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OXIDATIVE STRESS INDUCED BY A DI-SCHIFF BASE COPPER COMPLEX IS BOTH MEDIATED AND MODULATED BY GLUTATHIONE

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Abstract—The reductive activation of *N,N'*-bis(2-pyridylmethylene)-1-4-butanediamine (*N,N',N'',N'''*)-Cu(II)-diperchlorate (CuPUPY), a di-Schiff base copper complex with antineoplastic properties, was investigated *in vitro* in the presence of glutathione, ascorbate, NADH or NADPH. Glutathione and ascorbate but not the pyridine dinucleotides were able to reduce the compound. The apparent second order rate constants of the reduction reaction ($9.6 \pm 2.0\ M^{-1}\ sec^{-1}$ for ascorbate and $94.7 \pm 1.9\ M^{-1}\ sec^{-1}$ for glutathione) indicate that glutathione is more effective by about one order of magnitude in reducing CuPUPY than ascorbate. Reduction by glutathione triggered a CuPUPY-supported redox-cycle with oxygen yielding H_2O_2. Whereas reduction by ascorbate was reversible, CuPUPY reduced by glutathione reacted with excess reduced glutathione (GSH) in a ligand exchange reaction yielding a GSH–Cu(I) complex which was reoxidized by O_2, forming a complex between copper(II) and oxidized glutathione. These results suggest a dual role for the reduced thiol tripeptide; promoting oxidative stress induced by CuPUPY at low concentrations and inhibiting it at high concentrations. This hypothesis was verified by showing that optimum glutathione/CuPUPY ratios are needed in order to obtain maximum oxidative damage to both DNA and albumin *in vitro*. Evidence was obtained for the occurrence of the same reaction pathway in human K562 erythroleukemia cells: CuPUPY was more toxic to cells in which glutathione synthesis was inhibited by buthionine sulfoximine. Moreover, ESR spectroscopy revealed alterations in the hyperfine structure of the Cu(II) spectrum, consistent with the occurrence of ligand-exchange reactions in K562 cells.

Copper and its coordination compounds are known to produce severe cytotoxicity [1, 2]. Intriguingly, some copper complexes have been shown to have selective toxicity towards malignant cells [3–7]. Aspects of the molecular site of the antineoplastic effects of copper compounds have been elucidated and it appears that the transition metal undergoes reduction and subsequent reoxidation by molecular oxygen [2, 6]. This process leads to the formation of reactive oxygen species which are known to exert detrimental effects on cells by damaging biomolecules such as lipids [8], DNA [9] and proteins [10]. As many solid tumors and several tumor cell lines have been found to be deficient in enzymes that scavenge oxygen free radicals [11], this mechanism also provides a reasonable explanation for the observed tumor cell specific toxicity of some copper compounds. Accordingly, copper compounds need reductive activation before exerting their cytotoxic action. In some cases, thiols have been shown to play a prominent role in this context [2, 6, 7] but no detailed studies have been made so far. However, if thiol compounds are involved it is reasonable to expect glutathione to be a key compound because it is known to be of crucial importance in the cellular defense mechanisms against xenobiotics, as a radical scavenger and as a major intracellular low M_r copper chelator [12, 13].

The subject of this study is the interaction of glutathione with CuPUPY‖, a copper complex with pronounced superoxide dismutase activity *in vitro* [14] but not *in vivo* [6]. This compound was shown to have selective toxicity towards tumor cells *in vitro* [5, 6] and to produce increased host survival and reduction of tumor size in rats bearing Walker carcinoma [7]. Previous studies have shown that the complex induces both H_2O_2 production and glutathione oxidation in cells [6]. We present here the results from *in vitro* and cell culture studies indicating that glutathione both triggers and modulates oxidative stress induced by CuPUPY in a concentration-dependent fashion.

MATERIALS AND METHODS

Chemicals. All reagents used were of analytical

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‖ Abbreviations: BSO, (*d,l*)-buthionine-(*S,R*)-sulfoximine; CuPUPY, *N,N'*-bis(2-pyridylmethylene)-1,4-butanediamine (*N,N',N'',N'''*)-Cu(II)-diperchlorate; DMSO, dimethylsulfoxide; ESR, electron spin resonance; GSH, reduced glutathione; GSSG, oxidized glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; PBS, phosphate buffered saline; SOD, superoxide dismutase (EC 1.15.1.1); TBA, thiobarbituric acid.

grade purity. CuPUPY was prepared as described previously [14]. Catalase (EC 1.11.1.6), calf thymus DNA and BSO were purchased from the Sigma Chemical Co. (Deisenhofen, F.R.G.); and glutathione, NADPH and NADP were from Boehringer (Mannheim, F.R.G.). RPMI 1640, penicillin, streptomycin, Trypan blue and fetal calf serum were obtained from Flow Laboratories (Irvine, U.K.). TBA was bought from the Aldrich Chemical Co. (Steinheim, F.R.G.). All other chemicals were supplied by Merck (Darmstadt, F.R.G.).

Cell culture. K562 erythroleukemia cells were grown in RPMI 1640 medium supplemented with 100 units/mL penicillin and streptomycin, 2 mM glutamine, 20 mM HEPES and 10% heat-inactivated fetal calf serum (complete medium) at 37° in a humidified atmosphere containing 5% CO_2. Cells were routinely passaged twice a week in order to ascertain exponential growth. For glutathione depletion experiments, K562 cells were grown to a cell density of 0.5×10^6 per mL, 1 mM BSO was then added and cells were incubated for an additional 22 hr. Control cells were grown under the same conditions in BSO-free medium. Cells were harvested by centrifugation and resuspended in fresh complete medium (control cells) or fresh complete medium containing 10 μM BSO (GSH-depleted cells) at a cell density of 2×10^6 per mL. Glutathione was determined in each experiment using the method of Griffith [15] in order to control depletion efficiency. To determine cell viability at different time points, a 100 μL cell suspension was mixed with an equal volume of Trypan blue solution [0.16% (w/v) Trypan blue in 0.9% (w/v) NaCl], incubated for 5 min at room temperature and counted under a standard microscope using a Neubauer chamber. For ESR experiments, cells were grown as described above, washed twice with PBS buffer (20 mM sodium phosphate, pH 7.4, 140 mM NaCl) and resuspended in PBS.

Optical and ESR spectroscopy. Optical measurements of reduction kinetics were made at 25° with a Perkin–Elmer Lambda 9 spectrophotometer, monitoring the ligand field band of Cu(II)PUPY at 712 nm. Typically, the lag time between sample mixing and the beginning of measurement was 5 sec. Experiments were evaluated by calculating the initial slope of the absorbance decrease using a Lambda computing unit.

Fluorescence spectra were recorded with a modified Jobin Yvon JY3 spectrofluorimeter. Excitation at 336 nm was used to measure the Cu(I)-thiolate emission spectrum between 500 and 800 nm [16, 17]. An edge filter (440 nm) was used to suppress the second order emission of the excitation source.

Electron spin resonance measurements were made with a Bruker ESP 300 instrument. For spectral characterization, samples were contained in flat quartz ESR cells placed in a standard TE_{102} cavity operating at 9.79 GHz. Spectra were recorded using 20 mW microwave power, 1000 G scan width, a modulation amplitude of 10 G at 100 kHz, 64 msec time constant and a scan time of 4 min. Since manipulation of the flat cell is relatively slow, kinetics studies were done with 40 μL samples in 1.10 mm i.d. high precision capillary tubes. A circular TMH_{110}-mode cavity was used to compensate for the signal intensity loss due to decreased sample volume. The dead time from sample mixing to initiation of scanning could be kept within 40 sec. To avoid time-dependent spectral distortions, 500 G sweeps were recorded in 10 sec using a time constant of 10 msec; 4 consecutive scans were accumulated to improve the signal to noise ratio. Cu^{2+} signal intensities were calculated after double integration and comparison with a CuEDTA standard.

Oxidative damage to DNA and albumin. DNA damage was assayed by monitoring the release of TBA-reactive material [18]. Calf thymus DNA was dissolved in TKM buffer (50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 2.5 mM KCl) by gentle shaking overnight at 25°, further purified by phenol extraction, precipitated with ethanol and resolubilized in TKM. DNA solutions used for experiments had an E_{260}/E_{280} ratio of 1.9. The final DNA concentrations were calculated by measuring the absorbance of the solutions at 260 nm, assuming an absorption coefficient of 6.6 mM^{-1} [18]. Copper compounds were added to solutions containing 4 mM DNA and varying concentrations of GSH in TKM buffer (final volume 1 mL). The samples were incubated for 20 min at room temperature and the reaction was stopped by addition of EDTA to a final concentration of 10 mM. One mililiter 20% (v/v) acetic acid and 1 mL 1% (w/v) TBA in 0.05 M NaOH were added (pH of the final solution was 3.0). The samples were heated for 15 min at 100° and cooled on ice. The TBA-reactive product was extracted into *n*-butanol : pyridine (15 : 1) and optical spectra were recorded between 450 and 650 nm. The TBA-reactive product was identified by its absorbance maximum at 532 nm and quantified using an absorption coefficient of 1.36×10^5 M^{-1} [18]. No TBA-reactive material was detectable in any of the assay components (including acetic acid) when assayed alone.

Oxidative damage to albumin was measured as described previously [19] with some modifications: to solutions of albumin in PBS (1 mg/mL) containing varying amounts of GSH, CuPUPY was added to a final concentration of 0.5 mM. The samples were incubated for 20 min at room temperature and the reaction was stopped by addition of EDTA to a final concentration of 10 mM. A 5 μL aliquot of each sample was used for electrophoresis on a 7.5% polyacrylamide gel under non-denaturing conditions. The gel was stained with Coomassie blue, and the band intensities were determined by laser densitometry using an LKB densitometer with a Gelscan software package.

RESULTS

Reaction of CuPUPY with biological reductants

ESR and optical measurements of the reaction between CuPUPY and a series of biological reductants showed that glutathione was more efficient by about one order of magnitude in reducing CuPUPY than ascorbate, whereas both NADH and NADPH failed to reduce the compound. The second order rate constants were found to be

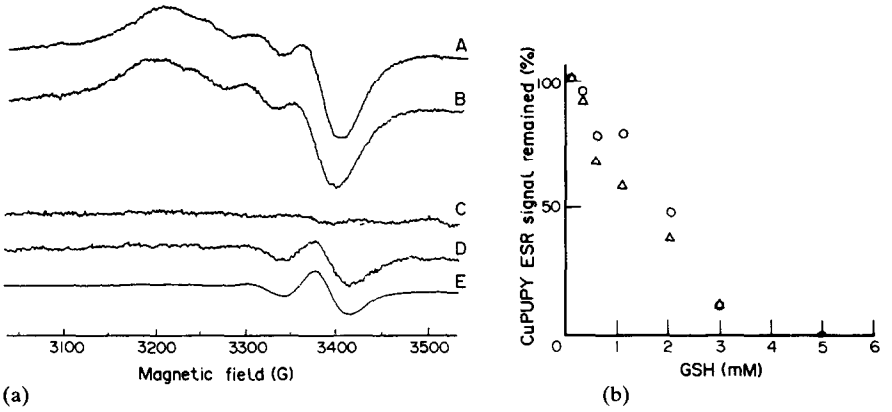

Fig. 1. Room temperature ESR spectroscopy of the reaction between CuPUPY and GSH. (a) ESR spectra of CuPUPY in the presence of glutathione, (A) 0.5 mM CuPUPY in PBS, 20 mM phosphate pH 7.4, 140 mM NaCl; (B) 0.5 mM CuPUPY in PBS after 2 hr incubation at room temperature in the presence of 1 mM GSSG; (C) 0.5 mM CuPUPY in PBS 1 min after addition of 5 mM GSH; (D) 0.5 mM CuPUPY in PBS after 1 hr incubation at room temperature with 5 mM GSH; (E) 2.5 mM Cu_2 GSSG. (b) Quantitation of reoxidizable CuPUPY after reduction by GSH. 0.5 mM CuPUPY was incubated at room temperature in PBS in the presence of different amounts of GSH. After 20 min the solutions were purged with air and ESR spectra were recorded (○). The measurement was repeated after 19 hr (△). The resulting Cu(II)–GSSG ESR signal was subtracted and the remaining Cu(II)PUPY was quantified by double integration against a Cu(II)EDTA standard. Spectrometer settings were as described in Materials and Methods.

$94.7 \pm 1.9\,M^{-1}\,sec^{-1}$ for glutathione and $9.6 \pm 2.0\,M^{-1}\,sec^{-1}$ for ascorbate.

ESR spectroscopy revealed that CuPUPY reduced by ascorbate was about 80% reoxidizable when the solution was purged with air and no qualitative changes in the ESR spectrum of the reoxidized complex were found, indicating reversibility of the observed redox reaction. On the other hand, reoxidation of CuPUPY after reduction by a 10-fold excess of glutathione produced a strikingly altered ESR spectrum, which proved to be identical to the spectrum of Cu(II)–GSSG (Fig. 1a). Thus, reduction of CuPUPY by GSH resulted in the formation of a complex between copper II and oxidized glutathione. Cu^{2+}PUPY was stable for hours in the presence of GSSG (Fig. 1a), suggesting that copper transfer occurred between reduced CuPUPY and reduced glutathione.

The reduction process was found to have a GSH:CuPUPY stoichiometry of approximately 8:1 in air-saturated samples (Fig. 1b). The large surplus of GSG indicated that a redox cycle reaction competed with the formation of the Cu(II)–GSSG complex. The reduction of CuPUPY reached its final level within a few minutes and no further changes were seen after several hours of exposure to air. In contrast, formation of the Cu(II)–GSSG complex occurred slowly and took several hours to reach completion (results not shown). During this time the typical Cu^+-thiolate luminescence spectrum could be detected (Fig. 2), indicating that Cu^+ was present as a relatively stable Cu(I)–GSH complex.

Damage to biomolecules induced by CuPUPY and GSH

In a previous study CuPUPY was shown to produce H_2O_2 *in vitro* in the presence of glutathione [6]. We have investigated the effect of different GSH concentrations on the CuPUPY-mediated oxidative damage to albumin and DNA (Figs 3 and 4). In both cases, a specific GSH:CuPUPY ratio was required in order to obtain the maximum effect; higher or lower GSH concentrations caused a decrease in oxidative damage to the biomolecules. $CuSO_4$ caused a smaller production of TBA-reactive material from DNA than CuPUPY (Fig. 4), although it showed the same dose–response pattern as CuPUPY. In contrast, Cu(II)(phenanthroline)$_2$, a well-studied

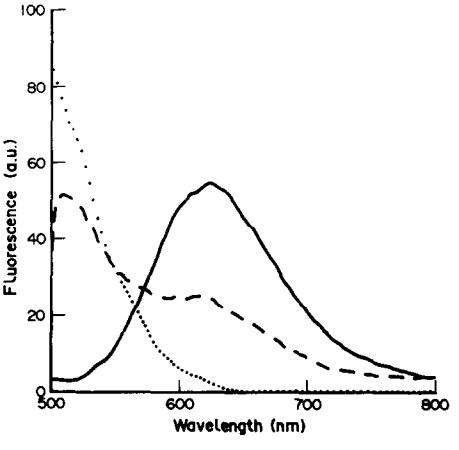

Fig. 2. Fluorescence emission spectra of Cu complexes at 293°K. 1 mM GSH + 0.5 mM $CuSO_4$ (full line), 0.5 mM CuPUPY (dotted line) 10 mM GSH + 0.5 mM CuPUPY (dashed line). Exitation was at 336 nm. All measurements were done in PBS buffer; the spectra were recorded within 2 min of mixing of the sample. No fluorescence could be detected from a sample of 6 mM GSH in PBS.

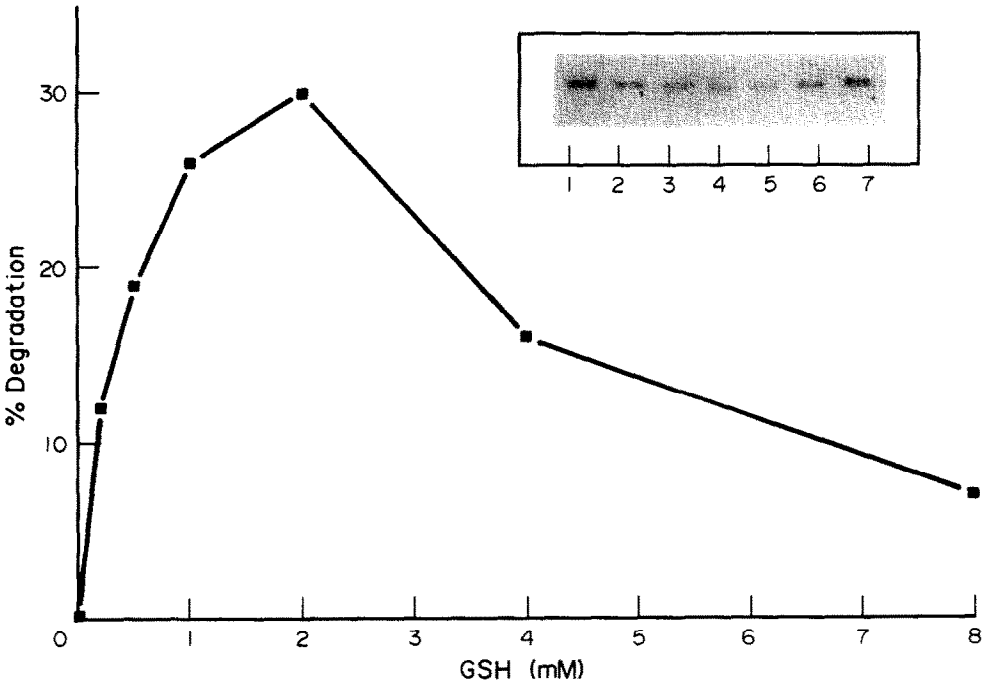

Fig. 3. Oxidative degradation of albumin by CuPUPY in the presence of GSH. The percentage protein degradation in samples containing 1 mg/mL albumin and 0.5 mM CuPUPY was determined at different GSH concentrations through densitometric analysis of polyacrylamide gels, as described in Materials and Methods. The inset shows the bands on a gel from a typical experiment. Lane 1: control, lane 2: +0.2 mM GSH, lane 3: +0.5 mM GSH, lane 4: +1.0 mM GSH, lane 5: +2.0 mM GSH, lane 6: +4.0 mM GSH, lane 7: +8.0 mM GSH. The presence of GSH alone had no effect on the band intensities.

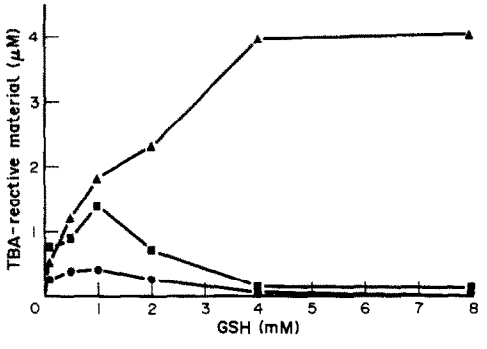

Fig. 4. Release of TBA-reactive material from DNA in the presence of CuPUPY and GSH. To solutions containing 4 mM DNA in TKM buffer (50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 2.5 mM KCl) and either 0.5 mM CuPUPY (■), 0.5 mM $CuSO_4$ (●) or 0.5 mM Cu(phenanthroline)$_2$ (▲) were added varying amounts of GSH. After 20 min incubation at room temperature the production of TBA-reactive material was determined as described in Materials and Methods. Data are mean values from four different experiments (SD was less than 5%).

Table 1. Effect of oxygen free radical scavengers on CuPUPY/GSH-mediated DNA-damage

Addition	TBA-reactive material (μM)
—	1.95 ± 0.05
Catalase (250 units/mL)	1.05 ± 0.04
Catalase (inactivated)	1.95 ± 0.15
DMSO (15 mM)	1.70 ± 0.10
Ethanol (10 mM)	1.78 ± 0.19
Mannitol (10 mM)	1.78 ± 0.18
Mannitol (50 mM)	1.57 ± 0.15
SOD (10 μM)	2.02 ± 0.08

The assay system contained 4 mM DNA and 2 mM GSH in 1 mL TKM buffer. After admixture of the indicated amount of scavenger, the reaction was started by addition of CuPUPY to a final concentration of 1 mM. After 20 min incubation at room temperature, TBA-reactive material was determined as described in Materials and Methods. Inactivation of catalase was done by incubating a solution containing 25,000 units/mL catalase for 5 min at 95°. Ten microliters of this solution were then added to 1 mL assay solution. Data are mean values ± SD of three separate experiments.

copper compound with endonuclease activity [20–22], showed increased production of TBA-reactive material from DNA with increasing GSH concentration, reaching a plateau at a GSH:Cu ratio of 4:1 (Fig. 4).

Removal of H_2O_2 by catalase, as well as addition of $OH^{\cdot}$ radical scavengers, reduced the yield of TBA-reactive material from DNA in the presence of CuPUPY and GSH, while addition of SOD had no effect (Table 1).

Taken together these findings indicate that low GSH concentrations promote a CuPUPY-driven Fenton chemistry which leads to oxidative damage to biomolecules, whereas high GSH concentrations inhibit oxidative damage.

Effects of CuPUPY on K562 cells

The *in vitro* studies suggest that CuPUPY should show increased toxicity towards cells with low glutatione levels. To verify this hypothesis the GSH synthesis in K562 cells was inhibited by addition of BSO. The amount of GSH in control cells was 3.2 ± 0.7 nmol/10^6 cells ($N = 3$), whereas BSO treatment reduced the GSH content to levels below 0.1 nmol/10^6 cells. Figure 5 shows that BSO-treated cells were significantly more susceptible to toxicity induced by the copper coordination compound than control cells.

A previous study showed that the hyperfine structure of the Cu(II)PUPY ESR spectrum is only slightly affected when the compound is incubated with K562 cells at a low cell density [6]. However, after 45 min incubation at 37° of 0.5 mM CuPUPY in a more concentrated cell suspension (5×10^7 K562 cells/mL), a second component appeared superimposed on the CuPUPY spectrum. Time course experiments revealed that the formation of a new Cu(II)–complex species, not identical to a Cu(II)–GSSG complex, occurred after prior reduction of CuPUPY (Fig. 6) and the kinetics of the disappearance of the Cu(II)PUPY ESR spectrum correlated with the formation of the new compound, suggesting a direct interconversion between the two species.

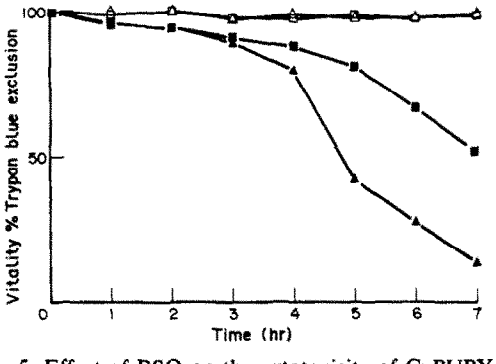

Fig. 5. Effect of BSO on the cytotoxicity of CuPUPY in K562 cells. Control or BSO-treated cells (2×10^6) were incubated at 37° in 1 mL RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum. At the indicated time points vitality was determined by Trypan blue exclusion. (□) 2×10^6 K562/mL, (△) 2×10^6 BSO-treated K562/mL, (■) 2×10^6 K562/mL + 0.5 mM CuPUPY, (▲) 2×10^6 BSO-treated K562/mL + 0.5 mM CuPUPY. Data are mean values from three different experiments (SD was less than 7%).

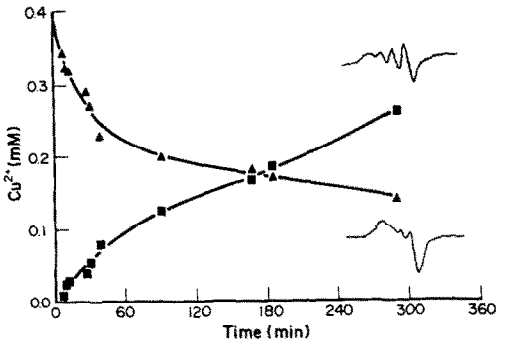

Fig. 6. Room temperature ESR spectroscopy of the interaction of CuPUPY with K562 cells. K562 cells in PBS (5×10^7/mL) were incubated at 37° in the presence of 0.5 mM CuPUPY. At the indicated time points ESR spectra were recorded. (▲) CuPUPY, (■) new Cu-complex. The two components were separated spectroscopically, as described in Materials and Methods, and quantified by double integration.

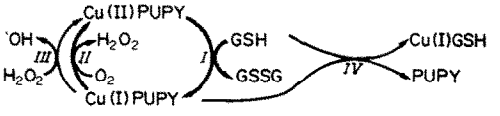

Fig. 7. Suggested reaction scheme of the interactions between CuPUPY and GSH.

DISCUSSION

Comparison of a series of biological reductants revealed that GSH is the most effective in reducing the cytotoxic copper complex CuPUPY *in vitro*. We have observed previously that oxygen consumption, H_2O_2 production and GSH oxidation occur both *in vitro* and in K562 cells during the reduction of CuPUPY by glutathione [5, 6]. Thus, the *in vitro* reduction effectively mimics the process that has been observed in cells [6] and *in vivo* [7], and which has been taken as evidence for a CuPUPY-supported redox-cycle, involving O_2 consumption and H_2O_2 production as the rationale for the antitumor activity of the compound.

We here show that glutathione, besides causing reductive activation of the compound, reacts with the reduced Cu(I)PUPY in a ligand-exchange reaction, transiently yielding a Cu(I)–GSH complex that could be identified by its characteristic luminescence spectrum. A suggested reaction scheme is drawn in Fig. 7. Reduction of the compound in reaction I triggers the copper-mediated redox-cycle and the associated production of reactive oxygen species in reactions II and III, while the ligand-exchange reaction IV can be regarded as a true detoxification reaction. Detoxification results partly from degradation of CuPUPY and partly from the formation of a Cu(I)–GSH complex which has been shown to be redox-inert and to be capable of transporting copper into Cu-binding apoproteins

[23], as well as into metallothionein, recently identified as a resistance factor towards copper toxicity [24].

From this reaction scheme it follows that at low GSH concentrations the thiol is likely to act primarily as the driving force of the redox cycle, whereas at high GSH concentrations the ligand-exchange reaction (IV) can compete efficiently with the reoxidation reactions (II and III). In the latter case, detoxification prevails over the activation of the redox cycle. The dual role of glutathione as both trigger and attenuator of oxygen free radical production by CuPUPY explains the fact that specific GSH concentrations are needed to obtain maximum oxidative damage to biomolecules.

We have found evidence for the occurrence of this reaction scheme in malignant cells. In fact, depletion of glutathione through inhibition of its synthesis by BSO increased the cytotoxicity of CuPUPY. Furthermore ESR spectroscopy revealed the occurrence of ligand-exchange reactions in K562 cells. Unlike the *in vitro* reaction between CuPUPY and GSH, which resulted in the formation of an ESR-detectable Cu(II)–GSSG complex, in the presence of K562 cells the ligand-exchange reaction would cause the formation of another small copper complex which was found extracellularly only (not shown). Its formation, however, occurred after prior reduction of CuPUPY, a process that has been shown previously to happen intracellularly [6]. It, thus, appears conceivable that intracellular ligand-exchange reactions involving CuPUPY cause copper to be excreted from the cell, thereby causing additional detoxification. In a subsequent study from this laboratory the new copper complex was identified as a Cu(II)–amino acid complex; the results from this work will be presented elsewhere.

Malignancy is often accompanied by an impairment of the cell capacity to detoxify oxygen free radicals. In this context, low glutathione levels have been reported to occur in some tumor cells [6]. Intriguingly, CuPUPY was shown by a previous report to be less toxic to non-malignant cells in which glutathione levels were higher than to tumor cells in which low levels of glutathione could be detected [6]. A similar effect is observed in the GSH-depletion experiments reported in this work. The fact that CuPUPY does not show the same pattern of endonuclease activity as $Cu(phenanthroline)_2$ is consistent with the idea that its cytotoxic effect is mediated essentially through interference with the glutathione status of the cell.

The dual role of glutathione as both metabolic activator of cytotoxic agents at low concentration and as detoxicant at high concentrations is probably not restricted to cytotoxic copper complexes. In fact, GSH has been reported to be a multidrug resistance factor in several tumor cell lines [25] as well as a metabolic activator of established chemotherapeutic drugs such as Pt(IV) complexes [26].

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