Cu(II) Transfer into Apo-Cu_2Zn_2-superoxide Dismutase from Cu–Thionein Oxidized by Activated Leukocytes†

HANS-JÜRGEN HARTMANN, THOMAS SCHECHINGER and ULRICH WESER*

Anorganische Biochemie, Physiologisch-chemisches Institut der Universität Tübingen, Hoppe-Seyler-Strasse 1, D-7400 Tübingen, F.R.G.

(Received November 12, 1986)

Abstract

The leukocyte-induced oxidative cleavage of yeast Cu(I)–thionein was examined. Oxidation was followed by the progressive decline of the specific Cotton bands attributed to the Cu(I)–thiolate chromophores between 400 and 270 nm. Despite many potent and competitive copper binding sites certainly present in leukocytes, the reconstitution of apo-Cu_2Zn_2-superoxide dismutase was expected due to its higher thermodynamic stability. Both enzymic activity measurements and characteristic Cu(II) EPR** properties of Cu_2Zn_2-superoxide dismutase supported a successful reconstitution. The most favoured pathway for releasing Cu(II) from Cu–thionein was suggested to be an enzyme-controlled oxidation.

Introduction

Little is known on the molecular side of copper metabolism. The most likely candidates in controlling the cellular copper levels are the metallothioneins. These ubiquitously present metal–thiolate-rich proteins are proposed to play an important role in copper transport and/or storage. In these proteins, copper is sequestered in the form of stable oligonuclear Cu(I)–thiolates [1]. Thus, the many undesired reactions of freely diffusing low M_r copper complexes are under control [2].

There is a striking physiological phenomenon in that the copper concentration of pre- and neonatal hepatic tissues can be 10–20 times higher compared to that of adult liver [3]. Essentially all of the copper is bound to metallothionein. It is suggested that this high content is required for the enhanced biosynthesis of Cu-enzymes needed for aerobic life.

One important question remains as to how copper is set free again from these Cu–thioneins. For example, during systemic inflammation in rats the concentration of blood plasma copper rises to 300% [4]. It may be proposed that this elevated copper level originates from catabolized Cu–thionein [5]. Cu(I)–thionein is extremely resistant towards proteolysis. Only oxidized or metal-free thioneins are digested [6]. Using some type I and type II copper proteins, the direct Cu(I) transfer from Cu–thionein into the respective apo-proteins was shown [7–9]. Furthermore, an enzyme-controlled oxidative breakdown of Cu–thionein was demonstrated. Xanthine oxidase and other H_2O_2 producing enzymes were able to oxidize the Cu(I)–thiolate chromophore of Cu–thionein [10].

During the search for a specific Cu–thionein oxidase, liver homogenate and different cell compartments, including the nuclear, mitochondrial, microsomal, lysosomal and cytosolic fractions, were investigated. Unfortunately, no detectable enzymic activity was noticed. As a result of these discouraging results, activated leukocytes were used as an oxidizing means on a cellular level. They were able to cleave the Cu–thiolate centres quite efficiently. The Cu(II) generated effectively inhibited the depolymerization of both hyaluronic acid and synovial fluid [5].

In this context it was intriguing to examine the possibility of whether or not simultaneously present apo-proteins of many copper enzymes could coordinate Cu(II) from deteriorated Cu–thionein. It is quite easy to measure the incorporation into the active centre of apo-proteins using simple buffered solutions. However, the reconstitution is expected to be considerably disturbed when using the above-mentioned cellular system. At the same time, the biological impact will be more pronounced. Cu_2Zn_2-superoxide dismutase was used as an appropriate Cu-enzyme as the chemical environment around the coordinated copper can be easily detected by EPR spectroscopy. Activated porcine leukocytes proved to be convenient to cleave copper–thionein. The successful release of Cu was monitored by following the changes of the dichroic properties of the Cu–thiolate chromophores. In addition, the formation of the holoenzyme was assayed by measuring the superoxide dismutase activity.

†Dedicated to Prof. Dr. E. Bayer on the occasion of his 60th birthday.

*Author to whom correspondence should be addressed.

**Abbreviation: EPR, electron paramagnetic resonance.

0020-1693/87/$3.50

Experimental

Materials

4-Nitroblue tetrazolium chloride (NBT) and xanthine were purchased from Serva (Heidelberg). Xanthine oxidase was obtained from Boehringer (Mannheim) and phorbol myristate acetate (PMA) was from Sigma (Heidelberg).

Spectrometry

Electronic absorption was measured using a Beckman DU-40 spectrophotometer. Circular dichroism measurements were performed on a JASCO 20A spectropolarimeter and EPR spectra at X-band frequency and 100 K were run on an E-109 Varian spectrometer. Cu and Zn were quantitated on a Perkin-Elmer 400 S atomic absorption spectrometer furnished with an HGA 76B unit.

Preparative Procedures

Yeast Cu–thionein was isolated using the methods described in both refs. 11 and 12 and contained 50 ± 2 μg Cu per mg protein. No EPR signal indicative for Cu(II) was detectable. Cu_2Zn_2-superoxide dismutase was isolated from bovine blood and converted into the apo-protein by gel filtration in the presence of EDTA [13]. Porcine leukocytes were isolated and activated as described in refs. 5 and 14.

Assays

Superoxide dismutase activity was assayed employing the Nitroblue tetrazolium method [15] in the presence of 1 mM EDTA to avoid unspecific activities derived from low M_r Cu-complexes.

Results and Discussion

The oxidative cleavage of Cu–thionein was examined on a cellular level. Yeast Cu–thionein which is known to bind exclusively Cu(I) was chosen. Although this Cu–thionein is different in its primary structure, the metal is coordinated in essentially the same manner as in the vertebrate Cu, Zn–thioneins [16]. Upon incubation of Cu–thionein with activated leukocytes and in the presence of EDTA, oxidation of the Cu(I)–thiolate chromophores was observed. Progress of the degradation was followed by observing the characteristic Cotton bands between 400 and 270 nm. The molar θ_{Cu} values were taken as a measure of the amount of native Cu(I)–thiolates (Fig. 1).

The time course of decomposition was dependent on both the cell number and the concentration of Cu–thionein. Two hours proved sufficient to cleave 50% of the Cu(I)–thiolate clusters. Concomitant with the decrease of the Cotton extrema the formation of unspecifically bound Cu(II) was detected by

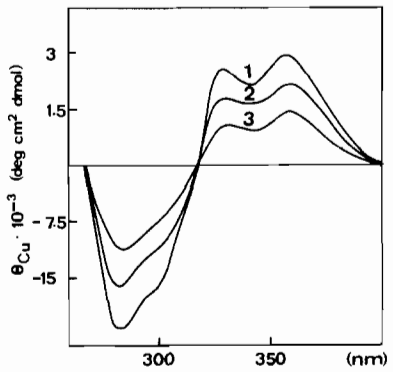

Fig. 1. Circular dichroism of Cu–thionein incubated with leukocytes in the presence of EDTA: (1) after 5 min, (2) 1 h, (3) 2 h. Incubation conditions: 1.5 ml leukocyte suspension (15×10^7 cells) in a buffer composed of: 140 mM NaCl, 10 mM Na_2HPO_4, 3 mM K_2HPO_4, 2.7 mM KCl, 5.5 mM D-glucose, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.3 0.75 ml Cu–thionein (3×10^{-4} M Cu), 25 μl 10^{-2} M EDTA, 270 μl H_2O, 30 μl phorbol myristate acetate (3 μg) (total 2.575 ml), temperature 20 °C. Stirring was maintained during incubation. 800 μl aliquots were centrifuged and circular dichroism measurements were carried out in the supernatant.

EPR spectrometry. In the incubation mixture, EDTA was essential to prevent inhibition of the leukocytes by this extraneously bound Cu(II) [5].

The specific trapping of generated Cu(II), regardless of possibly disturbing cellular components, was examined using apo-Cu_2Zn_2-superoxide dismutase. A very effective reconstitution of the metal-free protein by Cu(II) was seen (Table I). The simultaneous incubation of both apo-Cu_2Zn_2-superoxide dismutase and Cu–thionein and in the presence of leukocytes resulted in a marked superoxide dismutase activity compared to the cell-free control.

Only negligible superoxide dismutase activity was measured when either apo-Cu_2Zn_2-superoxide dismutase or Cu–thionein was omitted. Under the above experimental conditions the reaction was completed within approximately 1 min. Upon lowering the temperature to 20 °C and in the presence of 1/8 of the original cell number, a time-dependent formation of Cu_2Zn_2-superoxide dismutase was noticed (Fig. 2). The Zn(II) concentration of leukocytes, usually about 10 μM [17], is sufficient for reconstituting the 20 nM of Cu_2Zn_2-superoxide dismutase. Furthermore, the Zn-free enzyme yields the same enzymic activity [18]. Maximum reactivity of the enzyme was measured after 30 min with 66% inhibition of NBT-reduction. The 50% value was reached after 6 min. This is in accordance with a Cu_2Zn_2-superoxide dismutase concentration of 2×10^{-8} M, which is usually needed for 50% inhibition [19]. The slight activity in the control experiment devoid of any leukocytes derives from a negligible amount of extraneously bound thionein–copper(II) or Cu(I)–SR [1].

TABLE I. Reconstitution of Apo-Cu_2Zn_2-superoxide Dismutase in the Presence of Cu–Thionein and Leukocytes[a]

Assay	A_{540}	Inhibition of NBT-reduction (%)
Full incubation	0.194	67.0
Controls, omission of		
leukocyte activation	0.505	14.4
leukocytes	0.525	11.0
apo-SOD	0.576	2.4
Cu–thionein	0.580	1.7

[a]Superoxide dismutase activity was measured using the Nitroblue tetrazolium chloride (NBT)-reduction assay. Incubation conditions: 400 μl cell suspension (8×10^7 cells), 50 μl 5×10^{-7} M apo-Cu_2Zn_2-superoxide dismutase (apo-SOD), 50 μl Cu–thionein (10^{-5} M Cu), 10 μl phorbol myristate acetate (1 μg). Samples were incubated for 20 min at 37 °C. Prior to the superoxide dismutase activity assay the leukocyte-containing samples were centrifuged and the respective supernatants subjected to the test mixture composed of 750 μM NBT, 0.25% gelatine and 1 mM EDTA in 6 mM phosphate buffer, pH 7.4. To 410 μl of the reagent mixture 40 μl of the test solution and 25 μl of 4×10^{-6} M xanthine oxidase were added. The reaction was started with 25 μl 2 mM xanthine. A_{540} was recorded until the reaction was completed.

It is well known that superoxide dismutase activity can be measured with copper complexes other than Cu_2Zn_2-superoxide dismutase [18]. Thus, in support of the enzymic data it seemed desirable to gain structural information regarding the proper reconstitution. The high concentration of leukocytes may possibly interfere with the specific copper binding. The chemical environment around the Cu(II)

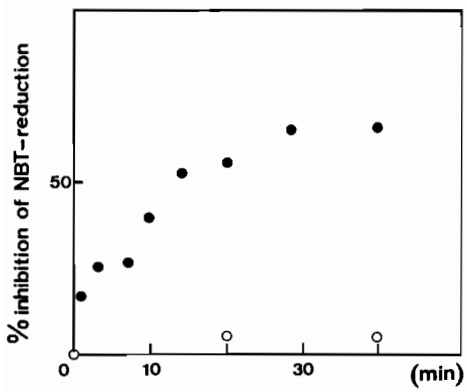

Fig. 2. Time-dependent formation of superoxide dismutase measured as inhibition of NBT-reduction. Incubation conditions: 50 μl leukocytes (10^7 cells), 350 μl buffer (see legend to Fig. 1), 50 μl apo-Cu_2Zn_2-superoxide dismutase (5×10^{-7} M), 50 μl Cu–thionein (10^{-5} M Cu), 20 °C. The reaction was started with 10 μl phorbol myristate acetate (1 μg). The NBT-assay was performed in the supernatant (see legend to Table I). •, full incubation; ○, control (without cells).

in Cu_2Zn_2-superoxide dismutase can be conveniently monitored by EPR spectrometry. The concentration of apo-Cu_2Zn_2-superoxide dismutase was raised to 0.1 mM to allow recording of the Cu(II) EPR parameters typical for the native enzyme. Contrary to the former enzymic assays, Zn(II) was added to ascertain full reconstitution of the holoprotein (Fig. 3). Essentially all copper is coordinated in the active centre. The EPR parameters are characteristic for a native Cu_2Zn_2-superoxide dismutase ($A_\parallel = 140$ G, $g_\perp = 2.060, g_\parallel = 2.262$) [13].

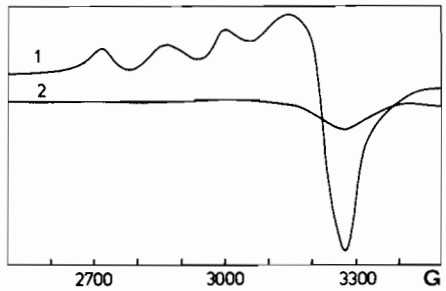

Fig. 3. EPR of reconstituted apo-Cu_2Zn_2-superoxide dismutase simultaneously incubated with Cu–thionein and activated leukocytes. (1) Incubation mixture: 250 μl apo-Cu_2Zn_2-superoxide dismutase (1.56×10^{-4} M), 60 μl Cu–thionein (6×10^{-4} M Cu), 100 μl leukocyte cell suspension (10^8 cells), 10 μl 10^{-2} M Zn^{2+}, 10 μl phorbol myristate acetate (1 μg). (2) Control (omission of leukocytes). EPR spectra were recorded after 60 min of incubation at 100 K; scan range 1000 G; modulation frequency 100 kHz; microwave power 20 mW; microwave frequency 9.24 GHz.

In erythrocytes, essentially all copper is coordinated in Cu_2Zn_2-superoxide dismutase [19]. No metallothionein has been detected so far. Thus, the present study bears more or less a model character. However, an immunologically identical cytosolic Cu_2Zn_2-superoxide dismutase is found in the liver. In the cytosol the major portion of Cu–thionein is simultaneously present. Both copper proteins are expected to be synthesized in the liver, and, if Cu–thionein acts as a copper storage and/or transports protein the subsequent release of copper should occur. As proteolysis of Cu–thionein appears to be not convincing, the oxidative cleavage is the more favoured pathway. Although no specific Cu–thionein oxidase has been characterized, the presently observed leukocyte-dependent oxidative breakdown is a promising step to elucidate the mechanism of the biological turnover of Cu–thionein.

Acknowledgements

This study was aided by a grant from the Deutsche Forschungsgemeinschaft We 401/18-1 and in part by the Fonds der Chemischen Industrie. Thanks go to Helga Heinemann for expert technical assistance.

References

1 U. Weser and H.-J. Hartmann, in R. Lontie (ed.), 'Copper Proteins and Copper Enzymes', CRC Press, Boca Raton, Fla., 1984, pp. 151–173.
2 H.-J. Hartmann and U. Weser, in P. Brätter and P. Schramel (eds.), 'Trace Element–Analytical Chemistry in Medicine and Biology', Walter de Gruyter, Berlin, 1984, pp. 267–281.
3 M. J. Ettinger, in R. Lontie (ed.), 'Copper Proteins and Copper Enzymes', CRC Press, Boca Raton, Fla., 1984, pp. 175–229.
4 U. Deuschle and U. Weser, *Prog. Clin. Biochem., 2,* 99 (1986).
5 H.-J. Hartmann, A. Gärtner and U. Weser, *Clin. Chim. Acta, 152*, 95 (1985).
6 U. Weser, W. Mutter and H.-J. Harmann, *FEBS Lett., 197*, 258 (1986).
7 H.-J. Hartmann, L. Morpurgo, A. Desideri, G. Rotilio and U. Weser, *FEBS Lett., 152*, 94 (1983).
8 L. Morpurgo, G. Rotilio, H.-J. Hartmann and U. Weser, *Biochem. J., 221*, 923 (1984).
9 S. Brütsch, H.-J. Hartmann and U. Weser, *Inorg. Chim. Acta, 92*, 147 (1984).
10 H.-J. Hartmann, A. Gärtner and U. Weser, *Z. Physiol. Chem., 365*, 1355 (1984).
11 U. Weser, H.-J. Hartmann, A. Fretzdorff and G. Strobel, *Biochim. Biophys. Acta, 493*, 465 (1977).
12 D. R. Winge, K. B. Nielson, W. R. Gray and D. H. Hamer, *J. Biol. Chem., 260*, 14464 (1985).
13 U. Weser, G. Barth, C. Djerassi, H.-J. Hartmann, P. Krauss, G. Voelcker, W. Voelter and W. Voetsch, *Biochim. Biophys. Acta, 278*, 28 (1972).
14 R. A. Greenwald and W. W. Moy, *Arthritis Rheum., 23*, 455 (1980).
15 M. Younes and U. Weser, *FEBS Lett., 61*, 209 (1976).
16 H.-J. Hartmann and U. Weser, *Biochem. Biophys. Res. Commun., 132*, 277 (1985).
17 K. H. Falchuk, in A. V. Xavier (ed.), 'Frontiers in Bioinorganic Chemistry', VCH Verlagsgemeinschaft, Weinheim, 1986, pp. 355–365.
18 A. Gärtner and U. Weser, *Top. Current Chem., 132*, 1 (1986).
19 A. Gärtner and U. Weser, *FEBS Lett., 155*, 15 (1983).