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CYTOTOXICITY OF A LOW MOLECULAR WEIGHT Cu_2Zn_2 SUPEROXIDE DISMUTASE ACTIVE CENTER ANALOG IN HUMAN ERYTHROLEUKEMIA CELLS

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Abstract—The cytotoxicity of SOD-mimics was studied in human K562 erythroleukemia cells. CuPUPY, a low molecular weight copper complex with properties typical of a Cu_2Zn_2 SOD active center analog was shown to display pronounced toxicity upon incubation with human K562 erythroleukemia cells, while the ligand, $CuSO_4$ or CuEDTA did not affect vitality. Externally added catalase decreased the cytotoxic effects of CuPUPY by 50% indicating an involvement of hydrogen peroxide in toxicity. An increased oxygen uptake and glutathione oxidation by K562 cells in the presence of CuPUPY suggested that toxicity might be due to a copper-mediated redox-cycle. In fact addition of glutathione to a solution of CuPUPY resulted in glutathione oxidation, O_2-consumption and H_2O_2-generation. CuPUPY proved to be less toxic to human lymphocytes than to K562 cells. This selectivity may be related to the low content of antioxidative enzymes in K562 cells.

Increasing interest concerning the antineoplastic effects of some metals has arisen in recent years, leading to the development of chemotherapeutically effective drugs such as *cis*-diammine dichloroplatinum (II) or to the assessment of the antitumor activity of bleomycin [1, 2]. Moreover several copper compounds have drawn interest due to their toxic effects on tumor cells [3–10]. However, little mechanistic information is available as yet. In this context an intriguing proposal has been made by Oberley and co-workers [6–8], who claimed that the antitumor activity of some low molecular weight copper compounds might be correlated with their superoxide dismutase activity.

Superoxide dismutases (SODs¶) are ubiquitous enzymes found in all aerobic cells, which are thought to provide a primary defence against the deleterious effects of O_2^- by dismuting it to H_2O_2 and O_2 [11]. H_2O_2 thus formed is potentially hazardous [12] and is readily removed by glutathione peroxidase (GSHPx) and catalase.

Tumor cells are known to often have deficient antioxidative defences [13–16], which results in higher susceptibility towards oxidative damage, although increased antoxidant levels are sometimes found in malignant cells and have been reported to be associated with drug resistance [17, 18].

Attempts to restore "lost" antioxidant levels in tumors by administration of CuDIPS**, a lipophilic, membrane-permeable low molecular weight copper complex with superoxide dismutase activity *in vitro* resulted in reduced growth of Ehrlich ascites in mice and increased average host survival [6, 7].

Oberley and co-workers claimed that the cytostatic action of the SOD-mimic was due to H_2O_2-generation. In fact, GSH depletion through inhibition of glutathione reductase by BCNU augmented the antineoplastic activity of the complex, while injection of GSH had the opposite effect [8] by indirectly affecting the H_2O_2-removing activity of GSHPx. The authors proposed that H_2O_2 might be formed in a CuDIPS catalysed dismutation of O_2^-, even though they did not rule out alternative mechanisms. In fact copper complexes with SOD activity *in vitro* might act as radical producers under *in vivo* conditions through performance of redox cycles with oxygen [19]. Both mechanisms, either radical scavenging or radical production, could account for the observed H_2O_2-generation [7].

CuDIPS might not be a good model to examine the antitumor action of SOD-active copper compounds because of its low specific SOD activity and its low ability to survive biological chelators including albumin [7, 10].

In order to investigate the mechanism of the antitumor activity of low molecular weight SOD-mimics we used a Cu_2Zn_2SOD active center analog, CuPUPY, which is a di-Schiff-base of 1,4 butanediamine and pyridine-2-aldehyde. Unlike in

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¶ Enzymes: catalase (EC 1.11.1.6), glutathione peroxidase (EC 1.11.1.9), glutathione reductase (EC 1.6.4.2) and superoxide dismutase (EC 1.15.1.1).

** Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CuDIPS, 3,5-diisopropylsalicylate-Cu(II); CuIM, 1,8-di(2-imidazoyl)-2,7-diazoctadiene-1,7-(*N,N',N'',N'''*)-Cu(II)-diperchlorate; CuPUPY, *N,N'*-bis(2-pyridylmethylene)-1,4-butanediamine (*N,N',N'',N'''*)-Cu(II)-diperchlorate; DMSO, dimethylsulfoxide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GSHPx, glutathione peroxidase; GSSG, oxidized glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MEM, minimal essential medium; NEM, *N*-ethylmaleineimide; PBS, phosphate buffered saline; SOD, superoxide dismutase.

CuDIPS, which is a salicylate-type complex, copper in CuPUPY is coordinated to four nitrogen atoms which are part of an unsaturated system. This coordination sphere is quite similar to that of Cu_2Zn_2SOD [11]. As a matter of fact CuPUPY displays a high specific SOD-activity [20] and remains intact in the presence of biological chelators [21].

We studied the reactivity of this compound in human K562 erythroleukemia cells and in human lymphocytes.

MATERIALS AND METHODS

Reagents and materials

CuPUPY and CuIM were prepared as previously described [22]. Bovine Cu_2Zn_2SOD was purified according to McCord and Fridovich [23]. Liposomes (composition: dipalmitoylphosphatidylcholine, cholesterol, stearylamine, 7:1:2, phospholipid concentration 50 μM), prepared according to Gruner *et al.* [24], were a generous gift from Dr Lucia Marcocci (University of Rome, La Sapienza). Catalase, NEM and DTNB were purchased from the Sigma Chemical Co. (Deisenhofen, F.R.G.). Glutathione, glutathione peroxidase and NADPH were from Boehringer (Mannheim, F.R.G.). Eagle's MEM, penicillin, streptomycin, Trypan blue and fetal calf serum were obtained from Gibco (Uxbridge, U.K.). All other chemicals were obtained from Merck (Darmstadt, F.R.G.).

Cell preparations

K562 erythroleukemia cells were grown in Eagle's minimal essential medium supplemented with 100 units/mL penicillin and streptomycin, 2 mM glutamine, 2 g/L $NaHCO_3$, 10 mM HEPES and 10% heat inactivated fetal calf serum (complete MEM) at 37° in humidified atmosphere containing 5% CO_2. Prior to the experiments cells were harvested by centrifugation and incubated in fresh complete MEM. Human lymphocytes were prepared according to Boeyum [25] from 20 mL fresh blood obtained from healthy volunteers (yield approx. 20×10^6 cells). Before experiments the lymphocytes were centrifuged at 150 *g*, washed twice in MEM and resuspended in complete MEM at a density of 10^6 cells/mL.

Assay of cell viability

After 5 hr incubation of cell suspensions (2×10^6 cells/mL) in the presence of Cu compounds Trypan blue uptake was determined by diluting the cell suspension 1:2 in Trypan blue solution (0.05% w/v) and incubating for 5 min at room temperature. At least 100 cells were then counted under a standard microscope using a Neubauer chamber.

EPR

Room temperature EPR-spectra were recorded with an ESP 300 instrument (Bruker) operating at 9.79 GHz, in standard flat EPR cells. EPR-settings for all spectra were: 1000 G scan width, 10 G modulation, 20 mW microwave power, 640 sec time constant, 240 sec scan time. The first derivative EPR signals which are indicative of the Cu (II) concentration in the sample, were quantitized by double integration against a Cu(II)EDTA standard.

Oxygen uptake

Oxygen uptake measurements were carried out with a Gilson oxygraph (Gilson, Middleton), using a Clark electrode covered with a Teflon membrane in a 2-mL cell kept at constant temperature (25° or 37°). All measurements were performed in PBS 20 mM phosphate, 140 mM NaCl, pH 7.4, which was supplemented with 5 mM glucose when respiration of K562 cells (2.5×10^6 cells/mL) was assayed.

Glutathione determinations

Total glutathione. Total glutathione was assayed according to Griffith [26]. Control experiments demonstrated no interference of CuPUPY on the accuracy of the assay.

Oxidized glutathione. Suspensions of 2×10^6 K562 cells/mL were incubated in complete MEM or in complete MEM supplemented with 1 mM PUPY or CuEDTA at 37° in humidified atmosphere containing 5% CO_2. After 0, 1 and 2 hr 10 mL cell suspension were collected to which 2 mL 0.25 M NEM was immediately added. Prior to addition of NEM a 10-μL aliquot was collected for determination of cell density and viability by Trypan blue uptake. Samples containing NEM were incubated for 10 min on ice. TCA (30%, 4 mL) was added and the samples were centrifuged at 2000 *g* for 15 min. The supernatants were collected and extracted 10 times with equal volumes of ether, neutralized with a few μL 6 N NaOH, frozen in liquid nitrogen and lyophilized overnight. The lyophilized samples were resuspended in 900 μL H_2O containing 3 mM EDTA. Oxidized glutathione was assayed according to Beutler [27]. When GSSG was added to the cell suspension as an internal standard full recovery was obtained.

Enzyme determinations

Cu_2Zn_2SOD was assayed with a polarographic method as CN^--sensitive activity at pH 9.6 [28]. Catalase was measured by a UV method according to Lück [29]. GSHPx was determined spectrophotometrically [30] with H_2O_2 as substrate, which is specific for the selenium containing enzyme.

RESULTS

Stability and membrane permeability of CuPUPY

EPR spectra of CuPUPY remained virtually unchanged in the presence of 10^7 K562 cells/mL after 1 hr incubation at 37°, indicating that the complex was stable under these conditions (Fig. 1).

Upon 10 min anaerobic incubation with high concentrations of K562 cells (95% viability, determined by Trypan blue uptake) 30% of the initial Cu(II)PUPY signal disappeared, most likely because of reduction to the EPR-undetectable Cu(I)-derivative, while anaerobic incubation with sonicated cells would cause complete reduction within the same time interval (Fig. 1).

Thus reduction of CuPUPY might be accomplished by intracellular reductants, suggesting

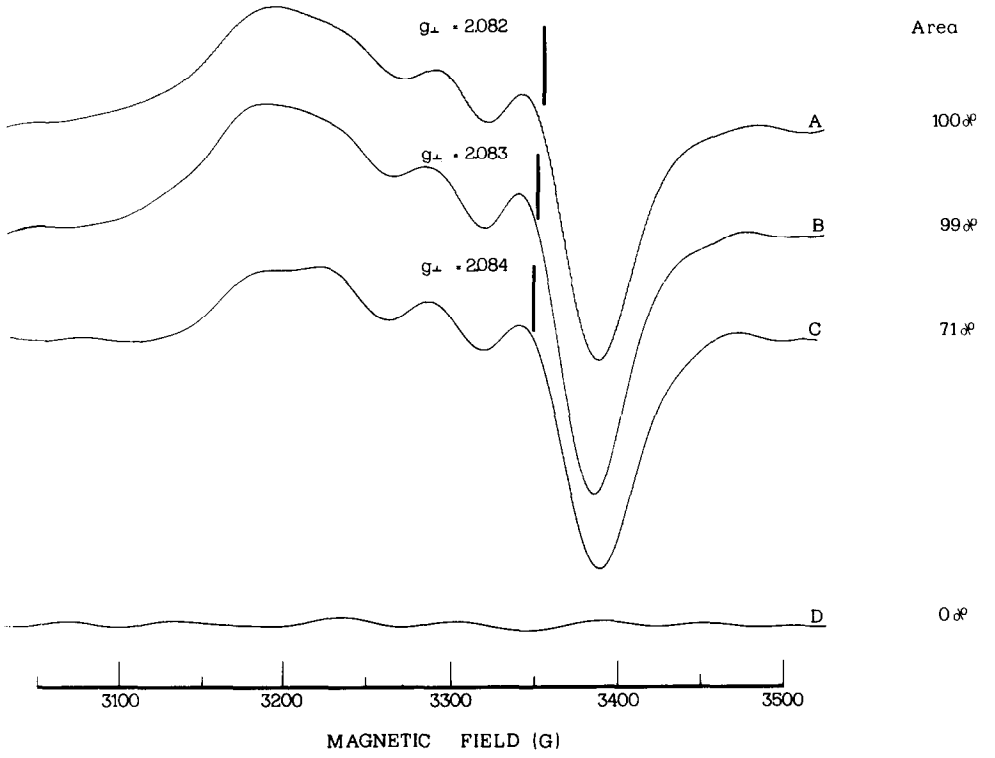


Fig. 1. Room temperature EPR spectra of CuPUPY in the presence of K562 cells. (A) CuPUPY (0.5 mM) in PBS 20 mM phosphate, 140 mM NaCl, pH 7.4; (B) 0.5 mM CuPUPY in PBS and 10^7 K562 cells/mL after 1 hr incubation at 37°; (C) 0.5 mM CuPUPY in PBS and 4.2×10^7 K562 cells/mL after 10 min incubation at 37° under anaerobic conditions; (D) 0.5 mM CuPUPY in PBS and 4.2×10^7 sonicated K562 cells/mL after 10 min incubation at 37° under anaerobic conditions. Double integration of spectra was performed and Cu(II)-concentration was determined with reference to a Cu(II)EDTA standard. All spectrometer settings were as described under Materials and Methods.

permeation of the compound through the cell membrane. However, the possibility that CuPUPY never enters the cell and is slowly reduced by groups on the cell membrane cannot be ruled out on the basis of these experimental data. We therefore used a liposome model in order to evaluate membrane permeability of CuPUPY.

All incubations with CuPUPY were carried out at the phase transition temperature of the liposomes ($T = 48°$) previously reported [31]. Subsequent additions and EPR measurements were performed at room temperature.

Figure 2 shows that ascorbate reduces Cu(II) in CuPUPY (curve A), while reduction time course was markedly slowed down by liposomes (curve B). Ascorbate is known not to permeate through liposome membranes [32] thus being able to reduce only the amount of CuPUPY not entrapped within the liposomes. In the presence of 0.2% Triton-X-100 (curve C), which disrupts the liposome membranes, reduction time course was accelerated when compared to experiment B, reaching an intermediate value between those observed in experiments A and B.

Differences between curves A and C could be due either to incomplete disrupture of the membranes by the detergent or to an interaction of the complex with the lipophilic environment which could render it less accessible to reduction by hydrophilic ascorbate.

The entrapment capacity of the liposomes was about 10% [33], which is in the same order of magnitude as the differences observed between the time courses of the experiments A and B.

Thus, CuPUPY displays pronounced lipophilicity, being able to freely diffuse across biological membranes regardless of any protein-linked transport system.

Toxicity of CuPUPY and CuIM

Incubation of K562 erythroleukemia cells with various concentrations of CuPUPY lead to a decrease in cell viability as detected by increased permeability to Trypan blue (Table 1). CuIm, a copper complex which was reported to have an SOD-activity comparable to CuPUPY [20–22] displayed analogous toxicity towards K562 cells. $CuSO_4$, copper-free PUPY or IM, or CuEDTA did not affect viability of K562 cells.

No reduction of mortality was produced by addition of $OH^{\cdot}$-scavengers such as mannitol or DMSO, while externally added catalase gave significant protection, indicating an involvement of H_2O_2-production in the mechanism of toxicity as suggested by Oberley and co-workers [6, 8] (Table 1).

Table 1. Toxicity of CuPUPY and CuIM to K562 cells and effects of oxygen radical scavengers

Addition	Cytotoxicity (% Trypan blue uptake) None	Catalase (1000 units/mL)	Catalase (inactivated)	Mannitol (50 mM)	DMSO (200 mM)
None*	4 ± 2	4 ± 1	2 ± 0.5	3 ± 1	5 ± 1
CuPUPY (1 mM)	89 ± 7	40 ± 5	ND	91 ± 5	90 ± 7
CuPUPY (0.5 mM)	53 ± 2	29 ± 4	52 ± 5	ND	ND
CuIM (0.5 mM)	66 ± 7	25 ± 0.5	56 ± 11	ND	ND

K562 cells were suspended in 2 mL MEM 10% fetal calf serum at a cell density of 2×10^6/mL in the presence of the copper compounds or their ligands. After 5 hr incubation at 37° in humidified atmosphere containing 5% CO_2 mortality was measured by Trypan blue uptake. Inactivation of catalase was performed by heating a solution containing 100,000 units/mL of active enzyme at 95° for 10 min. Twenty μL of this solution were added to 2 mL cell suspension. All data are mean values ± SD of three separate experiments.

* The same values were obtained in the presence of 1 mM CuEDTA, 1 mM $CuSO_4$ or 1 mM ligands of either CuPUPY or CuIM.

ND, not determined.

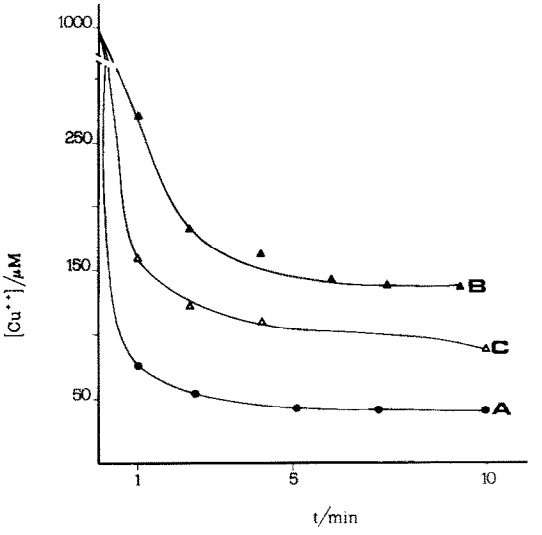


Fig. 2. Permeability of liposome membranes to CuPUPY. CuPUPY (1 mM) was incubated for 20 min at 48° with a liposome suspension (phospholipid concentration 50 μM) in PBS 20 mm phosphate, 140 mM NaCl, pH 7.4. Serial room temperature EPR spectra were registered for 10 min after addition of 10 mM ascorbate or 10 mM ascorbate and 0.2% Triton-X-100. Spectra were integrated and Cu(II)-concentrations were calculated with reference to Cu(II)EDTA standard solutions. Curve A: 1 mM CuPUPY + 10 mM ascorbate (●). Curve B: 1 mM CuPUPY + liposomes + 10 mM ascorbate (▲). Curve C: 1 mM CuPUPY + liposomes + 0.2% Triton-X-100 + 10 mM ascorbate (△).

Human lymphocytes were significantly less susceptible to the complexes-mediated toxicity than K562 cells (Table 2). This finding is likely to rely upon the lymphocytes higher capacity to detoxify oxygen free radicals. Accordingly, when compared to lymphocytes, K562 cells had low contents of glutathione, GSHPx and catalase (Table 3).

Table 2. Toxicity of CuPUPY or CuIM to human lymphocytes and to K562 erythroleukemia cells

	Cytotoxicity (% Trypan blue uptake) K562 cells	Lymphocytes
Control	3 ± 1	2 ± 1
CuPUPY (0.5 mM)	91 ± 7	49 ± 6
CuIM (0.5 mM)	90 ± 5	19 ± 3

Lymphocytes or K562 cells were incubated in 2 mL complete MEM or in complete MEM supplemented with either 0.5 mM CuPUPY or 0.5 mM CuIM at a cell density of 10^6 cells/mL at 37° in humidified atmosphere containing 5% CO_2. After 5 hr cell death was determined by Trypan blue uptake. Data reported are mean values ± SD of three separate experiments.

Oxygen consumption

An augmented O_2-uptake by K562 cells in the presence of 100 μM CuPUPY but not in the presence of CuEDTA was noticed (Table 4). To test whether this result might be related to redox cycling of the complex in the presence of biological reductants the reactivity of CuPUPY in the presence of GSH as a reducing agent was studied in a cell free system:

Figure 3 shows the O_2-uptake curve of a solution containing 100 μM CuPUPY and 300 μM GSH. The O_2-uptake comes to a halt after 30 sec. Addition of 50 μM CuPUPY at this point would not produce any further O_2-uptake. However, subsequent addition of NADPH and GSSG-reductase would cause O_2-consumption to start again, suggesting that glutathione oxidation had occurred. Final addition of catalase resulted in O_2-production, indicating that in the course of the reaction H_2O_2 had been formed.

Effects of CuPUPY on glutathione levels in K562 cells

The data reported in Table 4 and in Fig. 3 strongly

Table 3. Oxygen free radical scavengers in K562 erythroleukemia cells and in human lymphocytes

	Lymphocytes	K562
Cu_2Zn_2 SOD	0.65 ± 0.01	1.16 ± 0.06 (N = 3)
Catalase	81.2 ± 2	40 ± 5 (N = 3)
GSHPx	86.3 ± 6.3	2.5 ± 0.2 (N = 3)
Glutathione	30 ± 6	5.6 ± 0.6 (N = 3)

Cu_2Zn_2SOD is expressed in μg/mg of protein, catalase in units/mg of protein, GSHPx in units × 10^3/mg of protein and total glutathione content in nmol/mg of protein. Enzymes and total glutathione were determined as described in Materials and Methods.

Table 4. Oxygen uptake of K562 cells in the presence of copper compounds

	O_2 consumed (nmoles O_2/min 10^{-6} cells)
Control	12.8 ± 0.5
CuPUPY (0.1 mM)	25.2 ± 2.2
CuIM (0.1 mM)	32.2 ± 4.3
CuEDTA (0.1 mM)	13.3 ± 0.8

O_2-uptake was measured using a Clark electrode in 2 mL PBS 20 mM phosphate, 140 mM NaCl, pH 7.4, supplemented with 5 mM glucose at a cell density of 2.5 × 10^6 cells/mL and 37°. Data are mean values ± SD of three separate determinations.

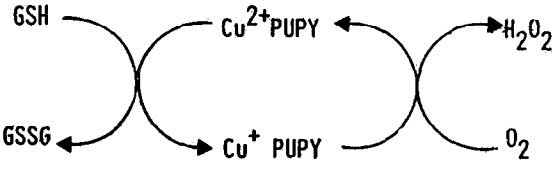
support the hypothesis of a redox-cycling mechanism which may involve glutathione in cells. This prompted us to investigate the effects of CuPUPY on glutathione in K562 cells.

No significant time-dependent changes in total glutathione content of erythroleukemia cells (1.47 ± 0.15 nmoles 10^{-6} cells (N = 3)) occurred during 2 hr incubation with either 1 mM CuEDTA or 1 mM CuPUPY (data not shown).

Oxidized glutathione was below the detection limit of our analytical procedure in either control cells or cells exposed to 1 mM CuEDTA for 2 hr, while a time-dependent increase in GSSG was observed in cells exposed to 1 mM CuPUPY: thus after 1 hr and 2 hr incubation GSSG levels were 0.15 ± 0.03 nmoles 10^{-6} cells and 0.28 ± 0.05 nmoles 10^{-6} cells (N = 3), respectively. This means that about 20% of total intracellular glutathione was present in the oxidized form after 2 hr incubation with CuPUPY. Remarkably, there was no Trypan blue detectable decrease in cell viability (≥90%) within 2 hr incubation with 1 mM CuPUPY, indicating that considerable glutathione oxidation had taken place prior to cell death.

DISCUSSION

In this study the mechanism of the interaction of the SOD-mimic CuPUPY with human erythroleukemia cells was investigated. The compound was taken up by cells and reduced, concomitantly enhancing O_2-uptake and causing oxidation of intracellular GSH. The whole process ultimately led to cell death, the extent of which was reduced by 50% by external addition of catalase, suggesting H_2O_2 formation and its implication in toxicity. These findings indicate that CuPUPY is unlikely to act as a SOD-mimic in cells but primarily performs a deleterious redox-cycle which we could reproduce *in vitro* by simply adding GSH to buffered solutions of CuPUPY:



Possibly this reaction scheme is common to most SOD-mimics once exposed to physiological conditions. In fact SOD-activity of a low molecular weight copper complex *in vitro* is related to its ability to coordinate both Cu(I) and Cu(II) and to the availability of at least one free coordination site [20, 34]. These features however do not imply a specificity for O_2^- as substrate. Thus the lack of an electrostatic barrier around the active site metal makes the copper in low molecular weight SOD mimics reactive towards oxygen, which is not the

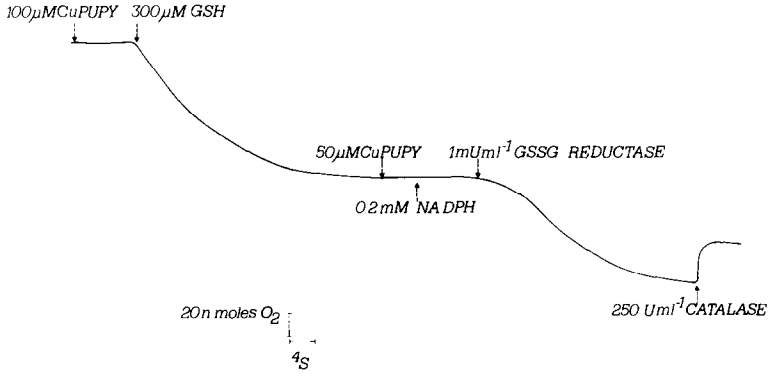


Fig. 3. Oxygen uptake curve of CuPUPY in the presence of GSH. O_2-consumption was measured in 2 mL PBS 20 mM phosphate, 140 mM NaCl, pH 7.4 with a Clark electrode at 25°.

case for Cu_2Zn_2 SOD [19, 35]. Furthermore, the lack of a protein moiety is likely to render the copper in CuPUPY accessible to bulky reductants such as glutathione which encounters sterical hindrances in reducing Cu(II) in Cu_2Zn_2 SOD.

$CuSO_4$ or CuEDTA are unable to perform redox-cycling reactions under our experimental conditions. In fact, unlike CuPUPY which proved to be stable, free Cu ions are readily chelated by biological ligands whereas CuEDTA has no free coordination sites through which oxygen or reductants could gain access to the copper. Accordingly both compounds were non-toxic at concentrations up to 1 mM.

Conceivably GSH is not the only agent able to reduce CuPUPY in cells but its oxidation is likely to play a role in the mechanism of toxicity of the copper complex. In fact H_2O_2 produced by CuPUPY could not be removed by GSHPx unless there is enough reduced glutathione at its disposal. Thus decreased GSH levels are likely to remarkably enhance H_2O_2 toxicity.

The capacity to detoxify oxygen free radicals is often impaired in tumor cells with respect to non-malignant cells. In fact, we have shown human lymphocytes to have higher activities of both GSHPx and catalase and a higher GSH content than our erythroleukemia cell line. Cu_2Zn_2SOD activity, on the other hand, proved to be twice as high in K562 cells than in human lymphocytes. As a result of this particular enzymatic pattern K562 cells should be characterized by a high H_2O_2/O_2^- ratio when compared to lymphocytes. This should render K562 cells much more susceptible to H_2O_2 induced cell-killing than human lymphocytes. Accordingly, we found CuPUPY and CuIM to be more toxic to K562 cells than to lymphocytes. Whether CuPUPY exerts differential cytotoxic action on tumor cells *in vivo* awaits further investigation.

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