Inhibition of cytosolic glutathione *S*-transferase activity from rat liver by copper

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Abstract

H_2O_2 inactivation of particular GST isoforms has been reported, with no information regarding the overall effect of other ROS on cytosolic GST activity. The present work describes the inactivation of total cytosolic GST activity from liver rats by the oxygen radical-generating system Cu^{2+}/ascorbate. We have previously shown that this system may change some enzymatic activities of thiol proteins through two mechanisms: ROS-induced oxidation and non-specific Cu^{2+} binding to protein thiol groups. In the present study, we show that nanomolar Cu^{2+} in the absence of ascorbate did not modify total cytosolic GST activity; the same concentrations of Cu^{2+} in the presence of ascorbate, however, inhibited this activity. Micromolar Cu^{2+} in either the absence or presence of ascorbate inhibited cytosolic GST activity. Kinetic studies show that GSH but no 1-chloro-2,4-dinitrobenzene prevent the inhibition on cytosolic GST induced by micromolar Cu^{2+} either in the absence or presence of ascorbate. On the other hand, NEM and mersalyl acid, both thiol-alkylating agents, inhibited GST activity with differential reactivity in a dose-dependent manner. Taken together, these results suggest that an inhibitory Cu^{2+}-binding effect is likely to be negligible on the overall inhibition of cytosolic GST activity observed by the Cu^{2+}/ascorbate system. We discuss how modification of GST-thiol groups is related to the inhibition of cytosolic GST activity.

Keywords: Cytosolic GST–Cu; Cytosolic GST–Cu/ascorbate; GST-thiols

1. Introduction

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are a family of isoenzymes involved in the xenobiotic detoxication. GSTs catalyze the nucleophilic attack of glutathione (GSH) on electrophilic/lipophilic substrates, thereby decreasing their reactivity with cellular macromolecules [1]. These substrates display an electrophilic carbon as the site of attack by GSH; the relative lack of specificity of the enzyme however, permit that atoms other than carbon could serve as the point of nucleophilic attack such as the nitrogen atom of organic nitrate esters or the sulfur atom of organic thiocyanates [2–4]. Although these isoenzymes display low specificity towards their substrates, GSTs show an absolute specificity towards its endogenous cofactor, GSH [5].

Several roles in cellular processes have been described for mammalian liver GST isoenzymes, between them drug metabolism [1,4,6–8], intracellular transport [5,9–11], antioxidant defense [12–18], leukotriene biosynthesis [19–21], cell survival [22–24] and drug multi-resistance [11,25,26]. As many as eight

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classes of cytosolic GSTs, designated Alpha, Mu, Pi, Sigma, Theta, Zeta, Kappa and Omega have been identified in mammals [27]. In rat liver, GSTs occur as four cytosolic and one microsomal isoforms. These isoenzymes display differences in their apparent molecular weights, induction by xenobiotics, and immunochemical properties. Remarkably, these isoenzymes are distinguishable by their response towards detergents or sulfhydryl group-reducing agents [14,28]. The active form of rat liver microsomal GST is thought to be a dimeric conformation of two identical protein chains bound by their unique cysteine residue [29–31]. On the other hand, it has been reported that rat glutathione transferase P-form is inactivated by hydrogen peroxide (H_2O_2). The authors suggest that cysteine 47 and cysteine-101 may be involved in the formation of intra- or inter-subunit disulfides and these residues may be located in an important region for GSH binding, disulphide bond formation between these residues resulting in steric hindrance [32]. Similar results were observed with human placenta GSH-transferase π form and the authors demonstrated that the oxidative inactivation was reversed by 2,4-dithiotreitol (DTT) treatment [33]. Thus, it has been generally accepted that reactive oxygen species (ROS) lead to the activation of microsomal GST and to the inactivation of cytosolic isoenzymes.

Copper toxicity is thought to be a consequence of the generation of reactive oxygen species (ROS) via Fenton and/or Haber–Weiss reactions, in which copper ions catalyze the formation of ROS such as hydroxyl ($HO^{\bullet}$) and superoxide anion radicals ($O_2^{\bullet -}$) [34]. Copper ions, however, display high affinity for thiol and amino groups occurring in proteins. We have recently been able to identify two different mechanisms for copper-induced modification of thiol groups in microsomal proteins, i.e. oxidation and protein binding [35]. In the present work, therefore, we investigated the effects of Cu^{2+} alone and the oxygen free radical-generating system Cu^{2+}/ascorbate on the overall GST activity from rat liver cytosol.

Treatment of the cytosolic fraction with micromolar copper concentrations either in the absence or in the presence of ascorbate inhibited the cytosolic GST activity; the major component of this inhibitory effect was, apparently, the pro-oxidant effect of copper ions. Further study of cytosolic GST inhibition by copper in terms of changes in kinetic parameters showed a decrease in V_{max}, an increase in the K_m for GSH without significant changes for the xenobiotic 1-chloro-2,4-dinitrobenzene. We also showed a clear relationship between thiol group's loss and GST activity inhibition induced by copper. We discuss the mechanisms by which copper display inhibitory effects on the overall cytosolic GST activity, in relation to conformational changes induced by the pro-oxidant effect of copper and the binding of this metal *per se* to GST thiol groups.

2. Materials and methods

2.1. Chemicals

1-Chloro-2,4-dinitrobenzene was purchased from ACROS Organics (New Jersey, NJ, USA). Glutathione (GSH); *N*-ethylmaleimide (NEM); mersalyl acid; 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB); 2,4-dithiothreitol (DTT) and iminodiacetic acid sodium form in polystyrene matrix (CHELEX-100), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). $CuSO_4{\cdot}5H_2O$ was obtained from Merck, Chile. All other chemicals used were of analytical grade. All these compounds were prepared in buffer solution previously treated with CHELEX-100.

2.2. Animals

Adult male Sprague–Dawley rats (200–250 g), derived from a stock maintained at the University of Chile, were used. They were allowed free access to pelleted food, maintained with controlled temperature (22 °C) and photoperiod (lights on from 07:00 to 19:00 h). All procedures were performed using protocols approved by the Institutional Ethical Committee of the Chemical and Pharmaceutical Sciences School and Medicine School, University of Chile.

2.3. Liver cytosolic fractionation

Rats were fasted for 15 h with water *ad libitum*, and sacrificed by decapitation. Livers were perfused in situ with four volumes of 25 ml 0.9% (w/v) NaCl, excised, and placed on ice. All homogenization and fractionation procedures were performed at 4 °C and all centrifugations were performed using either a Suprafuge 22 Heraeus centrifuge or an XL-90 Beckmann ultracentrifuge. Liver tissue (9–11 g wet weight), devoid of connective and vascular tissue, was homogenized with five volumes of 0.154 M KCl, with eight strokes in a Dounce Wheaton B homogenizer. Homogenates were centrifuged at $9000 \times g$ for 15 min and sediments were discarded. Supernatants were then centrifuged at $105{,}000 \times g$ for 60 min. Supernatants (cytosol fraction) were stored at −80 °C until use. Protein

determinations were performed according to Lowry et al. [36].

2.4. GST activity

Glutathione *S*-transferase activity was assayed by the method described by Habig et al. [4], using 10 μg of cytosolic protein, 1-chloro-2,4-dinitrobenzene as substrate, and GSH (1 and 4 mM final concentration, respectively), in 100 mM sodium phosphate buffer, pH 6.5. Conjugated-substrate apparition was continuously recorded for 2 min at 25 °C, at 340 nm ($\varepsilon_{340} = 9.6\,mM^{-1}\,cm^{-1}$) in a UV3 Unicam UV-VIS spectrophotometer. All GST activity assays were realized in conditions of linearity respect to incubation time and protein concentration. To obtain kinetic parameters, GST activity of samples was determined with different concentrations of GSH (0.1–4 mM) or 1-chloro-2,4-dinitrobenzene (0.2–2 mM). K_m and V_{max} data was calculated from Lineweaver–Burk plots.

2.5. Treatments with mersalyl and NEM

Cytosolic fraction (10 μg/ml) was incubated with or without different concentrations of NEM or mersalyl acid during 10 min at 25 °C under constant agitation (100 rpm), before assaying GST activities, in a Julabo SW-21C thermoregulated bath.

2.6. Cytosolic thiol content

Cytosolic thiol groups were titrated with DTNB before and after a Cu^{2+}/ascorbate pre-incubation, as described by Letelier et al. [37]. Thiol concentration was estimated by the equimolar apparition of 5-thio-2-nitrobenzoic-acid ($\varepsilon_{410} = 13{,}600\,M^{-1}\,cm^{-1}$).

2.7. Oxygen consumption

Oxygen consumption extent was polarographically determined during 5 min (continuous) with a Clark electrode No. 5331 (Yellows Springs instrument) in a Gilson 5/6 oxygraph.

2.8. Statistical analysis

Data presented in this work correspond to the arithmetical mean of at least four independent experiments ± S.E.M values. Statistical significance (ANOVA) and regression analyses were performed using the GraphPad® Prism Software. Differences were considered significant when $p < 0.05$.

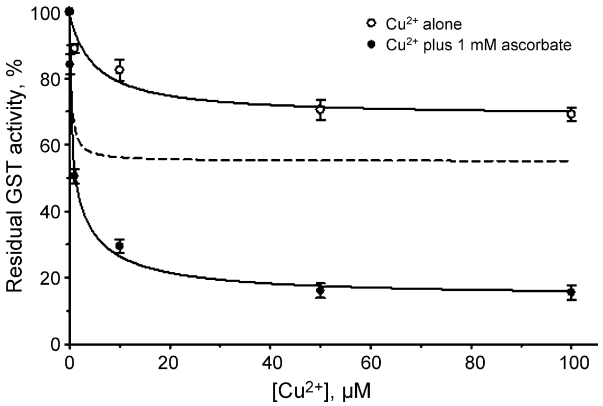

Fig. 1. Inhibition of cytosolic GST activity by Cu^{2+} in the absence or presence of ascorbate is dose-dependent. Cytosolic protein was pre-incubated for 15 min with Cu^{2+} in the absence or presence of 1 mM ascorbate, prior to determination of GST activity according to Section 2. Data points depict residual GST activity expressed in % of control activity (100% activity: 1004 ± 6 nmol of conjugate/min mg protein), determined in the absence of Cu^{2+}. Data represent the mean of at least four independent experiments ± S.D. Depicted curves were obtained by regression analysis, performed using the equation $y = A \times K_{0.5}/(x + K_{0.5}) + C$, where y corresponds to the residual GST activity, x to the Cu^{2+} concentration, $K_{0.5}$ to the concentration at which Cu^{2+} elicit a half-maximum effect, and C to a constant. The discontinuous line represents the difference curve obtained from subtracting the "Cu^{2+} alone" from the "Cu^{2+} plus 1 mM ascorbate" curve.

3. Results

3.1. Cu^{2+} and Cu^{2+}/ascorbate effect on the cytosolic GST activity

Pre-incubation of the cytosolic fraction with 25 and 100 nM Cu^{2+} in the absence of ascorbate and 25 nM Cu^{2+}/1 mM ascorbate, did not affect the cytosolic GST activity. As shown in Fig. 1, micromolar Cu^{2+} concentrations, either in the absence and presence of ascorbate, displayed a Cu^{2+} dose-dependent significant decrease in cytosolic GST activity. Regression analysis of the data was performed shows a single component for Cu^{2+} inhibition of cytosolic GST activity, with 30.6% maximum inhibitory effect and an IC_{50} value of 5.1 ± 1.2 μM. On the other hand, Cu^{2+}/ascorbate-induced displayed a more complex inhibitory effect on GST activity; it displayed two components, with maximum inhibitory values of 55.2% and 30.6% and IC_{50} values of 0.23 ± 0.01 and 5.0 ± 1.3 μM, respectively; the first of this components is plotted as a non-continuous line. Both Cu^{2+} alone and Cu^{2+}/ascorbate showed a time-dependent inhibitory effect on GST activity, as depicted in Fig. 2; again, such effects displayed single and double components, respectively. GST activity showed a single exponential inhibition with time when exposed to Cu^{2+} alone,

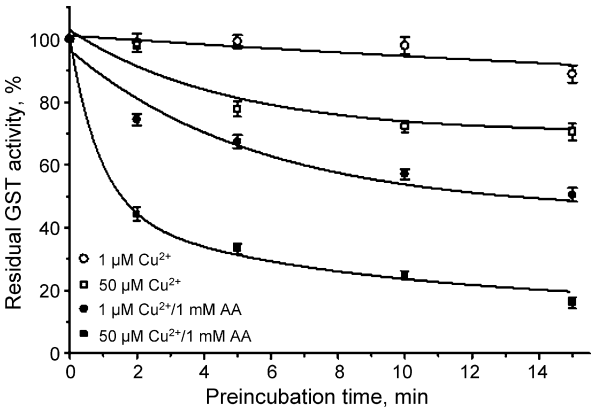

Fig. 2. Inhibition of cytosolic GST activity by Cu^{2+} in the absence or presence of ascorbate is time-dependent. Cytosolic protein was pre-incubated for different periods of time with Cu^{2+} (1 or 50 μM) in the absence or presence of 1 mM ascorbate, prior to determination of GST activity according to Section 2. Data points depict residual GST activity expressed in % of control activity (100% activity: 1004 ± 6 nmol of conjugate/min mg protein). Data represent the mean of at least four independent experiments ± S.D. Depicted curves were obtained by single-exponential decay regression analysis.

reaching a ∼30% maximum effect regardless of the ion concentration used; time constants for the inhibitory effect were 0.02 and 0.21 s^{-1}, for 1 and 50 μM Cu^{2+}, respectively. On the other hand, GST activity displayed a double exponential inhibition with time when incubated with Cu^{2+}/ascorbate, reaching ∼50% and ∼30% maximum effect components, regardless of the ion concentration used; time constant components were 0.01 and 0.18 s^{-1} in the case of 1 μM Cu^{2+}, and 0.12 and 1.0 s^{-1} in the case of 50 μM Cu^{2+} (both in the presence of 1 mM ascorbate).

3.2. Cu^{2+} effect on cytosolic GST kinetic parameters

In order to further evaluate the mechanisms underlying inhibition of GST activity induced by Cu^{2+} alone or Cu^{2+}/ascorbate we estimated the apparent kinetic constants of this enzyme under inhibitory conditions. Such conditions, i.e. 1 μM Cu^{2+} or Cu^{2+}/ascorbate for 15 min, and 50 μM Cu^{2+} or Cu^{2+}/ascorbate for 2 min, were selected in terms of obtaining significant activity values with which the kinetic constants were measurable; these constants are summarized in Table 1. In all conditions assayed but one, both Cu^{2+} and Cu^{2+}/ascorbate increased GST K_m for GSH without significant changes in the K_m value for 1-chloro-2,4-dinitrobenzene. The exception was the condition in which cytosolic fraction was pre-incubated during 15 min with 1 μM Cu^{2+}/ascorbate; in this case, 1-chloro-2,4-dinitrobenzene K_m was ∼2-fold higher than the control value. V_{max} values for 1-chloro-2,4-dinitrobenzene and GSH were equivalent and are shown as an average in Table 1. The decrease in GST V_{max} value was only marginal (about 10%) when the cytosol fraction was exposed to Cu^{2+} alone; in the presence of ascorbate, in contrast, V_{max} was decreased in approximately 50% compared to control value. Catalytic efficiencies for GSH and 1-chloro-2,4-dinitrobenzene were diminished in 50% and 20%, respectively, by treatment with 1 and 50 μM Cu^{2+} alone; in the presence of ascorbate, these values were decreased to about 25% of control value.

3.3. Cytosolic GST activity and thiol groups

Different authors have shown that GST thiol groups are involved in GST catalytic activity. Because NEM

Table 1
Kinetic constants following treatment of rat liver cytosolic GST with Cu^{2+} and Cu^{2+}/ascorbate

	Control	1 μM Cu^{2+} (15 min)	1 μM Cu^{2+}/AA (15 min)	50 μM Cu^{2+} (2 min)	50 μM Cu^{2+}/AA (2 min)
K_m GSH (mM)	0.19 ± 0.01	0.33 ± 0.02	0.43 ± 0.02	0.32 ± 0.02	0.41 ± 0.02
K_m 1-Cl-2,4-DNB (mM)	0.11 ± 0.01	0.12 ± 0.01	0.22 ± 0.01	0.12 ± 0.01	0.10 ± 0.01
V_{max}	1004 ± 6	897 ± 8	507 ± 16	905 ± 10	447 ± 12
Catalytic efficiency for GSH (V_{max}/K_m)	5284	2718	1179	2828	1090
Catalytic efficiency for 1-Cl-2,4-DNB (V_{max}/K_m)	9127	7475*	2305	7542*	4470

Cytosolic GST activities (10 μg/ml) were measured at different concentrations of GSH or 1-chloro-2,4-dinitrobenzene and kinetic parameters were calculated as described in Section 2. V_{max} values are expressed in nanomoles of conjugate per minute per milligram of cytosolic protein. All values represent the mean of at least four independent experiments ± S.D. Cu/AA: depicted Cu^{2+} concentration plus 1 mM ascorbate; 1-Cl-2,4-DNB: 1-chloro-2,4-dinitrobenzene. One standard unit catalyze the conjugation of 1.0 μmol of 1-chloro-2,4-dinitrobenzene and reduced glutathione per min at pH 6.5 at 25 °C.

* Values not statistically different ($p > 0.05$).

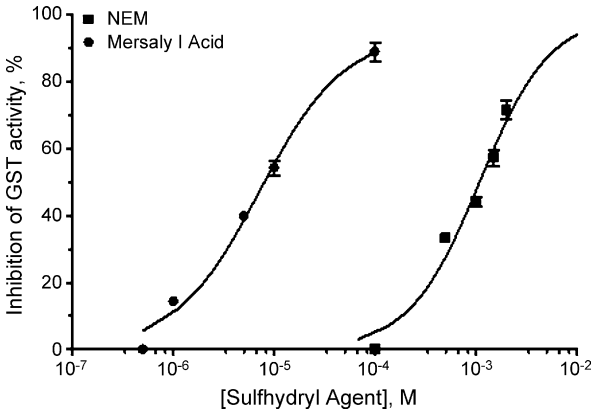

Fig. 3. Cytosolic GST activity is differentially inhibited by *S*-alkylation with NEM or mersalyl acid. Cytosolic protein was pre-incubated with different concentrations of NEM or mersalyl acid for 10 min at 25 °C before to add GSH to initiate the determination of GST activity according to Section 2. Data points depict % inhibition of GST activity compared to control activity (0% inhibition: 1004 ± 6 nmol of conjugate/min mg protein). Data represent the mean of at least four independent experiments ± S.D. Depicted curves were obtained by regression analysis using the Hill equation.

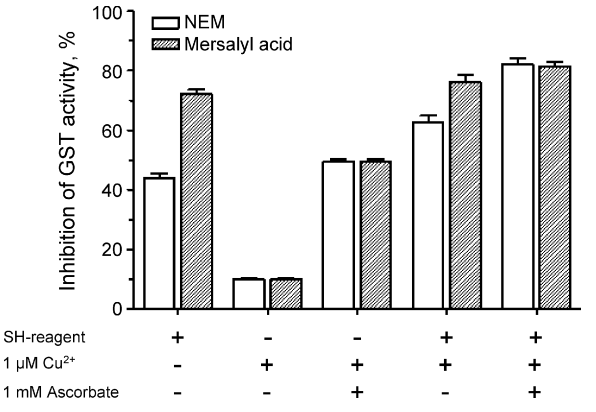

Fig. 4. Inhibition of cytosolic GST activity by Cu^{2+} in the absence or presence of ascorbate is differentially affected by *S*-alkylating agents. Cytosolic protein was pre-incubated with 1 mM NEM (open bars) or 5 μM mersalyl acid (hatched bars) for 10 min at 25 °C, prior to incubation with 1 μM Cu^{2+} in the presence or absence of 1 mM ascorbate for 15 min at 25 °C. Determination of GST activity was according to Section 2. Bars depict % inhibition of GST activity compared to control activity (0% inhibition: 1004 ± 6 nmol of conjugate/min mg protein). Data represent the mean of at least four independent experiments ± S.D.

and mersalyl acid are recognized *S*-alkylating agents, we assayed the effect of these compounds on cytosolic GST activity. As shown in Fig. 3, pre-incubation of cytosolic protein during 2 min with these agents inhibited GST activity in a dose-dependent manner. This figure depicts the significant difference in inhibitory potency between these agents, with mersalyl acid displaying the highest effect, with an IC_{50} of 4.5 μM as compared to NEM IC_{50} value of 0.2 mM.

In order to evaluate the relationship between the loss of thiol groups and the inhibition of cytosolic GST activity, we assayed the effects of NEM (1 mM) or mersalyl acid (5 μM) in the absence or presence of Cu^{2+} or Cu^{2+}/ascorbate; these results are shown in Fig. 4. Pre-incubation of cytosolic fraction with 1 mM NEM decreased GST activity in 44.1%; on the other hand, pre-incubation with 1 μM Cu^{2+} in the absence or presence of ascorbate provoked a 10.3% and 49.5% decrease in cytosolic GST activity, respectively; in the presence of 1 mM NEM, however, these values increased to 62.6% and 82.1%, respectively. Likewise, pre-incubation of cytosolic fraction with 5 μM mersalyl decreased GST activity in 72%. On the other hand, pre-incubation with 1 μM Cu^{2+} in the absence or presence of ascorbate and 5 μM mersalyl, increased the inhibition percentage induced by 1 μM Cu^{2+} and 1 μM Cu^{2+}/ascorbate since 10.3% and 49.5% to 76.3% and 81.4%, respectively (Fig. 4).

3.4. Oxygen consumption induced by Cu^{2+}/ascorbate: effect of cytosolic protein

To evaluate the oxidative and binding copper effects on the cytosolic fraction from rat liver, we assayed oxygen consumption induced by Cu^{2+}/ascorbate in the absence and presence of this fraction. Cu^{2+}/ascorbate promoted oxygen consumption, which rate can be polarographically determined as described in Section 2. Presence of cytosolic protein in the cuvette inhibited oxygen consumption elicited by Cu^{2+}/ascorbate in a dose-dependent manner, in agreement with our previous studies [35] (not shown). As depicted in Fig. 5, in either the absence or presence of cytosolic protein, oxygen consumption rates were still dependent on Cu^{2+} concentration, even though they were decreased in the latter condition. Interestingly, the Cu^{2+}-dependence of this decrease, obtained by subtracting the curve in presence of cytosolic protein from the one in its absence resulted in a highly saturable curve, with a $K_{0.5}$ of ∼10 μM for Cu^{2+}. To investigate the participation of thiol-groups in this phenomenon, we pre-incubated samples with NEM and mersalyl acid; our results are shown in Fig. 6. Pre-incubation of the cytosolic protein with NEM (0.1 and 1.0 mM) during 10 min, completely abolished the inhibition of the oxygen consumption induced by the cytosolic protein. In the same experimental conditions, mersalyl acid (0.5 and 5 μM),

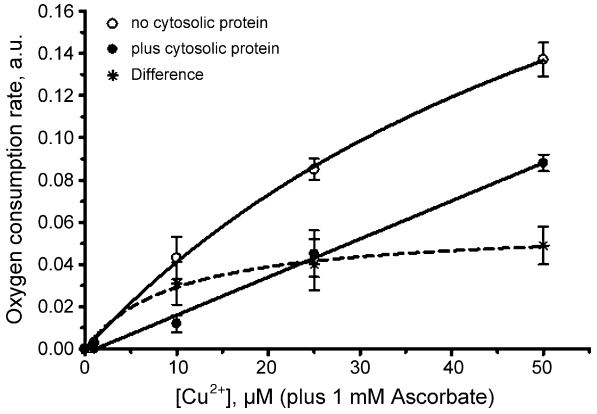

Fig. 5. Oxygen consumption elicited by Cu^{2+}/ascorbate is inhibited by the presence of cytosolic proteins. Oxygen consumption was determined as described in Section 2. Data points depict oxygen-consumption rates from experiments in the absence (open circles) or presence (closed circles) of cytosolic protein (0.1 mg/ml). Solid curves were obtained by hyperbolic regression analysis; the discontinuous curve was obtained by subtracting the curve in the presence of cytosolic protein from the curve in its absence. Determination of oxygen consumption was according to Section 2. Data represent the mean of at least four independent experiments ± S.D.

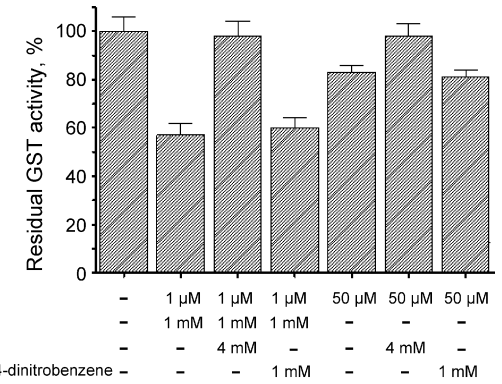

Fig. 7. GSH but not 1-chloro-2,4-dinitrobenzene protects cytosolic GST activity from inhibition by Cu^{2+} in the absence or presence of ascorbate. Cytosolic fractions (10 μg/ml) were pre-incubated in the absence or presence of either 4 mM GSH or 1 mM 1-chloro-2,4-dinitrobenzene for 20 min at 25 °C prior to incubation with either 1 μM Cu^{2+} plus 1 mM ascorbate or 50 μM Cu^{2+} alone for 10 min at 25 °C. GST activity was determined as described in Section 2. Bars depict residual GST activity expressed in % of control activity (100% activity: 1004 ± 6 nmol of conjugate/min mg protein). Data represent the mean of at least four independent experiments ± S.D.

however, did not display the expected analogous effect (Fig. 6).

3.5. Cytosolic GST activity in the presence of Cu^{2+}/ascorbate and Cu^{2+}: effect of pre-incubation with GSH and 1-chloro-2,4-dinitrobenzene

It has been suggested that critical thiol groups in cytosolic GST enzymes can be protected by pre-incubation with it cofactor GSH, because the redox-reactive cysteine residues may be located in the region for GSH binding [27,32]. Thus, to further evaluate this protective effect, we investigated whether pre-incubation (20 min) of the cytosolic fraction with the cofactor GSH and the xenobiotic 1-chloro-2,4-dinitrobenzene were able to protect cytosolic GST activity from Cu^{2+}-induced inhibition. As shown in Fig. 7, the pre-incubation of cytosolic fraction with GSH completely prevented the Cu^{2+}-induced inhibition on cytosolic GST induced by 1 μM Cu^{2+}/ascorbate and 50 μM Cu^{2+}. On the other hand, pre-incubation of cytosolic fraction with the substrate 1-chloro-2,4-dinitrobenzene did not modify the Cu^{2+}-induced inhibition on cytosolic GST (Fig. 7).

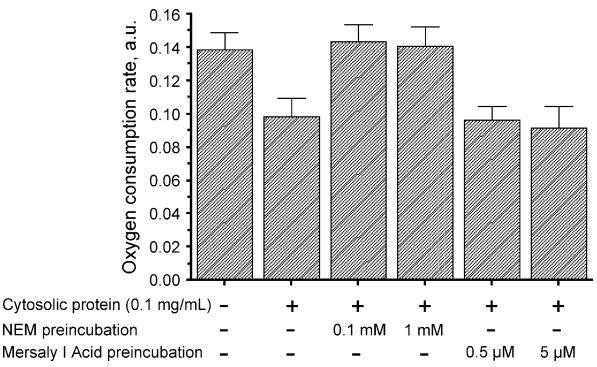

Fig. 6. Oxygen consumption elicited by Cu^{2+}/ascorbate-induced is not inhibited by cytosolic proteins treated with *S*-alkylating agents. Cytosolic fraction was pre-incubated in the absence or presence of NEM (0.1 or 1 mM) or mersalyl acid (0.5 or 5 μM) for 10 min at 25 °C. Then, oxygen consumption elicited by 50 μM Cu^{2+} and 1 mM ascorbate was determined in the absence or presence of cytosolic protein samples, as described in Section 2. Data represent the mean of at least four independent experiments ± S.D.

4. Discussion

4.1. Inhibition of GST activity by copper

It has been reported that H_2O_2 leads to the formation of intra- or inter-subunit disulfide bonds between particular cysteine residues within GST amino acid sequence, leading to the inactivation of the cytosolic enzymes [27,28,32,33]. The effect of other ROS on this activity, however, has not been thoroughly investigated; thus, we set to explore the effects of oxygen free radical superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$) on the overall cytosolic GST activity. With this purpose, we chose the Cu^{2+}/ascorbate system because it

generates ROS (see Fig. 5A for oxygen consumption by this system), and it also displays an non-discriminating Cu^{2+}-binding effect to thiol groups [35]; the latter effect enables us to better discriminate the mechanism by which cysteinyl lateral chains in these isoenzymes are crucial to GST activity. In other words, the pro-oxidant copper activity, through oxygen free radicals generation, will propitiate the formation of disulfide bonds between reactive cysteine residues, while Cu^{2+}-binding activity is likely to elicit the blockade of such residues. Our results showed that micromolar copper concentrations were able to inhibit hepatic cytosolic GST activity, in either the presence or absence of ascorbate; this phenomenon was dependent on both the pre-incubation time of cytosolic fraction with Cu^{2+} and Cu^{2+} concentration (Figs. 1–3). Interestingly, close examination of the inhibition parameters of these inhibition effects (Fig. 1) shows that one of the components in Cu^{2+}/ascorbate-induced inhibition of GST activity (30%) is identical to the one-component inhibition effect displayed by Cu^{2+} alone. This strongly suggests that the Cu^{2+}/ascorbate system elicits both the pro-oxidant and Cu^{2+}-binding effect simultaneously; the subtracted line shown in Fig. 1 (non-continuous line) further illustrates this point. Further studies on purified GST isoenzymes are required to evaluate whether the effect of Cu^{2+} on cytosolic GST activity is the result of inhibiting all cytosolic GST isoenzymes to the same extent or is the consequence of differential effects on different isoenzymes.

On the other hand, GST kinetic parameters showed that both Cu^{2+} and Cu^{2+}/ascorbate modified significantly the affinity of the enzyme for its endogenous cofactor, GSH, while the xenobiotic 1-chloro-2,4-dinitrobenzene affinity remained unchanged (Table 1). Thus, the decrease in V_{max} and catalytic efficiency of the enzyme induced by the systems tested can be explained in terms of the changes in its affinity to GSH. Kinetics studies with purified GSTs show high differences in the specific activity for a same substrate as also for different substrates; K_m values for 1-chloro-2,4-dinitrobenzene can differ 100 or more fold depending of the GST isoform assayed. Likewise, the GST isoforms K_m values for GSH described seem to be lower than 1 mM. It is necessary to note that comparative values of these kinetic constants are difficult to obtain because the assay conditions used by the investigators are not the same [38–42]. Although we measured the overall cytosolic GST activity, the apparent kinetic constant values obtained are in the range of data described for purified GST isoforms; so they may represent values average of the all GST isoforms' kinetic constants present in our cytosolic preparation (Table 1).

The information concerning to the precise enzyme-substrate interactions responsible for the catalytic activity has been greatly increased by the determinations of many three-dimensional structures of GST isoforms. These studies have shown that protein–protein non-covalent interactions are involved in the catalytically active dimer of the different cytosolic GST isoforms. The GSH (G site) and the xenobiotic-substrate (H site)-binding sites are constituted principally by hydrophobic aminoacid residues [27,32,33]. However, oxidative inactivation of some GST seems involve some cysteine residues which are involved in disulphide formation; these cysteinyl residues seem be located in the G site [27,32,43]. Moreover, these studies reported the existence of two reactive thiol groups per dimer [27,28,32,33]. Our data also suggest that the cytosolic GST isoenzymes' cysteinyl residues might have differential reactivity to modification by Cu^{2+}/ascorbate. To test this postulate, we turned to two well-known thiol group-alkylating agents: NEM and mersalyl acid; there was a clear difference in the dose-dependence effect of these agents even though they are both *S*-alkylating compounds (Fig. 3). This difference may be explained by difference in the accessibility of the GST thiol groups to these agents. Moreover, NEM pre-incubation appeared to increase the ability of Cu^{2+} and Cu^{2+}/ascorbate to inhibit GST activity. This synergistic effect may be a consequence of putative NEM-induced conformational changes of the protein that expose novel GST thiol groups to the action of Cu^{2+} (Fig. 4). The inhibitory effects induced by mersalyl on cytosolic GST activity, however, seem to be independent of that induced by Cu^{2+} and Cu^{2+}/ascorbate (Fig. 4). This finding may be indicative of the relative position of the reactive thiol groups of the GST towards Cu^{2+} and/or ROS. The lower effect of Cu^{2+} alone on cytosolic GSTs kinetic parameters compared to that of the Cu^{2+}/ascorbate system seems to enforce the idea that Cu^{2+} binding to GST thiol groups is a minor phenomenon compared to the pro-oxidative action of this ion in the presence of ascorbate. This further supports the idea of differential reactivity of thiol groups present in the cytosolic GST isoenzymes.

At this point, our results suggests that: (1) modification of thiol groups, regardless of the mechanism – i.e. oxidation or Cu^{2+} binding – inhibits cytosolic GST activity; (2) there are likely at least two groups of cysteine residues with different reactivity to the Cu^{2+}/ascorbate system; (3) the pro-oxidant action of Cu^{2+} has a more significant effect on GST activity than its binding property; (4) both effects are summative and not synergic. Such finding represents a novel mechanism for cytosolic GST isoenzymes inhibition, which appears to be additional to

that of intra- or inter-molecular cross-linking, mediated by modifications of it thiols residues.

To elucidate whether the effects shown in our study were due only to modification of different thiol, we performed additional studies. First, we addressed the oxygen consumption pattern generated by Cu^{2+}/ascorbate (Fig. 5); cytosolic protein inhibited the oxygen consumption elicited by Cu^{2+}/ascorbate in a dose-dependent manner (Fig. 5). In agreement with our previous study, Cu^{2+} binding to thiol groups in proteins is likely to decrease the availability of Cu^{2+} ions to undergo Haber-Weiss and/or Fenton reactions [34,35]. Indeed, when pre-incubated with NEM, but not with mersalyl acid, the cytosolic fraction is no longer able to decrease the oxygen consumption elicited by Cu^{2+}/ascorbate; this clearly demonstrate the critical property of thiol groups, reactive only to NEM, in Cu^{2+} binding. Taken together, these results show that although both NEM and mersalyl acid inhibited GST activity (Fig. 3), these agent may *S*-alkylate different thiol groups in the cytosolic GST isoenzymes. Furthermore, this evidence suggests that NEM, and not mersalyl acid, may target solely thiol groups of GSTs which will display Cu^{2+} binding. As mentioned above, our results hinted the possibility that critical thiol groups may be located close to the GSH-binding site of the cytosolic GSTs. This is suggested by the fact that Cu^{2+}/ascorbate (and Cu^{2+} alone to a lesser extent) decreased the affinity of cytosolic GST isoenzymes to GSH, without changing their affinity for the xenobiotic substrate (Table 1). This postulate was further strengthened by the total protection of cytosolic GST activity towards both Cu^{2+} or Cu^{2+}/ascorbate by pre-incubating the cytosolic fraction with GSH, while pre-incubation with 1-chloro-2,4-dinitrobenzene displayed negligible effects (Fig. 7). According with other authors, our results seem indicate that binding of GSH to the GST active site seem to hinder this type of critical cysteinyl residues preventing either its oxidation or Cu^{2+} binding, thus protecting GST activity [27,28,32,33]. Since free GSH may reduce Cu^{2+} ions like those that ascorbate does, our results also suggest that binding of GSH to the GSTs active site may also abolish the redox reactivity of GSH; this phenomenon has also been reported in other systems [44] and requires further testing.

In summary, our data suggest that the major mechanism underlying copper-induced inhibitory effect on cytosolic GST activity is likely to reside in this ion pro-oxidant properties, leading to the oxidation of protein thiol groups. Binding of Cu^{2+} to cytosolic GST thiol groups of the monomeric or dimeric isoforms also may occur, but only micromolar copper concentrations may induce a significant inhibition of overall cytosolic GST activity. Thus, it is likely that the cytosolic GSTs thiol groups capable of binding copper ions are not exposed to the environment.

In humans, physiological copper plasma concentrations range between 14 and 19 μM [45–47]; in hepatic cells, copper ions are sequestered principally by metallotionein and GSH [47]. High cellular copper concentration however, such as those propitiated in copper-related pathologies, can saturate the capacity of these molecules to bind copper ions; in this condition, copper may induce on one side, oxidative stress, and on the other, binding of this metal to critical cysteinyl residues of cellular proteins, leading to cytotoxicity. Our experimental K_m of cytosolic GST for GSH was 0.19 mM; since cytosolic GSH concentration ranges between 0.5 and 10 mM and hepatic concentration ∼5 μmol/g of liver [48–50], GSH may be saturating GST molecules under physiological conditions, protecting its activity from Cu^{2+}-induced damage. GST represents 10% of total content of cytosolic protein and its role in detoxication mechanisms is well acknowledged [12–16,19,22,23]. The existence of this putative GST–GSH complex may represent a physiological mechanism to protect this remarkable cytosolic enzyme – and also GSH – from the oxidative stress-related damage.

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