions. No buffer was added so as to minimize uncontrolled interferences.

Results and Discussion

Cu(I)–Thionein and Cu(I)–Penicillamine

Cu(I) coordination to the thiolate sulphur in both Cu_8–thionein and Cu(I)–penicillamine was compared by fluorescence spectrometry and circular dichroism measurements. Upon excitation at λ_{max} = 295 nm a minor emission band at 350 nm and a strong luminescence emission at 610 nm were noticed. The small Stokes shift in the first emission band cannot be attributed to a charge-transfer transition. This luminescence must be assigned to the contribution of the polypeptide chain of the protein. However, the strong shift to 610 nm is typical for charge-transfer transition of Cu(I)–thiolate sulphur bonding (Figs. 1, 2).

The difference between the emission maxima at 610 nm measured for yeast Cu–thionein and the 565 nm emission obtained with *Neurospora crassa* Cu–thionein should be noticed [9, 12]. Whether this red shift is due to the different types of Cu–thionein or to the corrected and uncorrected emission needs to be elucidated. Furthermore, it was important to detect the similar fluorescence of Cu(I)–penicillamine at 293 K. At room temperature no such fluorescence was measured using Cu(I)–cysteine or Cu(I)–glutathione, respectively [12]. Using crystalline Cu(I)–penicillamine an emission maximum was observed at 665 nm with an excitation

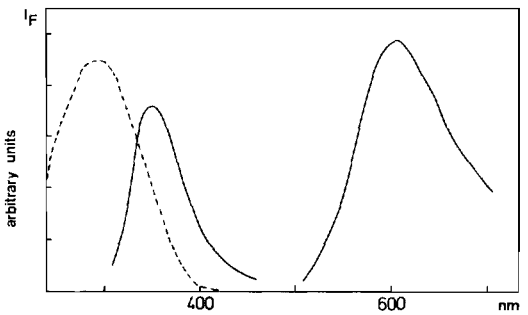

Fig. 1. Fluorescence intensity of Cu(I)–thionein at 293 K: (——) emission spectrum (λ_{ex} = 295 nm); (-----) excitation spectrum (λ_{em} = 610 nm). Aqueous Cu(I)–thionein (0.18 mM of Cu) was maintained aerobically.

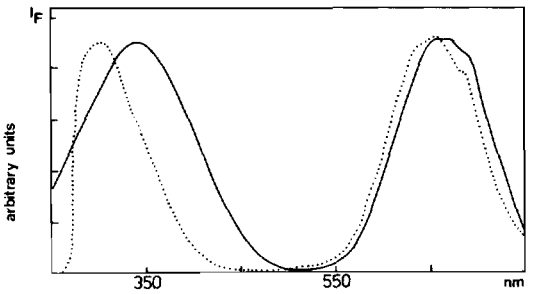

Fig. 2. Fluorescence intensity of Cu(I)–penicillamine at 293 K: (——) excitation and emission of crystalline Cu(I)–penicillamine; (·····) excitation and emission of Cu(I)–penicillamine (50 μM of Cu) dissolved in dimethyl formamide. The spectra were recorded under argon, λ_{ex} = 350 nm.

maximum at 335 nm. Cu(I)–penicillamine dissolved in dimethyl formamide yielded an emission at 655 nm (λ_{ex} = 350 nm).

The comparison of chiroptical properties between Cu_8–thionein and Cu(I)–penicillamine was very encouraging. The conclusion that Cu(I)–SR is similarly coordinated in both compounds gained considerable support (Fig. 3). Most of the Cotton bands were similar in both shape and position (Table I).

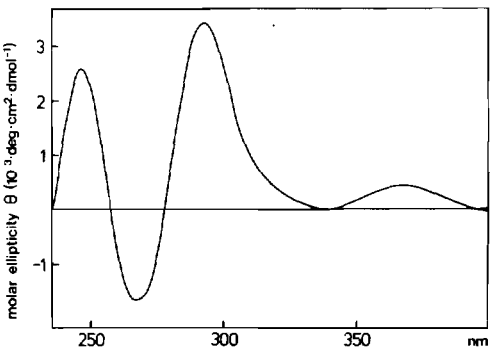

Fig. 3. Circular dichroism of Cu(I)–penicillamine. Cu(I)–penicillamine (0.2 mM of Cu) was dissolved in 0.1 N NaAc buffer, pH 4, and measured under argon at 298 K in 1-cm light path quartz cells.

TABLE I. Chiroptical Properties of Cu_8–Thionein and Cu(I)–Penicillamine

Cu(I)–SR complex	Position of Cotton extrema[a] nm			
Cu_8–thionein	248(+) (15)	283(–) (9)	329(+) (1.5)	354(+) (1.5)
Cu(I)–penicillamine	248(+) (2.6)	270(–) (1.7)	294(+) (3.4)	365(–) (0.5)

[a]Molecular ellipticities (× 10^3 grad cm^2/decimol) are given in parentheses.

Both types of spectroscopic measurements emphasize the usefulness of Cu(I)–penicillamine as a possible model for the Cu–thiolate binding centres in Cu_8–thionein. Unfortunately, unlike Cu_8–thionein, functional studies could not be carried out because of the extreme lability of Cu(I)–penicillamine under aerobic conditions.

Release of Copper from Cu_8–thionein

In earlier work the levelling off of the characteristic Cotton bands of Cu_8–thionein in the presence of oxidizing agents has been shown [6, 10]. It was of interest to examine the time dependency of the breakdown of these Cu–thiolate chromophores. Additions of H_2O_2 ranging from equimolar to a 5-fold excess resulted in the same type of decay (Fig. 4).

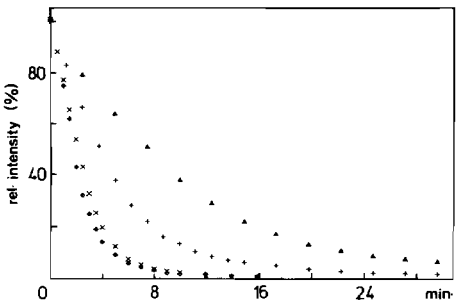

Fig. 4. Time-dependent oxidation of yeast Cu_8–thionein in the presence of H_2O_2. Dichroic recording was performed at 283 nm in a 1-cm light path cell. The H_2O_2 concentration used per mole of Cu was: (△) equimolar; (+) 2 mol; (×) 4 mol; and (◇) 5 mol. The Cu(I)–thionein (0.7 mM of Cu) was aerobically dissolved in H_2O at 298 K.

A kinetic analysis was carried out by measuring the relative θ_{283} values against time (Fig. 5); an exponential decay was seen. An attempt was made to elucidate the reaction type. As there was no linear relation, zero order could be fully excluded. In a first-order rate reaction all curves should be independent of the concentration of H_2O_2. Assuming pseudo-first-order kinetics according to

$$A + B \rightarrow P$$

the velocity can be calculated as

$$v = -d[A]/dt = k[A][B]$$

$[H_2O_2]$ may be considered constant while the other reaction component (*i.e.* Cu–thionein) varies. In other words, the reaction may be regarded as first order. The rate constants, however, depend on the H_2O_2 concentration:

[Cu–thionein] = [A]

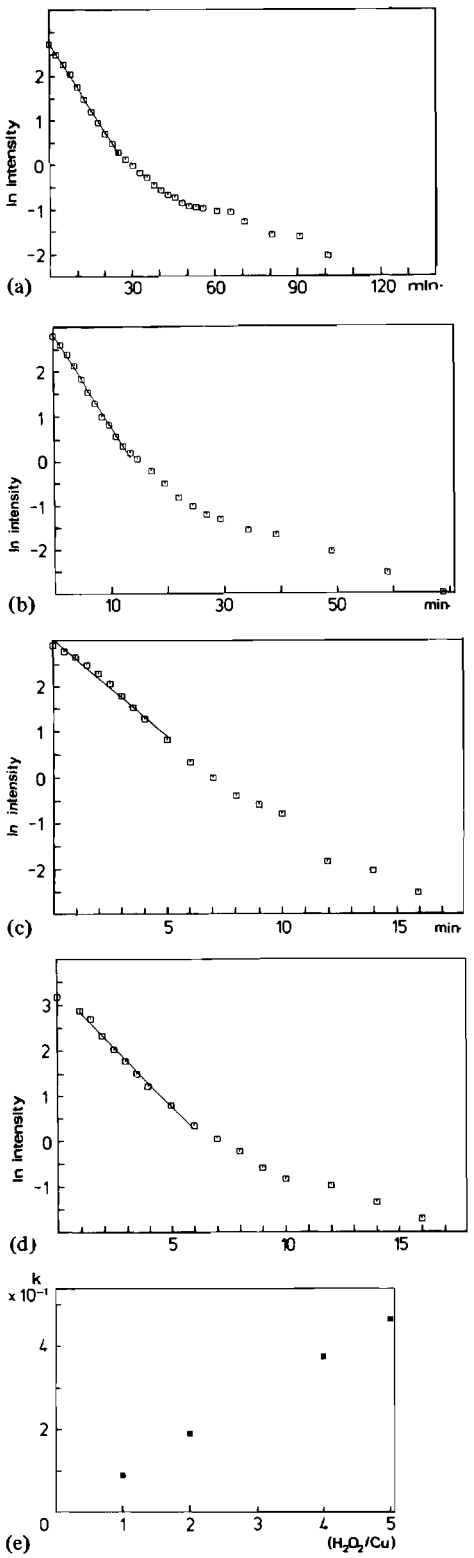

Fig. 5. θ_{283} decrease after H_2O_2 addition: (a) equimolar H_2O_2; (b) 2 mol; (c) 4 mol; (d) 5 mol. For experimental details see legend to Fig. 4. (e) Dependence of the rate constant on the amount of H_2O_2 addition averaged over the wavelengths of the three Cotton extrema (283, 329 and 354 nm).

$v = k'[A]$ while $k' = f[H_2O_2]$

$$\ln [A] = \ln [A_0] k't$$

Plotting ln [A] against time should result in a straight line. In fact, an initial straight line can be noticed which is indicative of a pseudo-first-order decay. When approximately 75% of the Cu–thiolate centres had deteriorated, the kinetics proceeded in an uncontrolled manner.

The oxidative release of Cu from Cu(I)–thionein was also followed by fluorescence spectrometry. Aqueous Cu_8–thionein was excited at 295 nm and the emission recorded at 610 nm (Fig. 6). Essentially the same mode of decay as in the circular dichroism studies was seen. Evaluation of the type of kinetics and the possible linear dependency of the rate constant on the H_2O_2 concentration was performed. For each curve a τ-value was determined, expressing the time at which the intensity I corresponds to $I_0 e^{-1}$.

$$I = I_0 e^{-kt}$$

at $t = \tau, I = I_0 e^{-1}$

$$I_0 e^{-1} = I_0 e^{-k\tau}$$

where k can be obtained from $k = 1/\tau$. From the linear relationship of $k[H_2O_2]$ the pseudo-first-order reaction rate can be deduced which proceeds in this mode.

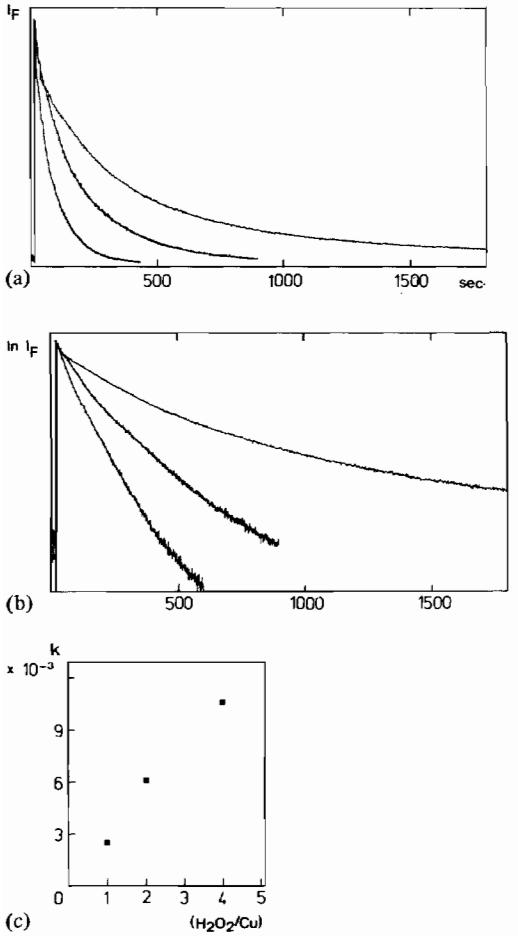

Fig. 6. Diminution of the fluorescence band of the copper–thiolate chromophore at 610 nm after addition of H_2O_2, monitored as: (a) normal plotting; (b) half-logarithmic plotting. The solution of the protein in water (0.5 mg/ml; 0.5 mM of Cu) was excited at 295 nm in the presence of air at 293 K. (c) Dependence of the rate constant on the concentration of H_2O_2.

Conclusions

The well-known oxidative cleavage of the copper–thiolate centres [2, 8, 10] was reexamined to shed some light on possible metastable intermediates. Employing both circular dichroism and fluorescence spectrometry a pseudo-first-order decay was elucidated. This mode of decay lasted a short time only and was followed by an uncontrolled decay. No detectable signs of interconversion reactions and/or metastable situations were attributed to the spectrometrically active copper–thiolate centres.

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