Regulation of Cu,Zn superoxide dismutase with copper

Caeruloplasmin maintains levels of functional enzyme activity during differentiation of K562 cells

Susan S. PERCIVAL* and Edward D. HARRIS†

Department of Biochemistry and Biophysics and the Texas Agricultural Experiment Station,
Texas A&M University College Station, TX 77843, U.S.A.

K562 cells, a human erythroleukaemic cell line blocked for differentiation, commit towards erythrocytes when exposed to haemin (20 μ M). The cells synthesize fetal haemoglobins and show site-specific binding of caeruloplasmin, a plasma copper protein. These events are set into motion by haemin. On the assumption that the binding of caeruloplasmin could reflect a greater need for copper, we sought to determine whether the transfer of ^{67}Cu from caeruloplasmin was accelerated in haemin-induced compared with non-induced K562 cells. Cu,Zn superoxide dismutase (CuZnSOD) was the recipient. Haemin induction caused the K562 cells to lose CuZnSOD activity. By 96 h, the level of SOD activity was less than 60 % of that of non-induced cells. The loss was confined entirely to the CuZn form, MnSOD activity staying essentially unchanged. Although CuZnSOD activity declined with the haemin induction, the incorporation of [4,5- ^3H]lysine into immunoprecipitable CuZnSOD protein was unaffected. There was also no change in CuZnSOD mRNA concentration in haemin-induced cells. Thus a loss of enzyme did not correlate with a decline in the synthesis *de novo* of CuZnSOD protein. When 48 h-induced cells were transferred to a medium supplemented with 0.2 μ M-caeruloplasmin, CuZnSOD activity was restored to control levels in 24 h. Caeruloplasmin also stimulated the incorporation of [^3H]lysine into immunoprecipitable CuZnSOD protein. Caeruloplasmin addition may have affected a post-translational regulatory site for CuZnSOD biosynthesis, possibly by providing copper for the newly synthesized enzyme.

INTRODUCTION

Human K562 cells, an uncommitted erythroleukaemic cell line, are especially suitable for studying the molecular events in cellular differentiation. For example, 20–50 μ M of haemin/l activates globin genes (Andersson *et al.* 1979; Rutherford *et al.*, 1979), increases the expression of specific globin mRNAs (Cioe *et al.*, 1981; Rutherford *et al.*, 1981), causes a reversible loss in surface-expressed transferrin receptors (Roualt, 1985; Petraki *et al.*, 1986), decreases the rate of transferrin receptor synthesis (Roualt, 1985; Pelosi *et al.*, 1986), induces a fetal-type membrane acetylcholinesterase (Bartha *et al.*, 1987) and leads to a marked accumulation of endogenous porphyrins (Malik *et al.*, 1988). Most, if not all, of these changes permit the K562 cell to approach the phenotype of a mature erythrocyte. Surface antigen profiles of non-induced K562 cells are distinctly different from those of mature erythrocytes (Fukada, 1980; Testa *et al.*, 1982; Ichiki *et al.*, 1986), showing that the membrane undergoes structural reorganization as differentiation proceeds. Adapting the erythroid phenotype could also increase the level of Cu,Zn superoxide dismutase (CuZnSOD) activity, since the enzyme is present at high concentrations in haemoglobin-producing cells, perhaps as a protection against the build-up of O_2 -derived radicals that characterize erythrocytes (Saltman, 1989). One may predict that the availability of copper, a cofactor for the enzyme, could be a determining factor in the synthesis and expression of the enzyme. Thus the haemin-induced K562 cell may be an excellent model to study the regulation of CuZnSOD by copper.

Previously, we showed that haemin enhanced the binding of caeruloplasmin to the surface of K562 cells (Percival & Harris, 1988). Caeruloplasmin, a plasma copper protein, has been shown

to donate copper atoms to CuZnSOD protein in liver and aorta (Marceau & Aspin, 1973; Dameron & Harris, 1987b). There are two types of SOD in mammalian tissues: a $\text{Cu}_2\text{Zn}_2\text{SOD}$ in the cytosol and mangano-SOD (MnSOD) in the mitochondria (Weisiger & Fridovich, 1973). One purpose of enhanced caeruloplasmin binding may be to ensure that adequate amounts of copper are present for CuZnSOD synthesis. We tested the hypothesis by monitoring both SOD types during differentiation with haemin. CuZnSOD protein and mRNA were also measured as a function of differentiation.

MATERIALS AND METHODS

Cells and caeruloplasmin

K562 cells were obtained from the American Type Culture Collection and grown routinely in RPMI 1640 medium (Mediatech, Herndon, VA, U.S.A.) supplemented with 10 % (v/v) fetal-calf serum. The cells were passaged when they had reached a concentration of 2×10^5 – 5×10^5 cells/ml. Before induction, the cells were adjusted to a concentration of 5×10^4 /ml to ensure exponential-phase growth. Haemin was added at a final concentration of 20 μ M. In this procedure it is important that the cells have the lower initial concentration. Viability determinations of haemin-induced and non-induced K562 cells have been described (Percival & Harris, 1988).

Crystalline human caeruloplasmin was obtained from Alpha Therapeutic Corp. (Los Angeles, CA, U.S.A.). $^{67}\text{CuCl}_2$ was purchased from Los Alamos National Laboratories (Los Alamos, NM, U.S.A.). ^{67}Cu -labelled human caeruloplasmin followed an ascorbate-catalysed exchange (0.05 % ascorbate) as described previously (Dameron & Harris, 1987b).

Abbreviations used: CuZnSOD, Cu,Zn superoxide dismutase; DPTA, diethylenetriaminepenta-acetic acid; SSC, 0.15 M-NaCl/0.015 M-sodium citrate buffer, pH 7.0; SSPE, 0.15 M-NaCl/0.01 M- NaH_2PO_4 /0.001 M-EDTA buffer, pH 7.4.

* Present address: Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL 32611, U.S.A.

† To whom all correspondence should be addressed.

Uptake of ^{67}Cu from caeruloplasmin into cells

Uptake of ^{67}Cu into haemin-induced and non-induced K562 cells followed a method described previously (Percival & Harris, 1989). In essence, cells were incubated with ^{67}Cu -labelled caeruloplasmin for the time indicated in Figs. 1–5 at 37 °C and collected by centrifugation. The cells were washed twice with phosphate-buffered saline (12 mM-sodium phosphate/0.15 M-NaCl buffer, pH 7.4) and once with 0.05 M-sodium acetate/0.15 M-NaCl buffer, pH 2.0, to remove surface-bound radioactivity. ^{67}Cu remaining with the cell layer was considered to be incorporated into the cells (Percival & Harris, 1990).

Subcellular fractionation

Mitochondria, nuclei and cytosolic fractions were prepared by differential centrifugation after rupturing of the cells by N_2 cavitation (Parr Instruments Co.) at 6900 kPa for 5 min. The lysed cells were centrifuged at 500 *g* for 8 min at 4 °C (Jouan CR412 refrigerated centrifuge) to collect the nuclei. The supernatant was centrifuged at 15000 *g* for 10 min at 4 °C (Beckman L2 65B, Ti 50 rotor) to collect the mitochondria. The supernatant constituted the crude cytosolic fraction.

Rocket immunoelectrophoresis

Measurements of CuZnSOD protein by rocket immunoelectrophoresis were performed on the soluble fraction obtained by centrifuging cell lysates at 100000 *g* for 60 min. Agarose (Pharmacia, Piscataway, NJ, U.S.A.) was boiled in 100 mM-Tris/30 mM-barbitone buffer, pH 8.6, containing 0.4 mM-calcium lactate and 1 mM- NaN_3 . After cooling to 55 °C, antiserum to CuZnSOD (1:20 titre) was added at a ratio of 100 μl per 10 ml of agar. Glass plates of area 49 cm^2 were used. Electrophoresis was performed at 15 mA for 4–6 h on a cooling plate. Gels were pressed, dried and then stained with Coomassie Blue. Quantification was based on peak heights relative to the known amounts of human erythrocyte CuZnSOD (Sigma Chemical Co.) run concurrently.

Assays of SOD activities

The method of Marklund & Marklund (1974) was followed with the following modifications. Assays were performed in 96-well micro-titre plates. About 10–25 μl of 100000 *g* supernatant from cell lysates was added to 50 μl of pyrogallol (1 mM-pyrogallol suspended in 1 mM-DPTA). The volume was adjusted to 200 μl with 50 mM-Tris/HCl/1 mM-DPTA buffer, pH 8.2. Pyrogallol (1,2,3-trihydroxybenzene) was obtained from BDH Chemicals. MnSOD activity was determined indirectly by conducting measurements in the presence of 1.0 mM-NaCN, an inhibitor of the CuZnSOD. Corrections were applied for effects of NaCN on the autoxidation of pyrogallol. The reaction was monitored at 25 °C by using a micro-plate reader (Vmax; Molecular Devices) at 405 nm for 2 min with an automatic 2 min lag programmed in. SOD activity is expressed in units/ 10^7 cells, where 1 unit equals the amount of enzyme producing a 50% inhibition in the autoxidation of pyrogallol.

Incorporation of [^3H]lysine into total cell proteins and CuZnSOD

K562 cells (haemin-treated and non-treated) were incubated in lysine-free RPMI 1640 medium for 24 h before analysis. No loss in viability was observed. Cultures were divided into 200 ml portions containing 50000 cells/ml and treated with 5 μCi of L-[4,5- ^3H]lysine (specific radioactivity 46 Ci/mol; ICN, Irvine, CA, U.S.A.)/ml of cells. Samples were taken at 0, 2 and 24 h and tested for lysine incorporation into CuZnSOD protein. Control studies showed that the rate of lysine uptake was linear for at

least 24 h. At specified times, samples representing 5×10^6 cells were removed and treated with 1 vol. of cold 16% (w/v) trichloroacetic acid. The precipitate was collected on cellulose nitrate filters and washed three times with 5 ml of 8% trichloroacetic acid containing 1% unlabelled lysine. The washed filters were transferred to scintillation vials to which was added 10 ml of scintillation cocktail (Optifluor; Packard Instruments). Radioactivity was quantified in a Beckman LS250 liquid-scintillation counter. Counting efficiency for tritium was 33%.

The incorporation of [^3H]lysine into CuZnSOD was determined at 24 h. The cells were collected by centrifugation and lysed by pressure homogenization as described above. The 100000 *g* supernatant from the lysate (100 μl) was incubated with rabbit anti-(human CuZnSOD) antibodies (kindly provided by Dr. Robert A. DiSilvestro, Ohio State University) for 1 h at 22 °C. A 5 μl portion of staphylococcal Protein A (Sigma Chemical Co.) was added to precipitate CuZnSOD-antibody complex. Corrections were made for radioactivity that was precipitated without antibody, about 25–35% of the quantity precipitated with antibody present. Purification and tests of antibody specificity have been described previously (Dameron & Harris, 1987b).

Measurements of CuZnSOD mRNA

Northern-blot and dot-blot analyses were used to detect and assess the concentrations of CuZnSOD mRNA in induced and non-induced cells. Cells were pelleted (800 *g* for 20 min) and the supernatant was discarded. The pellets were extracted with isothiocyanate and extracts were sheared with 15–20 up and down strokes through 18-gauge, 20-gauge and 22-gauge needles fitted to a 10 ml syringe as described previously (Chirgwin *et al.*, 1979). Extracts were layered over 5.7 M-CsCl and centrifuged at 23 °C for 16 h. The pellets were taken up in RNAase-free water, and, after being heated at 65 °C for 10 min, 1 μg of RNA was transferred to a 3 mm agarose gel and electrophoresed for 6 h on 1% agarose/2.2 M-formaldehyde gels at 100 mA total current. The agarose gel was blotted with a nylon membrane (Hybond-N) overnight and RNA was covalently cross-linked to the membrane by u.v. radiation. The RNA was stained with ethidium bromide to estimate the amounts that had been applied. The dot-blot analysis used the Schleicher and Schuell apparatus. Diluted samples of formaldehyde-denatured RNA in 15 $\times$ SSC buffer, 200 μl volume, were applied to the wells and washed twice with 200 μl each of the SSC buffer. Hybridization was performed at 65 °C for 18 h after a preliminary hybridization for 1 h. A plasmid containing a full-length cDNA was obtained from transformed human fibroblasts (Sherman *et al.*, 1983) and subcloned into bluescript as previously described (Fleming & Gitlin, 1990). The human CuZnSOD was random-primed with [γ - ^{32}P]CTP (3000 Ci/mmol; ICN) and used immediately. Hybridized membranes were washed in 1 litre of 2 $\times$ SSPE, 1 litre of 0.2 $\times$ SSPE and 1 litre of 0.2 $\times$ SSPE containing 1% SDS twice at 65 °C for 20 min. Blots were autoradiographed at –70 °C with intensifying screen for 5–6 h.

Statistical measurements

Differences between means were judged significant by using a one-way analysis of variance. The analysis was performed with the EPISTAT software program as described previously (Percival & Harris, 1989).

RESULTS

K562 cells grew with a doubling time of 24–26 h. In the presence of haemin, the doubling time was lengthened to around 36 h and the cells gradually took on a brownish appearance as

Table 1. Uptake of ^{67}Cu from caeruloplasmin by haemin-induced and non-induced K562 cells

Cells ($10^7/\text{ml}$) were incubated in phosphate-buffered saline supplemented with 3% (w/v) albumin for 1 h at 37°C . Haemin-treated cells were incubated with $20\ \mu\text{M}$ -haemin for 48 h before caeruloplasmin addition. ^{67}Cu appearing in the pellet fraction from acid-washed cells (see the Materials and methods section) was measured. Data are means $\pm$ s.d. for triplicate determinations.

Caeruloplasmin (nM)	^{67}Cu (pmol/ 10^7 cells)	
	Non-induced	Haemin-induced
50	2.27 ± 0.33	3.95 ± 0.42
100	7.21 ± 1.1	11.23 ± 1.8
200	18.34 ± 2.4	26.11 ± 2.5

Fig. 1. Effect of haemin on short-term uptake of ^{67}Cu from caeruloplasmin

Non-induced K562 cells ($10^7/\text{ml}$) in phosphate-buffered saline containing 3% (w/v) BSA were incubated with (●) and without (○) $40\ \mu\text{M}$ -haemin. Samples were taken at the times indicated and analysed for radioactivity in the pellet fraction. Error bars show $\pm$ s.d.

Table 2. Distribution of ^{67}Cu in subcellular fractions from induced and non-induced K562 cells

Induced and non-induced cells were incubated with ^{67}Cu -labelled caeruloplasmin. Induced cells had been treated with haemin ($20\ \mu\text{M}$) 48 h before the experiment. Values are for 10^7 cells after 24 h incubation. Crude fractions were prepared from lysates by differential centrifugation as described in the Materials and methods section.

	Distribution of ^{67}Cu (%)		
	Nuclei	Cytosol	Mitochondria
Non-induced	10.5	75.4	17.0
Induced	13.7	69.3	14.9

haemoglobin appeared in the cytosol. In agreement with earlier work, both haemin-treated and non-treated cells took up ^{67}Cu from ^{67}Cu -labelled caeruloplasmin and transferred the label to an acid-resistant fraction. The untreated cells took up about 8–12% of the ^{67}Cu added to the medium. The absolute amount was a function of the ^{67}Cu -caeruloplasmin added. Haemin-treated cells took up about 40% more ^{67}Cu than did non-induced cells. This differential was seen over a 4-fold range of ^{67}Cu -caeruloplasmin concentrations (Table 1). Control studies were run to test for spontaneous dissociation of ^{67}Cu from the protein. These studies showed that the ^{67}Cu -caeruloplasmin

Table 3. Incorporation of ^{67}Cu from caeruloplasmin into immunoprecipitable CuZnSOD in induced and non-induced K562 cells

Incubations with ^{67}Cu -labelled caeruloplasmin ($100\ \text{nM}$) were for 20 h at 37°C . Cells were lysed and the $100\,000\ \text{g}$ fraction was used to test for immunoprecipitable ^{67}Cu that was bound to CuZnSOD. Corrections have been applied for ^{67}Cu precipitated in the absence of antibody. Data are means $\pm$ s.d. for triplicate determinations.

	^{67}Cu (pmol/ 10^7 cells)	
	Cytosol	Immunoprecipitate
Non-induced	1.95 ± 0.24	1.08 ± 0.11 (55.4%)
Induced	2.50 ± 0.30	1.00 ± 0.09 (40.0%)

Table 4. Changes in total SOD, CuZnSOD and MnSOD dismutase activities in K562 cells with haemin treatment

Assays were performed in triplicate and represent the results of three experiments. CuZnSOD activity represents the difference in mean values for MnSOD and total SOD activity. Values in a column not having a common superscript are significantly different by a one-way analysis of variance. All data are means $\pm$ s.d.

Time of induction (h)	SOD activity (units/ 10^7 cells)		
	Total SOD	MnSOD	CuZnSOD
0	76.4 ± 4.5^a	16.8 ± 4.0^a	59.9
24	57.0 ± 7.8^b	17.3 ± 3.9^a	39.7
48	58.6 ± 5.1^b	15.2 ± 3.2^a	43.4
72	49.8 ± 6.4^b	16.3 ± 2.6^a	33.5
96	45.3 ± 5.9^b	14.6 ± 4.2^a	30.7

complex was stable in $40\ \mu\text{M}$ -haemin, i.e. about twice the concentration of haemin used in the experiments. Moreover, haemin did not alter the rate of ^{67}Cu uptake from caeruloplasmin (Fig. 1). The data show clearly that enhanced copper uptake was a function of the induced state of the cells and not the incubation conditions. In subsequent studies described here, haemin treatment refers to cells that have been incubated for 48 h in the presence of $20\ \mu\text{M}$ -haemin.

Subcellular distribution of ^{67}Cu from caeruloplasmin

Three subcellular fractions accounted for nearly all of the ^{67}Cu taken into the cells in the 60 min incubation. Two-thirds of the ^{67}Cu appeared in the cytosol and the remainder was divided evenly between the nuclei and mitochondria. The distribution in haemin-induced and non-induced cells was about the same for both (Table 2). It should be emphasized that these were crude subcellular fractions. The data do not imply that only three subcellular compartments took up ^{67}Cu from caeruloplasmin. Slightly more than half of the ^{67}Cu appearing in the cytosol was precipitated with antibodies to CuZnSOD (Table 3). Haemin treatment enhanced the amount of cytosolic ^{67}Cu derived from caeruloplasmin, but did not augment the amount in immunoprecipitable CuZnSOD protein. Although more ^{67}Cu entered the cell, proportionately less bound to the enzyme.

Effect of haemin on SOD activity

Haemin treatment resulted in a progressive decline in SOD activity in the cells (Table 4). After 96 h only about 60% of the level before treatment was detected. Table 4 shows that only the

activity of the CuZn form declined; the MnSOD activity was unchanged. In non-induced cells, CuZnSOD made up about 80 % of the total SOD activity. After 96 h in haemin, the ratio of CuZnSOD to MnSOD activity fell to about 2:1, representing a 50 % decline in the CuZnSOD activity. The sharpest loss occurred during the first 24 h of haemin treatment.

Effect of caeruloplasmin on SOD activity in induced and non-induced cells

When caeruloplasmin was added to the medium after 48 h, induced and non-induced cells showed more CuZnSOD activity at 72 h. The caeruloplasmin concentration that achieved activation was 0.2 μ M, about one-tenth the concentration found normally in plasma. The effect was more noticeable with induced cells, where the increase in CuZnSOD activity in response to caeruloplasmin was almost 40 % (Table 5). Caeruloplasmin exerted a smaller, but measurable, effect on non-induced cells. The increase in SOD activity (or reversal of its decline) occurred despite a small loss of MnSOD in the non-induced cells (Table 5). The decline in MnSOD activity was significant in non-induced cells and was noticeable only when a caeruloplasmin was present.

Effect of haemin on CuZnSOD protein content

To explore the caeruloplasmin effects more closely, CuZnSOD protein was measured in the haemin-induced and non-induced cells. Immunoreacting CuZnSOD protein as measured by rocket immunoelectrophoresis showed a significant increase with haemin (Table 6). Moreover, when caeruloplasmin was added to 48 h-induced cells, the immunoreacting CuZnSOD protein increased by nearly 60 % over cells treated with haemin alone. With no caeruloplasmin, haemin-treated cells showed a 46 % increase. The combined effects of haemin and caeruloplasmin raised the immunoreacting CuZnSOD protein content to 2–3-fold higher than that in non-induced cells. Table 6 further shows that the specific activity of CuZnSOD was decreased sharply by the haemin induction. Caeruloplasmin partially restored the specific activity but not the level of that in non-induced cells. These data tend to contradict earlier indications (Table 4) that haemin-treated cells no longer synthesize SOD. Rather the data now suggest that cells synthesize and accumulate a catalytically inactive form of the enzyme. Caeruloplasmin in the medium appeared to enhance the amount of the CuZnSOD protein that accumulated. Total SOD activity, although increased substantially by caeruloplasmin (Table 5), was only about half the specific activity of the enzyme extracted from non-induced cells (Table 6). Thus caeruloplasmin at the concentration tested (200 nM) did not restore full enzyme activity.

Effect of haemin on CuZnSOD protein synthesis

It still remained to be determined whether the haemin treatment stimulated cells to synthesize more CuZnSOD *de novo*. To answer the question, haemin-induced and non-induced cells were incubated with and without caeruloplasmin (100 nM). After temperature equilibration, [4,5- 3 H]lysine was added and radioactivity incorporated into trichloroacetic acid-insoluble material was determined. The amount of [3 H]lysine incorporated into CuZnSOD was measured by immunoprecipitation, with appropriate corrections applied for spurious precipitation (see the Materials and methods section). Table 7 shows that about 8 % of [3 H]lysine taken into non-induced cells was precipitated with CuZnSOD antibody. In induced cells this value was about the same. Haemin treatment, however, led to 1.5 times more radioactivity being incorporated into total cell protein, possibly reflecting the increase in haemoglobin synthesis. With caeruloplasmin added to the medium, total radioactivity also increased, but only in the non-induced cells. Caeruloplasmin, however,

Table 5. Comparison of total SOD, CuZnSOD and MnSOD activities in induced and non-induced cells: effect of caeruloplasmin

Results are for 72 h cells incubated with and without 20 μ M-haemin. Caeruloplasmin (Cp) (200 nM) was added after 48 h. CuZnSOD activity represents the difference in the mean values for MnSOD and total SOD activity. Values in a column not having a common superscript are significantly different by a one-way analysis of variance. Data are means $\pm$ S.D. for triplicate determinations.

	SOD activity (units/10 ⁷ cells)		
	Total SOD	MnSOD	CuZnSOD
Non-induced	64.7 $\pm$ 4.2 ^a	13.8 $\pm$ 2.7 ^a	50.9
Non-induced + Cp	70.1 $\pm$ 4.2 ^b	6.2 $\pm$ 1.9 ^b	63.9
Induced	44.1 $\pm$ 4.9 ^c	14.4 $\pm$ 2.2 ^a	29.7
Induced + Cp	66.9 $\pm$ 6.5 ^{ab}	10.7 $\pm$ 2.9 ^{ab}	59.7

Table 6. Comparison of CuZnSOD enzyme protein and specific activity in induced and non-induced K562 cells: effects of caeruloplasmin

Experimental design was similar to Table 5. Immunoreacting CuZnSOD protein was determined by quantitative rocket immunoelectrophoresis as described in the Materials and methods section. Values in a column not having a common superscript are significantly different by a one-way analysis of variance. Caeruloplasmin (Cp) concentration was 200 nM. Data are means $\pm$ S.D. for triplicate determinations.

	Immunoreactive protein (μ g/10 ⁷ cells)	Specific activity (units/ μ g of enzyme)
Non-induced	10.7 $\pm$ 2.6 ^a	4.8
Non-induced + Cp	12.3 $\pm$ 2.3 ^{ab}	5.2
Induced	15.6 $\pm$ 2.9 ^b	1.9
Induced + Cp	25.2 $\pm$ 4.2 ^c	2.4

Table 7. Incorporation of [4,5- 3 H]lysine into total cell proteins and immunoprecipitable CuZnSOD: effect of haemin and caeruloplasmin

The study used 48 h haemin-induced and non-induced cells. Caeruloplasmin (Cp) concentration was 100 nM. The cells were pulsed with [3 H]lysine for 24 h. Values in a column not having a common superscript are significantly different by a one-way analysis of variance. Data are means $\pm$ S.D. for triplicate determinations.

	Incorporation of [4,5- 3 H]lysine (%)		
	Acid-insoluble (A)	Immunoprecipitable (B)	A/B ratio
Non-induced	2.48 $\pm$ 0.13 ^a	0.203 $\pm$ 0.003 ^a	12.2
Non-induced + Cp	4.46 $\pm$ 0.88 ^b	0.213 $\pm$ 0.009 ^a	20.9
Haemin	4.56 $\pm$ 0.34 ^b	0.285 $\pm$ 0.005 ^b	16.0
Haemin + Cp	4.09 $\pm$ 0.43 ^b	0.348 $\pm$ 0.19 ^c	11.8

caused the fraction of 3 H incorporated into CuZnSOD to increase significantly in haemin-induced cells. Incorporation of 3 H into SOD of non-induced cells was unaffected. These data suggest that caeruloplasmin was causing more CuZnSOD protein to accumulate in haemin-induced cells.

Fig. 2. Concentration of CuZnSOD mRNA in extracts of induced and non-induced cells

³²P-labelled cDNA template to human CuZnSOD mRNA was used as a probe. Row A, Non-induced cells; row B, non-induced + caeruloplasmin; row C, induced; row D, induced + caeruloplasmin.

Effect of haemin and caeruloplasmin on CuZnSOD mRNA concentrations

To determine whether the haemin effects extended to the expression of CuZnSOD mRNA, total RNA from cells subjected to various treatments was probed with cDNA to CuZnSOD mRNA. A Northern-blot analysis showed the presence of a split band migrating beneath the 18S RNA band (results not shown). Dot-blot analysis of total RNA was used to quantify the amount in the haemin- and caeruloplasmin-exposed cells (Fig. 2). RNA that hybridized to the CuZnSOD probe was detectable after 96 h of induction and, as shown by dot-blot analysis, at nearly identical concentrations with mRNA in non-induced cells. Moreover, caeruloplasmin had no effect on the amount of CuZnSOD mRNA recovered from either induced or non-induced cells. Thus the CuZnSOD mRNA concentration did not decline with induction and the turnover of mRNA is not one of the causes of low CuZnSOD activity.

DISCUSSION

The data in this study show that in addition to promoting haemoglobin biosynthesis and modulating iron transport, haemin profoundly affects the metabolism of copper in K562 cells. Here we examined a specific link between copper and a key copper-containing enzyme, CuZnSOD. The enzyme allows mature erythrocytes to cope with oxygen metabolites and may be essential for the survival of cells engaged in haemoglobin biosynthesis (Ripalda *et al.*, 1989). Previously we showed that haemin-treated K562 cells bound greater amounts of caeruloplasmin to the cell surface (Percival & Harris, 1988). Now it seems probable that, by allowing more caeruloplasmin to bind, haemin also stimulated the cells to take up more copper from caeruloplasmin. It has not been established that enhanced binding accelerated copper incorporation, but such is implicated. The copper incorporated was localized mainly in the cytosol and did not divert preferentially to mitochondria or nuclei. Half of the ⁶⁷Cu in the cytosol was bound to CuZnSOD, a major and perhaps exclusive protein recipient of copper in these cells (S. S. Percival & E. D. Harris, unpublished work). Interestingly, haemin stimulated K562 cells to synthesize more CuZnSOD protein, yet the treated cells lost CuZnSOD activity progressively as induction proceeded. Caeruloplasmin was able to restore partial CuZnSOD activity to the differentiated cells.

SOD activity is known to change in cells undergoing differentiation. This has been seen in rat embryonic fibroblasts (Allen & Balin, 1988), mouse erythroleukaemic cells (Beckman *et al.*, 1989), plasmodial slime mould (Aller. *et al.*, 1988) and human leukaemic cells (Speier & Newberger, 1986). Why levels of SOD

activity vary is not fully understood, but could relate to the production of reduced oxygen species. Some have argued that elevated SOD activity may be the stimulus that sets differentiation into motion (Oberley, 1982). At first glance this principle may not apply to K562 cells, since SOD activity fell as the cells entered into advanced developmental states. On the other hand, SOD protein biosynthesis was clearly enhanced, suggesting that the cells were responding to signals to increase the amount of SOD. While CuZnSOD activity declined, MnSOD activity remained unchanged. An exception was in non-induced cells incubated with caeruloplasmin, where MnSOD activity was down significantly. The meaning of the decline is not clear, but could represent a compensatory response to keep the level of total SOD activity in the cells uniform.

As yet, we have no firm molecular reason for the decline in CuZnSOD activity. Whatever the reason, it is important to recognize that only one type of SOD, namely CuZnSOD, was affected. Having only one of the SOD enzymes lose activity in differentiation has been noted previously. For example, dimethylformamide-induced HL-60 cells show a decline in MnSOD activity; the CuZnSOD activity remains unchanged (Speier & Newberger, 1986). One possible cause for the decline is the concentration of mRNA for the enzyme. Auwerx *et al.* (1989) noted that prevailing levels of CuZnSOD activity in HL-60, THP-1 and HEL cell lines correlated with the concentration and transcriptional efficacy of CuZnSOD mRNA. A phorbol-induced decline worked through a decrease in the mRNA concentration in their study. A similar finding was reported by Saito *et al.* (1989) for U937 and HL-60 cells induced with phorbol. Diminished CuZnSOD mRNA may be a hallmark of differentiation in these cells. On the other hand, human B-cell leukaemia cells or normal diploid fibroblasts treated with phorbol retained their concentrations of CuZnSOD mRNA (Saito *et al.*, 1989). Both CuZnSOD mRNA and MnSOD mRNA concentrations go down in Morris hepatomas, the decline correlating with the degree of differentiation (Galeotti *et al.*, 1989). In the present study, we saw no discernible difference in the concentration of CuZnSOD mRNA for 48 h-induced and non-induced cells regardless of whether caeruloplasmin had been added or not. Moreover, the cells retained translational capacity, as evidenced by the unimpeded and somewhat stimulated incorporation of [³H]lysine into immunoprecipitable CuZnSOD protein. Thus the decline in SOD was not a pretranslational happening.

Whereas induced K562 cells may synthesize CuZnSOD at accelerated rates, the emergence of a functional enzyme will depend on an adequate supply of copper to the cells and at the site of enzyme synthesis. Conceivably, copper will be needed not only for catalytic activity, but also for metabolic stability, as judged by the susceptibility of some copper proteins to metabolic turnover when copper is not supplied to the medium (Merchant & Bogorad, 1986). Cu²⁺ ions stimulate production of CuZnSOD in aerobic steady-state chemostat cultures of *Saccharomyces cerevisiae* (Lee & Hassan, 1987) and, through induction, regulate the synthesis of CuZnSOD in yeast (Galiano *et al.*, 1988). A dietary deficiency in copper causes a decline in functional CuZnSOD in experimental animals (Bettger *et al.*, 1978; Dameron & Harris, 1987a; Chung *et al.*, 1988). This suggests that copper is a major determinant of an active stable enzyme.

To explain the initial loss of SOD activity, one must consider that the enhanced synthesis of CuZnSOD induced by the haemin may have accelerated the turnover of existing enzyme. We know very little about growth requirements of stressed cells, but, in general, one can predict *a priori* that they will not be the same as for non-stressed cells. The increase in synthesis may have brought on a demand for additional copper, a demand that was met in part by caeruloplasmin. We base this conclusion on the

observation that K562 cells have the capacity to extract copper atoms from caeruloplasmin and transfer the cofactor to CuZnSOD (Percival & Harris, 1989). That reaction has been studied in detail and found to occur with the exchange of copper at the cell membrane without holo-caeruloplasmin penetrating the cells (Percival & Harris, 1990).

In conclusion, the apparent loss of CuZnSOD activity upon differentiation was prevented or reversed by caeruloplasmin in the medium. Despite the decline, haemin-treated K562 cells maintained a capacity to synthesize CuZnSOD protein. Production of a functioning enzyme required an exogenous copper source, which was met in part by caeruloplasmin. It is hypothesized that the copper atoms, not the protein as such, were the factors that intervened to maintain or restore functional CuZnSOD. The data support the view that copper controls expression of CuZnSOD at the level of post-translational regulation.

This study was supported in parts by National Institutes of Health Grant DK35920, National Institutes of Health Postdoctoral Fellowship Award DK08170(SSP) and Project H-6621 of the Texas Agricultural Experiment Station. We acknowledge with gratitude the excellent technical assistance provided by Ms. Frances Stuart, Mr. Charles Landrum and Mr. Michael Marsh. We thank Dr. Robert A. DiSilvestro for supplying rabbit anti-(human CuZnSOD) serum. We also thank Dr. Johnathan Gitlin for making available human CuZnSOD cDNA and providing technical assistance for assays of mRNA concentrations.

REFERENCES

- Allen, R. G. & Balin, A. K. (1988) *J. Clin. Invest.* **82**, 731–734
- Allen, R. G., Balin, A. K., Reimer, R. J., Sohal, R. S. & Nations, C. (1988) *Arch. Biochem. Biophys.* **261**, 205–211
- Andersson, L. C., Jokinen, M. & Gahmberg, C. M. (1979) *Nature (London)* **278**, 364–365
- Auwerx, J. H., Chait, A., Wolfbauer, G. & Deeb, S. S. (1989) *Blood* **74**, 1807–1810
- Bartha, E., Olah, E., Szelenyi, J. G. & Hollan, S. R. (1987) *Blood Cells* **12**, 647–651
- Beckman, B. S., Bailin, A. K. & Allen, R. G. (1989) *J. Cell. Physiol.* **139**, 370–376
- Bettger, W. J., Fish, T. J. & O'Dell, B. L. (1978) *Proc. Soc. Exp. Biol. Med.* **158**, 279–282
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- Chung, K., Romero, N., Tinker, D., Keen, C. L., Amemiya, K. & Rucker, R. B. (1988) *J. Nutr.* **118**, 859–864
- Cioe, L., McNab, A., Hubbell, H. R., Meo, P., Curtis, P. & Rovera, G. (1981) *Cancer Res.* **41**, 237–241
- Dameron, C. T. & Harris, E. D. (1987a) *Biochem. J.* **248**, 663–668
- Dameron, C. T. & Harris, E. D. (1987b) *Biochem. J.* **248**, 669–675
- Fleming, R. E. & Gitlin, J. D. (1990) *J. Biol. Chem.* **265**, 7701–7707
- Fukada, M. (1980) *Nature (London)* **285**, 405–407
- Galeotti, T., Wohlrab, H., Borrello, S. & De Leo, M. E. (1989) *Biochem. Biophys. Res. Commun.* **165**, 581–589
- Galiuzzo, F., Schiesser, A. & Rotilio, G. (1988) *Biochim. Biophys. Acta* **965**, 46–51
- Ichiki, A. T., Bamberger, E. G., Wust, C. J. & Lozzio, C. B. (1986) *Leukemia Res.* **10**, 565–574
- Lee, F. S. & Hassan, H. M. (1987) *Appl. Microbiol. Biotechnol.* **26**, 531–536
- Malik, Z., Chitayat, S. D. & Langzam, Y. (1988) *Cancer Lett.* **41**, 203–209
- Marceau, N. & Aspin, N. (1973) *Biochim. Biophys. Acta* **293**, 338–350
- Marklund, S. L. & Marklund, G. (1974) *Eur. J. Biochem.* **47**, 469–474
- Merchant, S. & Bogorad, L. (1986) *J. Biol. Chem.* **261**, 15850–15853
- Oberley, L. W. (1982) in *Superoxide Dismutase* (Oberley, L. W., ed.), vol. 2, pp. 127–165, CRC Press, Boca Raton
- Pelosi, E., Testa, U., Louache, F., Thomopoulos, P., Salvo, G., Samoggia, P. & Peschle, C. (1986) *J. Biol. Chem.* **261**, 3036–3042
- Percival, S. S. & Harris, E. D. (1988) *J. Trace Elem. Exp. Med.* **1**, 63–70
- Percival, S. S. & Harris, E. D. (1989) *J. Nutr.* **119**, 779–784
- Percival, S. S. & Harris, E. D. (1990) *Am. J. Physiol.* **258**, C140–C146
- Petraki, H., Ionnides, C. G., Fillis, S. & Papamichail, M. (1986) *Exp. Cell Biol.* **54**, 80–88
- Ripalda, M. J., Rudolph, N. & Wong, S. L. (1989) *Pediatr. Res.* **26**, 366–369
- Roualt, T., Rao, K., Harford, J., Mattia, E. & Klausner, R. D. (1985) *J. Biol. Chem.* **260**, 14862–14866
- Rutherford, T., Clegg, J. B. & Weatherall, D. J. (1979) *Nature (London)* **280**, 164–165
- Rutherford, T., Clegg, J. B., Higgs, D. R., Jones, R. W., Thompson, J. & Weatherall, D. J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 348–352
- Saito, H., Kuroki, T. & Nose, K. (1989) *FEBS Lett.* **249**, 253–256
- Saltman, P. (1989) *Semin. Hematol.* **26**, 249–256
- Sherman, L., Dafni, N., Lieman-Hurwitz, J. & Groner, Y. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5465–5469
- Speier, C. & Newberger, P. E. (1986) *Arch. Biochem. Biophys.* **251**, 551–557
- Testa, U., Bettaieb, A., Titeux, M., Vainchenker, W., Tonthat, H., Docklear, M.-C. & Rochant, H. (1982) *Cancer Res.* **42**, 4694–4700
- Weisiger, R. A. & Fridovich, I. (1973) *J. Biol. Chem.* **248**, 3582–3592