Copper-dependent metabolism of Cu,Zn-superoxide dismutase in human K562 cells

Lack of specific transcriptional activation and accumulation of a partially inactivated enzyme

Christian STEINKÜHLER,* Maria Teresa CARRI,* Gioacchino MICHELI,† Lea KNOEPFEL,* Ulrich Weser§ and Giuseppe ROTILIO*‡||

*Department of Biology, University of Rome 'Tor Vergata', I-00173 Rome, Italy, C.N.R. Centres for †Nucleic Acid Research and ‡Molecular Biology, University of Rome 'La Sapienza', I-00185 Rome, Italy, and §Department of Inorganic Biochemistry, Institute of Physiological Chemistry, University of Tübingen, D-72076 Tübingen, Germany

The regulation of Cu,Zn-superoxide dismutase by copper was investigated in human K562 cells. Copper ions caused a dose- and time-dependent increase, up to 3-fold, of the steady-state level of Cu,Zn-superoxide dismutase mRNA. A comparable increase was also observed for actin and ribosomal protein L32 mRNAs, but not for metallothionein mRNA which was augmented more than 50-fold and showed a different induction pattern. The copper-induced mRNAs were actively translated as judged from their enhanced loading on polysomes, the concomitantly increased cellular protein levels and an augmented incorporation of [³H]lysine into acid-precipitable material. Cu,Zn-superoxide dismutase protein followed this general trend, as demonstrated by dose- and time-dependent increases in immunoreactive and enzymically active protein. However, a specific accumulation of Cu,Zn-superoxide dismutase was noticed in cells grown in the presence of copper, that was not detectable for other proteins. Purification of the enzyme demonstrated that Cu,Zn-superoxide dismutase was present as a reconstitutable,

copper-deficient protein with high specific activity ($k_{cat}/Cu = 0.89 \times 10^9 M^{-1} \cdot s^{-1}$) in untreated K562 cells and as a fully metallated protein with low specific activity ($k_{cat}/Cu = 0.54 \times 10^9 M^{-1} \cdot s^{-1}$) in copper-treated cells. Pulse-chase experiments using [³H]lysine indicated that turnover rates of Cu,Zn-superoxide dismutase in K562 cells were not affected by growth in copper-enriched medium, whereas turnover of total protein was significantly enhanced as a function of metal supplementation. From these results we conclude that: (i) unlike in yeast [Carri, Galiazzo, Ciriolo and Rotilio (1991) FEBS Lett. 278, 263–266] Cu,Zn-superoxide dismutase is not specifically regulated by copper at the transcriptional level in human K562 cells, suggesting that this type of regulation has not been conserved during the evolution of higher eukaryotes; (ii) copper ions cause an inactivation of the enzyme in intact K562 cells; and (iii) the metabolic stability of Cu,Zn-superoxide dismutase results in its relative accumulation under conditions that lead to increased protein turnover.

INTRODUCTION

Metalloproteins that catalytically react with superoxide radicals [superoxide dismutases (SODs)] are present in most aerobic cells as two distinct entities: one is a constantly expressed 'house-keeping' activity, while the other is adaptively expressed as response to a series of environmental stimuli. The members of this latter group of inducible proteins are the manganese-containing superoxide dismutases (Mn-SODs), which are found both in eukaryotes and in prokaryotes [1]. Mn-SOD expression is affected by molecular oxygen [2], chemical oxidants [3] or heat shock [4] in prokaryotes and by cytokines and phorbol esters [5,6] in mammalian cells.

In eukaryotes, intracellular copper- and zinc-containing SODs (Cu,Zn-SODs) are generally constitutively expressed [1] and have been identified as 'housekeeping' proteins in both mammalian and amphibian cells [7,8].

Recent work has pointed to a metal-dependent regulation of SODs. In *Escherichia coli*, both iron containing SOD (Fe-SOD) and Mn-SOD activities are regulated by iron at the transcriptional and at the post-translational levels [9–11]. Similarly, Cu,Zn-SOD activity has been shown to be regulated by copper at the transcriptional level in yeast [12] and at the post-translational level in both yeast [13] and human cells [14].

Copper-dependent transcriptional regulation of Cu,Zn-SOD in yeast cells involves the transcription factors ACE1 in *Saccharomyces cerevisiae* [12,15] and AMT1 in *Candida glabrata* [16]. In a copper-loaded form, these proteins bind with high affinity to specific regulatory DNA sequences present in the promoters of the metallothionein (MT) and Cu,Zn-SOD genes of yeasts, thereby activating gene transcription [15]. This causes a concerted expression of Cu,Zn-SOD and the metal-storage protein MT in yeast cells exposed to copper [12].

MT gene expression is regulated by copper (and other metal ions) via metal-dependent transcription factors in higher eukaryotes also [17]. Thus the aim of this work was to investigate whether or not copper-mediated transcriptional co-regulation of MT and Cu,Zn-SOD is a generalized feature of eukaryotic cells or is specific to the yeast system. This question was addressed using two eukaryotic cell lines, namely human K562 erythroleukaemia cells and *Xenopus laevis* kidney cells. The former cell line was chosen due to its capability to undergo changes in copper metabolism, including caeruloplasmin receptor synthesis, copper uptake and Cu,Zn-SOD levels in response to different stimuli [14], whereas *X. laevis* has been previously used as a model system for the study of Cu,Zn-SOD regulation during embryogenesis [9].

We here present evidence against a specific copper-dependent

Abbreviations used: MT, metallothionein; SOD, superoxide dismutase; ESB, PBS containing 0.3% gelatin; EWB, PBS containing 0.2% Tween-20; LB, (lysis buffer; 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 3% BSA, 10 mM Lys in 50 mM Tris/HCl, pH 7.5).

|| To whom correspondence should be addressed at: Dipartimento di Biologia, Università di Roma 'Tor Vergata', Via della Ricerca Scientifica, I-00173 Roma, Italy.

transcriptional regulation of Cu,Zn-SOD gene expression in human K562 cells and in *X. laevis* kidney cells. We further show that copper induces Cu,Zn-SOD in K562 cells by causing profound changes in protein and RNA metabolism that lead to the accumulation of Cu,Zn-SOD molecules with altered biochemical properties.

EXPERIMENTAL

Chemicals

All chemicals were of analytical grade quality and used without further purification. Cell culture media and medium additives were obtained from Flow. Lysine-deficient RPMI medium was purchased from Sigma. Radiochemicals were supplied by Amersham. All other chemicals were obtained from Merck.

Cell culture

Human K562 erythroleukaemia cells (clone A) were purchased from the American Type Culture Collection and grown as previously described [14]. *Xenopus laevis* kidney culture cells were grown as previously described [18]. Copper was added to cultures from freshly prepared stock solutions containing 10 mM CuSO₄ in 0.9% NaCl. Growth curves and EC₅₀ values were determined according to published procedures [19]. Cell homogenates were prepared by sonication as previously described [14]. Cell disruption for subcellular fractionation experiments was done with a Potter–Elvehjem homogenizer upon suspension of the cells in homogenization buffer (250 mM saccharose, 10 mM Hepes, pH 7.4, 1 mM EDTA).

Enzyme assays

Polarographic determination of Cu,Zn-SOD activity and reconstitution of inactive enzyme in homogenates was performed as described elsewhere [14]. Enzyme activity was expressed as ng of Cu,Zn-SOD/10⁶ cells using pure recombinant human Cu,Zn-SOD as standard. Catalase [20], lactate dehydrogenase [21] and acid phosphatase [22] were assayed according to published protocols. Protein was estimated according to Lowry et al. [23].

Copper concentration determinations

Cells (5×10^7) were harvested by centrifugation and washed once with each of the following buffer solutions, prepared with Chelex-100 (Bio-Rad)-treated water: PBS (50 ml; 20 mM sodium phosphate, 140 mM NaCl, pH 7.4) containing 1 mM CaCl₂; 50 ml of PBS containing 1 mM EDTA; and 50 ml of PBS without further additives. The pellet was resuspended in 300 μ l of PBS and transferred to an acid-washed vial containing 700 μ l of 65% ultrapure HNO₃ (Merck). Control samples consisted of PBS treated in the same way. After hydrolysis for 7 days at 25 °C, copper was determined with a Perkin–Elmer 3030 atomic absorption spectrometer equipped with a graphite furnace.

Total RNA extraction and Northern-blot analysis

Total poly(A⁺) RNA was extracted from 1×10^7 cells using a commercial kit (Pharmacia). Northern-blot analysis was performed according to standard protocols [8]. Nitrocellulose filters

were probed using the whole coding sequence for human Cu,Zn-SOD (a gift from Dr. Y. Groner, Weizman Institute of Science, Rehovot, Israel), L32 [24] or β -actin [25]; synthetic oligonucleotide 5'-GGGCAGCAGGAGCAGCAGCT-3' was used as a probe for human MT [26].

Polysome isolation

Polysome fractionation and RNA extraction from ribonucleo-protein fractions was performed according to [8] by running cell extracts on 15–50% linear sucrose gradients. The absorbance at 260 nm was recorded upon fractionation and purified RNAs were subjected to Northern-blot analysis as described above.

Cu,Zn-SOD e.i.s.a.

Polyclonal antibodies against human Cu,Zn-SOD (pAb α SOD) were raised in rabbits and purified by precipitation with 50% (w/v) (NH₄)₂SO₄ and DE-52 chromatography. Then 96-well microtitre plates were coated overnight with 50 μ l of a solution containing 160 μ g/ml of this antibody preparation in 100 mM sodium carbonate buffer, pH 9.5. After saturation of unspecific binding sites with 100 μ l of 1% gelatin in PBS for 1.5 h, samples, in 50 μ l of ESB solution (PBS containing 0.3% gelatin) were added to the antibody-coated wells. Plates were incubated for 1.5 h at room temperature after which unbound antigen was removed by extensive washing with EWB solution (PBS containing 0.2% Tween-20). A monoclonal antibody directed against human Cu,Zn-SOD (Sigma) was then added at a dilution of 1:300 in 50 μ l of ESB. After 1.5 h incubation plates were washed with EWB and incubated for a further hour in the presence of anti-(mouse IgG) conjugated with horseradish peroxidase (Sigma), diluted 1:1000 in ESB. After repeated plate washing with EWB the amount of horseradish peroxidase attached to the solid phase by the immune reaction was determined using 1,2-phenylenediamine as a substrate. The amount of SOD present in samples was determined by comparison with a standard curve between 100 pg and 3.3 ng of pure, recombinant human Cu,Zn-SOD. The detection limit of the assay was 50 pg of pure Cu,Zn-SOD. Both intra- and inter-assay CV-values were less than 10% (CV = ratio between standard deviation and mean value).

Purification of Cu,Zn-SOD from K562 cells

Cu,Zn-SOD was purified from cells grown for 72 h in the presence of 200 μ M CuSO₄. The protein was detected by e.i.s.a. throughout the purification procedure, which was performed using a Pharmacia f.p.l.c. unit. K562 cells (1×10^9) were disrupted by sonication and the resulting homogenate was centrifuged for 70 min at 110000 g at 4 °C. The supernatant was subsequently dialysed overnight against 5 mM Tris/HCl, pH 7.5, and applied to a HiLoad Q-Sepharose 16/10 column (Pharmacia) operating at a flow rate of 2 ml/min. A linear NaCl gradient (0–200 mM) was applied to elute Cu,Zn-SOD. The e.i.s.a.-positive fractions were pooled, concentrated to 200 μ l by ultrafiltration and chromatographed on a Superose-12 (HR10/30) gel-filtration column (Pharmacia) equilibrated with 50 mM Tris/HCl, pH 7.5, operating at 0.3 ml/min. The SOD-containing fractions were pooled, diluted 10-fold with bidistilled water and applied to a Mono Q (HR 5/5) ion-exchange column (Pharmacia) equilibrated with 5 mM Tris/HCl, pH 7.5, and operating at a flow rate of 1 ml/min. Cu,Zn-SOD from K562 cells grown in the presence of copper eluted as two distinct peaks from this column. The peaks were collected separately. About 80 μ g of purified protein were ob-

tained, as determined by e.l.i.s.a., of which 75% were eluted as the major peak (SODI, see the Results section). Homogeneity was assessed by PAGE. Gels were either Coomassie Blue- or activity-stained [27].

Labelling with [^3H]lysine and immunoprecipitation

K562 cells were labelled for 24 h in the presence of $5\ \mu\text{Ci/ml}$ [^3H]lysine (specific radioactivity $83\ \text{Ci/mmol}$) essentially as described by others [28]. The cells were washed with 50 ml of RPMI medium supplemented with 10% (v/v) fetal-calf serum and 5 mM unlabelled lysine and resuspended at 0.5×10^6 cells/ml in 100 ml of this medium. At different time points aliquots (25 ml) of the cell suspensions were collected, centrifuged and the cells were washed with 50 ml of PBS. To determine the amount of trichloroacetic acid-precipitable label, 0.5×10^6 cells were suspended in $100\ \mu\text{l}$ of PBS containing 7% trichloroacetic acid, incubated for 30 min at 4°C and centrifuged. The resulting pellet was washed four times with 1 ml of 5% trichloroacetic acid/200 mM KCl and once with 1 ml of acetone. The pellet was then solubilized by addition of 1 M NaOH and radioactivity was determined by liquid-scintillation counting using a Packard 1500 Tri-Carb liquid-scintillation analyser. To determine the amount of immunoprecipitable label, 6×10^6 cells were incubated for 30 min at 4°C in the presence of $100\ \mu\text{l}$ of lysis buffer (LB) containing: 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 3% BSA and 10 mM lysine in 50 mM Tris/HCl, pH 7.5. The lysate was centrifuged for 20 min at 17000 g and to one-half of the resulting supernatants a 100-fold excess of unlabelled recombinant human Cu,Zn-SOD (260 μg in 20 μl of water) was added. After 5 min incubation, 10 μl of pAb α SOD (17 mg/ml) was added to all samples. After further incubation for 1 h, 25 μl of a 1:1 (v/v) suspension of Protein A beads (Pharmacia) in LB were added and incubation was continued for an additional hour. The Protein-A beads were then collected by centrifugation and washed four times with 1 ml of LB. Immunoprecipitated Cu,Zn-SOD was detached from the beads by incubation for 10 min at 95°C in 50 μl of 2 $\times$ SDS sample buffer [29]. Radioactivity was determined by liquid-scintillation counting and immunoprecipitated Cu,Zn-SOD was expressed as the amount of radioactivity which was inhibitable by a 100-fold excess of unlabelled Cu,Zn-SOD. Generally 30–40% of total radioactivity were due to unspecific co-precipitation of unrelated proteins.

RESULTS

Effects of copper on the growth of K562 cells

In order to avoid copper precipitation in culture medium [19], we tested several amino acid- or synthetic bi-Schiff-bases as solubility-increasing ligands. However, growth inhibition of K562 cells by copper compounds varied considerably as a function of the specific metal ligand studied, the EC_{50} values ranging from $35\ \mu\text{M}$ to 5 mM (results not shown). The lowest toxic potential was displayed by Cu^{2+} -His $_2$ ($\text{EC}_{50} = 5\ \text{mM}$). This fact is probably related to an inhibition of metal uptake in the presence of histidine. In contrast with previous observations [19] we were not able to detect CuCO_3 precipitates in medium supplemented with up to $400\ \mu\text{M}$ CuSO_4 , possibly due to rapid accumulation of the metal by the cells. To avoid ligand effects, we therefore used this form of copper supplementation for the subsequent experiments.

CuSO_4 -supplementation produced both dose-dependent growth inhibition ($\text{EC}_{50} = 226\ \mu\text{M}$) and metal accumulation by

K562 cells (Figure 1), but no significant enhancement of the amount of Trypan Blue-positive cells (< 5% at all copper concentrations tested).

Copper distribution in K562 cells

Copper eluted in three major fractions upon size-exclusion chromatography of extracts from copper-exposed cells (Figure 2): one high-molecular-mass fraction (> 500 kDa), eluting with the void volume (containing about 30% of the total copper); one fraction corresponding to a molecular mass of 10 kDa (containing 50% of the total copper); and a fraction corresponding to < 1 kDa (20% of the cellular copper). No copper was detectable in the latter two fractions in the absence of the reducing agent 2-mercaptoethanol, indicating that the metal was bound to redox-sensitive components present in these fractions. The elution behaviour of the 10 kDa species on a Superose 12 size-exclusion f.p.l.c. column was identical with that of pure rabbit liver Cu-MT, indicating that this protein is a major copper-binding species in copper-exposed K562 cells. Further evidence for the role of MT in copper storage in K562 cells comes from the copper-dependent induction of MT mRNA in this cell line (see below). Less than 1% of copper co-eluted with immunoreactive Cu,Zn-SOD in K562 cells grown in the presence of $200\ \mu\text{M}$ Cu, whereas about 70% of cellular copper was found to be sequestered by this protein in control cells which were shown to contain very low levels of MT [14] and MT mRNA (see below).

Increase of Cu,Zn-SOD activity and protein levels in response to copper

Supplementation of growth medium for 72 h with 50–400 μM copper caused a dose-dependent increase of polarographically detectable SOD activity in K562 cells (Table 1). This activity was quantitatively attributable to an increase of the copper- and zinc-containing enzyme since: (i) it was unaffected by dialysis; (ii) it was inhibited by > 95% in the presence of 2 mM CN^- , a specific inhibitor of the copper-dependent SOD; and (iii) the activity

Figure 1 Effect of copper supplementation on growth rate and intracellular copper levels of K562 cells

K562 cells were grown for 72 h in the presence of increasing amounts of copper. Growth rates (a) were calculated upon determination of cell density during the growth period and expressed relative to the rate of untreated cells, which had a doubling time of 19.8 ± 1.7 h. Intracellular copper (b) was measured by atomic absorption spectroscopy of acid-hydrolysed cell pellets. Data are mean values $\pm$ S.D. of five different experiments.

Figure 2 Size-exclusion chromatography of extracts from K562 cells grown in the presence of copper

Supernatants (17 000 g) of homogenates from 3×10^6 K562 cells, grown for 72 h in the presence of 200 μM copper, were chromatographed on a Superose 12 (HR10/30) f.p.l.c. gel-filtration column equilibrated with Chelex-100-treated buffer containing: 20 mM sodium phosphate, 140 mM NaCl, 0.1% (v/v) 2-mercaptoethanol. Solid line, A_{254} ; dashed line, copper determined by atomic absorption spectroscopy; dotted line, immunoreactive Cu,Zn-SOD determined by e.l.i.s.a. Recovery of copper was 92% in this experiment.

Table 1 Increase of Cu,Zn-SOD activity and immunoreactive protein in K562 cells grown for 72 h in the presence of copper

Cu,Zn-SOD activity and immunoreactive protein content were determined in K562 cell extracts. In order to obtain directly comparable results both data were expressed as $\text{ng}/1 \times 10^6$ cells with reference to recombinant human Cu,Zn-SOD as a standard. To detect the presence of copper-deficient SOD cell extracts were incubated for 3 h with 2 mM Cu^{2+} and after extensive dialysis activity was re-measured. Data are mean values $\pm$ S.D. of the number of experiments indicated in parentheses. Data having different superscripts are significantly different as evaluated by Student's *t*-test ($P < 0.05$).

	Cu,Zn-SOD activity ($\text{ng}/1 \times 10^6$ cells)		Immunoreactive protein content ($\text{ng}/1 \times 10^6$ cells)
	Without copper	With copper	
Control	276 $\pm$ 29 ^(a) (5)	404 $\pm$ 48 ^(b) (3)	415 $\pm$ 61 ^(b) (5)
50 μM Cu^{2+}	372 $\pm$ 65 ^(b) (5)	371 $\pm$ 29 ^(a,b) (3)	453 $\pm$ 173 ^(a,b) (5)
100 μM Cu^{2+}	424 $\pm$ 30 ^(b) (5)	385 $\pm$ 25 ^(b) (3)	679 $\pm$ 102 ^(c,d) (5)
200 μM Cu^{2+}	579 $\pm$ 105 ^(c) (5)	599 $\pm$ 84 ^(c) (3)	964 $\pm$ 117 ^(e) (5)
400 μM Cu^{2+}	782 $\pm$ 115 ^(d) (5)	703 $\pm$ 99 ^(d) (3)	1167 $\pm$ 186 ^(e) (3)

quantitatively co-eluted with the immunoreactive Cu,Zn-SOD protein at an apparent molecular mass of 30 kDa upon size-exclusion chromatography (results not shown).

The increase of Cu,Zn-SOD activity was paralleled by an augmented immunoreactive SOD protein (Table 1). Time-course experiments showed that this latter increase became evident only after a lag phase of about 20 h (results not shown).

No differences between control and copper-exposed cells were measurable in the subcellular distribution of Cu,Zn-SOD, with 96% of both immunoreactive and enzymically active Cu,Zn-SOD being found in the cytosol, 3% co-purifying with nuclei and less than 1% being detectable in mitochondrial, lysosomal and microsomal fractions respectively. These findings confirm

Figure 3 Protein content of K562 cells grown in the presence of copper

K562 cells were cultured for 72 h in the presence of increasing amounts of copper, harvested and disrupted by sonication. Protein was determined in the crude homogenate (■) or in the supernatant after centrifugation for 15 min at 17 000 g (□).

previous observations concerning the subcellular localization of the enzyme in rat hepatocytes [30].

The apparent induction of Cu,Zn-SOD protein was specific, since it was not paralleled by other enzymes including catalase (a peroxisomal marker enzyme), lactate dehydrogenase (a cytosolic marker) and the lysosomal enzyme acid phosphatase, whereas the activity of glutathione peroxidase, an enzyme involved in oxygen metabolism, was below detection limits (results not shown). It was, however, accompanied by an accumulation of total cellular protein (Figure 3). The extent of Cu,Zn-SOD induction (> 2.5 -fold) exceeded this generalized increase of cellular protein (approx. 1.5-fold), becoming evident when expressed either on a per cell or on a per protein basis. Furthermore, whereas Cu,Zn-SOD was apparently restricted to the cytosolic fraction in copper-exposed cells also, the increase in total protein mainly affected insoluble protein molecules, as copper caused an increasing amount of protein to be found in the pellet after centrifugation of cell homogenates at 17 000 g (Figure 3).

A discrepancy between enzymically active and immunoreactive Cu,Zn-SOD was detectable at all copper concentrations tested, including control cells (compare columns 1 and 3, Table 1). Previous studies have shown K562 cells to contain a less active, copper-deficient Cu,Zn-SOD [14]. As a matter of fact, addition of copper to extracts from control K562 cells, followed by dialysis, resulted in reconstitution of this copper-deficient protein and abolished the discrepancy between enzymically active and immunoreactive SOD (Table 1). The same treatment, however, had no detectable effect on the activity of the protein contained in extracts from cells cultured in the presence of copper. Thus the discrepancy between immunoreactive and enzymically active SOD in copper-exposed cells was not accounted for by the presence of a reconstitutable protein and was therefore further investigated.

Characterization of Cu,Zn-SOD from copper-exposed K562 cells

Although eluting as a single peak from size-exclusion-chromatography columns, Cu,Zn-SOD from K562 cells grown for 72 h in the presence of 200 μM copper showed two distinct peaks upon ion-exchange chromatography (Figure 4). This behaviour differed from that observed with Cu,Zn-SOD from control cells,

Figure 4 Ion-exchange chromatography of Cu,Zn-SOD in K562 cell extracts

K562 cells cultured for 72 h in the absence (a) or in the presence of 200 μM Cu^{2+} (b) were disrupted by sonication and 17000 g supernatants derived from 3×10^6 cells were loaded on a Mono Q (HR5/5) f.p.l.c. ion-exchange column equilibrated with 5 mM Tris/HCl, pH 7.5. Solid line, A_{280} ; dashed line, immunoreactive Cu,Zn-SOD determined by e.l.i.s.a.; dotted line, NaCl gradient from 0 to 200 mM.

Figure 5 Gel electrophoresis of Cu,Zn-SODs purified from K562 cells

Purified proteins were run on 7.5% non-denaturing polyacrylamide gels and visualized by Coomassie Blue- (lanes 1–3; 1 μg of protein/lane) or activity-staining (lanes 4–6; 0.3 μg of protein/lane). Lanes 1 and 4, recombinant human Cu,Zn-SOD; lanes 2 and 5, SOD I; lanes 3 and 6, SOD II.

which eluted as a single species under these conditions (Figure 4). The two distinct Cu,Zn-SOD proteins present in copper-exposed cells were isolated using f.p.l.c. The major form, with lower retention time, and the minor form, with higher retention time, will be referred to as SOD I and SOD II respectively. Both proteins were purified to apparent homogeneity, as judged from Coomassie Blue-stained non-denaturing PAGE (Figure 5). Both SOD I and SOD II displayed single bands, differing in electrophoretic mobility on non-denaturing gels. Pure recombinant

human Cu,Zn-SOD, used as a reference, showed two bands with mobilities comparable with those observed for SOD I and SOD II. Differences in electrophoretic and chromatographic mobility of human Cu,Zn-SOD have been ascribed to oxidative modifications of the single exposed cysteine residue present in this protein [31,32]. Accordingly, exposure of recombinant human Cu,Zn-SOD to the reductant 2-mercaptoethanol resulted in an increase in the intensity of the band with lower mobility (results not shown). The same treatment did, however, not affect the electrophoretic or chromatographic behaviour of SOD II, indicating a different underlying modification of the protein to be responsible for its altered electrophoretic mobility. Due to the limited amount of protein available no spectroscopic characterization has been carried out. To investigate further the reasons for the discrepancy between immunoreactive and enzymically active Cu,Zn-SOD in copper-treated K562 cells we therefore determined the k_{cat} values and the copper content of the purified proteins.

SOD I and SOD II had indistinguishable catalytic constants and were shown to contain stoichiometric amounts of copper (Table 2). When compared with Cu,Zn-SOD purified from control K562 cells, or to a recombinant protein, SOD I and SOD II had a 60.7% lower specific catalytic constant (k_{cat}/Cu) (Table 2, column 3). Significantly, this difference was of the same order of magnitude as the difference between the immunoreactive and the enzymically active protein (60.0%) in homogenates derived from K562 cells grown in the presence of 200 μM copper (compare Table 1). Thus our data indicate that copper causes the accumulation, in K562 cells, of a structurally altered Cu,Zn-SOD protein with a lower catalytic efficiency.

Effects of copper on Cu,Zn-SOD mRNA levels and polysomal distribution

To investigate whether copper induces Cu,Zn-SOD gene transcription in K562 cells, mRNA levels were measured in time-course and dose-dependence experiments. Northern-blot analysis revealed a time-dependent increase of Cu,Zn-SOD mRNA in K562 cells over exposure for 22 h to 200 μM copper (Figure 6). This increase was unspecific as it was paralleled by a comparable increment of β -actin and ribosomal protein L32 mRNAs. A maximum, about 3-fold, accumulation of those mRNA species was observed after 72 h exposure to copper (Figure 6). This increase was dose-dependent, reaching a plateau value at about 100 μM copper. On the other hand, MT mRNA, the transcription of which is specifically induced via metal-dependent transcription factors, augmented more than 50-fold under the same conditions. Moreover, maximum induction of this species was observed up to 100 μM copper and declined at higher metal concentrations, possibly reflecting metal toxicity at copper concentrations greater than 100 μM (see Figure 1).

Taken together, these results indicate a lack of specific activation of Cu,Zn-SOD gene transcription by copper ions in K562 cells. To test the general validity of these findings in higher eukaryotes, the same experiments were repeated using *Xenopus laevis* cultured kidney cells. Comparable results were obtained (Figure 6).

We further investigated the polysomal distribution of mRNAs as a means of estimating a possible translational activation mediated by copper. Growth in copper-supplemented medium induced a dose-dependent increase in the amount of polysome-bound mRNAs in K562 cells (Figure 7). This effect was again an indiscriminate one as it concerned Cu,Zn-SOD mRNA, as well as unrelated messengers such as β -actin mRNA (Figure 7). These data indicate that the mRNAs that are accumulated within K562

Table 2 Catalytic constants and copper content of Cu,Zn-SODs purified from K562 cells

Catalytic constants of purified proteins were determined polarographically and normalized with respect to bovine Cu,Zn-SOD, assumed to have a k_{cat} of $2.0 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$. Proteins were purified from control K562 cells or from cells grown for 72 h in the presence of $200 \mu\text{M Cu}^{2+}$.

	k_{cat} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Copper content (mol of Cu/mol of protein)	k_{cat}/Cu ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Control cells	0.82×10^9	0.92	0.89×10^9
Copper-induced SOD I	1.05×10^9	1.95	0.54×10^9
Copper-induced SOD II	1.05×10^9	1.96	0.53×10^9
Recombinant human SOD	0.54×10^9	0.63	0.87×10^9

Figure 6 Induction of mRNAs in K562 and *X. laevis* kidney cells in response to copper ions

Poly(A⁺) mRNA was extracted from 1×10^6 cells and subjected to Northern-blot analysis using cDNAs coding for human Cu,Zn-SOD, β -actin and ribosomal protein L32 or a 20mer oligonucleotide coding for human MT as probes. (a) Cells were treated for various times with $200 \mu\text{M Cu}^{2+}$. Left-hand panel: K562 cells; lanes 1–5, 0, 1, 3, 6, and 22 h respectively. Right-hand panel: *X. laevis* kidney cells; lanes 1–4: 0, 3, 24 and 72 h respectively. (b) K562 cells were treated for 72 h with increasing amounts of Cu^{2+} : lane 1, no copper addition; lane 2, 50; lane 3, 100; lane 4, 200; and lane 5, 400 $\mu\text{M Cu}^{2+}$.

cells as a consequence of copper supplementation are in fact actively translated, which is in line with the dose-dependent increase in total cellular protein (Figure 3).

Figure 7 Effect of copper on the translation of Cu,Zn-SOD mRNA

Total ribonucleoproteins extracted from K562 cells grown for 72 h in the presence of various amounts of copper were fractionated on a linear 15–50% sucrose gradient. Upper panel: Absorbance at 258 nm was recorded during fractionation. Arrows indicate different fractions collected for specific mRNAs analysis. Lower panel: Analysis of the distribution of human Cu,Zn-SOD mRNA (A) and β -actin mRNA (B) between ribonucleoparticles (fractions 5–7) and polysomal fractions (fractions 1–4) separated on sucrose gradients. Cells were grown in the absence (–) or in the presence (+) of $200 \mu\text{M Cu}^{2+}$.

Effects of copper on total protein and Cu,Zn-SOD turnover

To test the effects of copper on protein metabolism further, control or copper-exposed cells were labelled with [³H]lysine and the amounts of trichloroacetic acid-precipitable and pAB α (SOD)-precipitable label were quantified. Copper-exposed K562 cells incorporated significantly more trichloroacetic acid- and immuno-precipitable [³H]lysine than control cells (Figure 8).

To assess whether copper affects the turnover of Cu,Zn-SOD, K562 cells were pulsed for 3 or 24 h with [³H]lysine, followed by a 24-h-long chase in the presence of unlabelled lysine. During the chase period, trichloroacetic acid- and immuno-precipitable radioactivities were determined (Figure 8). Identical decay rates were obtained for either 3-h or 24-h pulses. From the slopes of the resulting curves the degradation half-time of Cu,Zn-SOD was calculated and shown to be unaffected by growth in copper-supplemented medium. Half-lives were $10.6 \pm 1.2 \text{ h}$ ($n = 6$) in control cells, compared with $9.8 \pm 1.0 \text{ h}$ ($n = 6$) in K562 cells

Figure 8 Total protein and Cu,Zn-SOD degradation rates in K562 cells

K562 cells grown for 72 h in medium with or without 200 μ M Cu^{2+} were labelled for 24 h with [^3H]lysine, followed by a chase in the presence of 5 mM unlabelled lysine. During the chase period cells samples were collected at different times. The label incorporated into total protein and into Cu,Zn-SOD was determined as trichloroacetic acid-precipitable (a) or immunoprecipitable (b) radioactivity respectively. Control cells, $\blacksquare$; copper-exposed cells, $\square$. Data are from one experiment representative of six, in which the same trend was consistently observed.

grown for 72 h in the presence of 200 μ M copper. Significantly, decay rates of trichloroacetic acid-precipitable label were higher in copper-exposed cells than in control cells, indicating higher total protein degradation rates in cells grown in the presence of copper.

DISCUSSION

We were unable to detect a specific increase of Cu,Zn-SOD mRNA in response to copper ions in either human K562 cells or in *X. laevis* kidney cells, when monitored at different metal concentrations over a 72 h period. Under the same experimental conditions MT mRNA levels were induced more than 50-fold in K562 cells. Furthermore, inspection of the 5'-untranslated region of the human Cu,Zn-SOD gene failed to demonstrate the presence of regions significantly homologous to the consensus sequences of the metal-responsive elements present in the MT promoter (C. Steinkühler, M. T. Carri, G. Micheli, L. Knoepfel, U. Weser and G. Rotilio, unpublished work). From our data we conclude that copper ions do not specifically activate Cu,Zn-SOD gene transcription in the cell lines used for this study, although they caused a generalized accumulation of several mRNAs. This suggests that the metal-dependent transcriptional regulation of this enzyme is restricted to lower eukaryotes such as yeast [12,15,16], possibly because this kind of regulation has not been conserved during the evolution of higher eukaryotes.

Although not specifically activating transcription, copper ions caused an increase of Cu,Zn-SOD activity and immunoreactive protein levels in K562 cells by affecting total protein and RNA metabolism in a complex way.

The effects of copper included: (i) an unspecific accumulation of several mRNAs, together with their loading on polysomes; (ii) a concomitant enhancement of total protein biosynthesis; and (iii) increased protein degradation rates. The Cu,Zn-SOD mRNA followed this general trend, showing a copper-dependent accumulation and loading on polysomes. This resulted in an increased biosynthesis of Cu,Zn-SOD. The metabolic stability of the SOD protein in intact cells was, however, unaffected by

copper supplementation. This fact lead to an accumulation of Cu,Zn-SOD relative to other proteins.

The mechanisms by which copper exerts these different effects on the cellular protein metabolism are not known and several alternative explanations are possible for any single observation. For instance, mRNA accumulation in response to copper may be due to a metal-dependent oxidative stress, resulting in the activation of the transcription machinery. A similar mechanism has been described in mouse epidermal cells, where extracellular generation of reactive oxygen species caused the induction of several mRNA species [33]. From our data it appears that copper ions may also interfere with processes that regulate critical steps of protein biosynthesis.

We have shown that total protein degradation rates are higher in copper-exposed cells than in control K562 cells. This fact may be linked to the ability of copper to cause oxidative damage to proteins in the presence of reducing agents and molecular oxygen [34]. This process is expected to result in structurally altered, inactive proteins that are recognized by some proteolytic system [35].

A strikingly analogous mechanism has been described for cadmium, which impairs protein structure *in vitro* through interaction with protein sulphhydryls [36]. As a matter of fact, cadmium supplementation leads to the induction of ubiquitin-dependent proteases that remove misfolded proteins in cadmium-exposed cells [37].

Indications for protein-damaging effects of copper in our experimental system come from the increased amount of insoluble proteins in copper-exposed cells and from the isolation, from those cells, of a structurally altered Cu,Zn-SOD with a diminished catalytic efficiency. We have not identified the molecular basis of the copper-induced inactivation of the enzyme. Whatever its nature, this inactivation was not linked to changes in the native molecular mass of the protein, as judged from gel-filtration experiments. Also, the two-peak pattern observed upon ion-exchange chromatography of Cu,Zn-SOD from copper-exposed K562 cells was an epi-phenomenon not linked to the loss of enzymic activity, since the specific activities of SOD I and SOD II were indistinguishable. Hence, one can speculate that the modification induced by copper may have affected either residues involved in the co-ordination of the active-site copper or residues playing a role in the electrostatic interaction with the substrate [1]. These effects of copper are apparently slow acting as they were observable only after more than 24 h of growth in the presence of the metal and were not reproducible upon 4 h incubation of control cell extracts with 2 mM CuSO_4 .

It has been shown that Cu,Zn-SOD exposed to oxidants is degraded more rapidly by proteolytic systems than the native protein, which is exceedingly stable towards proteolysis [38]. This apparently contradicts our findings of an unaltered turnover rate of the Cu,Zn-SOD from copper-treated cells. However, the proteolytic susceptibility of Cu,Zn-SOD is mainly a function of the metallation state of the protein, apoSOD being rapidly degraded by a series of proteases *in vitro* and the holo protein being completely stable under the same conditions [39], A. Battistoni, C. Steinkühler and G. Rotilio, unpublished work).

H_2O_2 has been proposed to inactivate Cu,Zn-SOD by causing the hydroxylation of a copper-ligating histidine [40,41]. This results in a reduced metal affinity and, consequently, in the detachment of copper [42]. In fact, proteolytic susceptibility of H_2O_2 -treated Cu,Zn-SOD has been shown to be preceded by an altered binding of copper ions to the protein's active site [38], suggesting that a metal-deprived protein is the actual protease substrate. Cu,Zn-SOD isolated from copper-treated K562 cells, on the other hand, was shown to contain stoichiometric amounts

of copper, which explains the resistance of this protein towards proteolysis. This finding is possibly related to the fact that K562 cells grown in the presence of copper contain about 20 % of the total cellular copper bound to a low-molecular-mass, redox-sensitive component, for which glutathione is a likely candidate [43]. This copper pool rapidly saturates Cu,Zn-SOD with the metal [44], and is likely to do so even if the metal affinity of the protein is reduced as a consequence of oxidative damage.

The overall picture presented here is reminiscent of the results obtained by Percival and Harris [28]. These authors reported the presence of a Cu,Zn-SOD with reduced activity in K562 cells induced to differentiate by a 96-h-long exposure to haemin. Concomitant addition of copper to the cells (supplemented as caeruloplasmin) did not drastically affect the specific activity, but significantly augmented immunoreactive Cu,Zn-SOD protein levels. The capability of iron ions contained in haemin to catalyse oxidative, Fenton-type reactions is comparable with that of copper ions. Hence, it is conceivable that haemin and copper cause similar oxidative modifications to protein, including Cu,Zn-SOD.

Interestingly, oxidative modifications of Cu,Zn-SOD apparently occur physiologically also in the absence of stressing conditions such as copper or haemin exposure. In fact, eight isoelectric variants of Cu,Zn-SOD have been isolated from rat liver, at least two of which have been shown to arise from the major form upon exposure to oxidants [45]. It is tempting to speculate that analysis of either isoelectric variants, or of Cu,Zn-SOD isoforms with reduced catalytic efficiency, may be a diagnostic tool to assess the extent of *in vivo* generation of reactive oxygen intermediates.

This work was supported by the Consiglio Nazionale delle Ricerche special project 'Superoxide dismutases as stress proteins', by the Deutsche Forschungsgemeinschaft grant We 401/24-3 and by the Fonds der Chemischen Industrie. C.S. was the recipient of a post-doctoral fellowship awarded by the Deutsche Forschungsgemeinschaft. We thank Prof. F. Amaldi for kindly providing β -actin and L32 probes.

REFERENCES

- Bannister, J. V., Bannister, W. H. and Rotilio, G. (1987) *CRC Crit. Rev. Biochem.* **22**, 111–180
- Hassan, H. M. and Fridovich, I. (1977) *J. Bacteriol.* **130**, 805–811
- Privalle, C. T. and Fridovich, I. (1990) *J. Biol. Chem.* **265**, 29166–29170
- Privalle, C. T. and Fridovich, I. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2723–2726
- Wong, G. and Goeddel, D. U. (1988) *Science* **242**, 941–944
- Fujii, J. and Taniguchi, N. (1991) *J. Biol. Chem.* **266**, 23142–23146
- Delabar, J. M., Nicole, A., D'Auriol, L., Jacob, Y., Meunier-Rotival, M., Galibert, F., Sinet, P. M. and Jérôme, H. (1987) *Eur. J. Biochem.* **166**, 181–187
- Montesano, L., Carri, M. T., Mariottini, P., Amaldi, F. and Rotilio, G. (1989) *Eur. J. Biochem.* **186**, 421–426
- Niederhoffer, E. C., Naranjo, C. M., Bradley, K. C. and Fee, J. A. (1990) *J. Bacteriol.* **172**, 1930–1938
- Privalle, C. T. and Fridovich, I. (1992) *J. Biol. Chem.* **267**, 9140–9145
- Beyer, W. F. and Fridovich, I. (1991) *J. Biol. Chem.* **266**, 303–308
- Carri, M. T., Galiasso, F., Ciriolo, M. R. and Rotilio, G. (1991) *FEBS Lett.* **278**, 263–266
- Galiasso, F., Ciriolo, M. R., Carri, M. T., Civitareale, P., Marcocci, L., Marmocchi, F. and Rotilio, G. (1991) *Eur. J. Biochem.* **196**, 545–549
- Steinkühler, C., Sapor, O., Carri, M. T., Nagel, W., Marcocci, L., Ciriolo, M. R., Weser, U. and Rotilio, G. (1991) *J. Biol. Chem.* **266**, 24580–24587
- Gralla, E. B., Thiele, D. J., Sillar, P. and Valentine, J. S. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8558–8562
- Thorvaldsen, J. L., Sewell, A. K., McCowen, C. L. and Winge, D. R. (1993) *J. Biol. Chem.* **268**, 12512–12518
- Sadhu, C. and Gedamu, L. (1988) *J. Biol. Chem.* **263**, 2679–2684
- Buongiorno-Nardelli, M., Micheli, G., Carri, M. T. and Marilley, M. (1982) *Nature (London)* **298**, 100–102
- Freedman, J. H., Weimer, R. J. and Peisach, J. (1986) *J. Biol. Chem.* **261**, 11840–11848
- Lück, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 885–888, Verlag Chemie, Academic Press, New York
- Howell, F. B., McCune, S. and Schaffer, R. (1979) *Clin. Chem.* **25**, 269–272
- Moss, D. W. (1981) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 92–106, Verlag Chemie, Academic Press, New York
- Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Bagni, C., Mariottini, P., Annesi, F. and Amaldi, F. (1990) *Nucleic Acids Res.* **18**, 4423–4426
- Tokunaga, K., Taniguchi, H., Yoda, K., Shimizu, M. and Sakiyama, S. (1986) *Nucleic Acids Res.* **14**, 2829
- Hanke, T., Tyers, M. and Harley, C. B. (1988) *FEBS Lett.* **241**, 159–163
- Beauchamp, L. and Fridovich, I. (1971) *Anal. Biochem.* **44**, 276–287
- Percival, S. S. and Harris, E. D. (1991) *Biochem. J.* **274**, 153–158
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Liou, W., Chang, L. Y., Geuze, H. J., Strous, G. J., Crapo, J. D. and Slot, J. W. (1993) *Free Radical Biol. Med.* **14**, 201–207
- Briggs, R. G. and Fee, J. A. (1978) *Biochim. Biophys. Acta* **537**, 86–99
- Briggs, R. G. and Fee, J. A. (1978) *Biochim. Biophys. Acta* **537**, 100–109
- Crawford, D., Zbinden, I., Arnstad, D. and Cerutti, P. (1988) *Oncogene* **3**, 27–32
- Marx, G. and Chevion, M. (1985) *Biochem. J.* **236**, 397–400
- Pacifici, R. E., Salo, D. C. and Davies, K. J. A. (1989) *Free Radical Biol. Med.* **7**, 521–536
- Vallee, B. J. and Ulmer, D. A. (1972) *Annu. Rev. Biochem.* **41**, 91–129
- Jungmann, J., Reins, H. A., Schobert, C. and Jentsch, S. (1993) *Nature (London)* **361**, 369–371
- Salo, D. C., Pacifici, R. E., Giulivi, C. and Davies, K. J. A. (1990) *J. Biol. Chem.* **265**, 11919–11927
- Forman, H. J. and Fridovich, I. (1973) *J. Biol. Chem.* **248**, 2645–2649
- Hodgson, E. K. and Fridovich, I. (1975) *Biochemistry* **14**, 5294–5303
- Bray, R. C., Cockle, S. A., Fielden, E. M., Roberts, P. B., Rotilio, G. and Calabrese, L. (1974) *Biochem. J.* **139**, 43–48
- Jewett, S. L., Cushing, S., Gillespie, F., Smith, D. and Sparks, S. (1989) *Eur. J. Biochem.* **180**, 569–575
- Freedman, J., Ciriolo, M. R. and Peisach, J. (1989) *J. Biol. Chem.* **264**, 5598–5605
- Ciriolo, M. R., Desideri, A., Paci, M. and Rotilio, G. (1990) *J. Biol. Chem.* **265**, 11030–11034
- Yano, S. (1990) *Arch. Biochem. Biophys.* **279**, 60–69