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Inactivation of Yeast Glutathione Reductase by Fenton Systems: Effect of Metal Chelators, Catecholamines and Thiol Compounds

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Oxygen radical generating systems, namely, Cu(II)/H_2O_2, Cu(II)/ascorbate, Cu(II)/NAD(P)H, Cu(II)/H_2O_2/catecholamine and Cu(II)/H_2O_2/SH-compounds irreversibly inhibited yeast glutathione reductase (GR) but Cu(II)/H_2O_2 enhanced the enzyme diaphorase activity. The time course of GR inactivation by Cu(II)/H_2O_2 depended on Cu(II) and H_2O_2 concentrations and was relatively slow, as compared with the effect of Cu(II)/ascorbate. The fluorescence of the enzyme Tyr and Trp residues was modified as a result of oxidative damage. Copper chelators, catalase, bovine serum albumin and $HO^\bullet$ scavengers prevented GR inactivation by Cu(II)/H_2O_2 and related systems. Cysteine, N-acetylcysteine, N-(2-dimercaptopropionylglycine and penicillamine enhanced the effect of Cu(II)/H_2O_2 in a concentration- and time-dependent manner. GSH, Captopril, dihydrolipoic acid and dithiotreitol also enhanced the Cu(II)/H_2O_2 effect, their actions involving the simultaneous operation of pro-oxidant and antioxidant reactions. GSSG and trypanothione disulfide effectively protected GR against Cu(II)/H_2O_2 inactivation. Thiol compounds prevented GR inactivation by the radical cation $ABTS^{\bullet+}$. GR inactivation by the systems assayed correlated with their capability for $HO^\bullet$ radical generation. The role of amino acid residues at GR active site as targets for oxygen radicals is discussed.

Keywords: Glutathione reductase, Fenton systems, copper, hydrogen peroxide, oxygen radicals, oxidative damage, catecholamines, antioxidants, thiol compounds, Captopril, trypanothione.

Abbreviations: Cu(II) and Cu(I), cupric and cuprous ions, respectively; GR, glutathione reductase; GSH and GSSG, reduced and oxidized glutathione, respectively; Cu(II)/H_2O_2, Cu(II)-Fenton system; Asc, ascorbate; RSH, thiol compounds; Cys, L-cysteine; NAC, N-acetylcysteine; MPG, N-(2-mercaptopropionylglycine); PAM, penicillamine (3-mercapto-D-valine); DHLA, dihydrolipoic acid; DTT, dithiothreitol; TS_2, trypanothione disulfide; CA, catecholamine; epinephrine, 1-(3,4-dihydroxyphenyl)-2-methylaminoethanol; norepinephrine, 1-(3,4-dihydroxyphenyl)-2-aminoethanol; dopamine, 3,4-dihydroxyphenylethylamine; L-DOPA, 3-(3,4-dihydroxyphenyl)-alanine; CPT, Captopril [1-(3-mercapto-2-methyl-1-oxopropyl-L-proline)]; ABTS, [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid]); SOD, superoxide dismutase; HRP, horse-radish peroxidase; DCI, dichlorophenol-indophenol.

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INTRODUCTION

Yeast GR (glutathione disulfide reductase (NADPH); EC 1.6,4.2) catalyses the virtually irreversible reduction of GSSG by NADPH

$$GSSG + NADPH + H^+ \longrightarrow 2\,GSH + NADP^+ \quad (1)$$

(Reaction 1).[1] GR is a member of the oxido reductase family of homodimeric flavoenzymes, characterized by having a FAD cofactor and a redox active disulfide in each monomer.[1] Two *half*-reactions must be considered: E_{ox} to EH_2 and EH_2 to E_{ox}, where EH_2 (the FAD oxidized, disulfide reduced GR) is stable under anaerobic conditions.[2] The active site acid-base catalyst His467 (amino acid numbering as in Reference 1) has an essential role in both the reductive and oxidative half-reactions.[1] The FAD-binding domain comprises the polypeptide segment that contains the redox reactive dithiol.[1] The 467-amino acid sequence of yeast GR exhibits about 50% sequence identity to human GR with conserved regions, including the redox active disulfide peptide and those residues assigned to the binding of NADPH.[3] Moreover, the active sites of yeast GR, human GR, and trypanosomatids TS_2 reductase are similar in topology,[4] thus allowing the use of the yeast enzyme as a target for structure-based drug design.[4,5] The information obtained from yeast GR may, therefore be useful for the development of specific metabolic inhibitors, herbicides and trypanocidal agents.[4,5]

Previous studies[6–8] demonstrated that myocardial dihydrolipoamide dehydrogenase, a flavoenzyme structurally related to GR,[1] is inactivated by Fe(II)/H_2O_2 and Cu(II)/H_2O_2 systems and therefore a similar effect could be expected to occur with GR. The observations reported here, using Cu(II)/H_2O_2 and related systems as oxygen radical generators, confirm this hypothesis and allows one to suggest that GR inactivation might contribute to Cu cytotoxicity in yeasts, since, by maintaining GSH at an adequate level, GR protects yeast cells against reactive oxygen species.[9]

MATERIALS AND METHODS

Materials

GR preparations were purchased from Boehringer-Mannheim GmbH, Germany or from Calbiochem-Novabiochem Corporation, La Jolla, CA, USA. Absorption spectra indicated that the enzyma samples were 92–95% pure and fully oxidized. Specific activities were as indicated under Results. Before use, GR suspensions were centrifuged for 3 min in an Eppendorf centrifuge 5414, the supernatant was discarded and the sedimented protein was dissolved in a volume of 50 mM KH_2PO_4–K_2HPO_4 pH 7.4 (henceforth "phosphate buffer") solution twice the original suspension volume. The suspension was filtered through an Ultrafree-MC (NMWL 30000) at 3000 g and 10°C, for 10 min. To the protein was added 1.2 ml of the phosphate buffer solution and refiltered twice, as described. Finally, the protein was dissolved in 50 mM K-phosphate buffer solution, in the original suspension volume. The purified preparations were diluted ten-fold with the K-phosphate buffer solution and kept at 4°C for further studies.

Reagents

GSH, GSSG, NADPH, NADH, thioctic acid (α-lipoic acid), L-histidine, EDTA, DETAPAC, Cys, NAC, MPG, PAM, CPT, L-ascorbic acid, epinephrine, norepinephrine, dopamine, 6OH-dopamine, L-DOPA, DOPAC and HRP were purchased from Sigma Chemical Co., St. Louis, MO, USA. ABTS was purchased from Fluka, Buchs, Switzerland. Other reagents were as described previously.[6–8]

Enzyme Assays

Unless stated otherwise, GR activity was measured by the rate of NADPH oxidation, using GSSG as electron acceptor. The standard GR reaction medium contained 50 mM KH_2PO_4–

K_2HPO_4, pH 7.4, 0.1 mM NADPH, 1.0 mM GSSG and GR as indicated under Results; total volume, 3.0 ml. The reaction was started by adding NADPH and the initial velocity of the GR reaction was measured by the slope of the recorded tracings. NADPH concentration in the reaction medium was measured spectrophotometrically, at 340 nm, at 30°C. GR diaphorase activity was measured by the rate of DCI reduction, using NADPH as electron donor.[10] The reaction medium for this assay contained 0.2 mM NADPH, 40 μM DCI, 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, and GR as indicated under Results. DCI reduction was measured spectrophotometrically at 600 nm ($\epsilon = 19\ mM^{-1} \cdot cm^{-1}$). Spectrophotometric measurements were performed using a Perkin-Elmer 550S UV/VIS spectrophotometer, at 30°C.

GR Inactivation

Unless stated otherwise, the standard inactivation medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 0.25 μM GR, 25 μM $CuSO_4$, 3.0 mM H_2O_2 and additions or omissions as indicated under Results; total volume, 0.2 ml. The inactivation medium was incubated in a test-tube at 30°C for the time period indicated under Results. Twenty μl duplicate samples were added to the assay medium and the residual GR or diaphorase activities were measured as described above. Control samples without free-radical generator were incubated simultaneously. Radical-cations used for GR inactivation and thiyl radical production were produced using the ABTS/HRP/H_2O_2 system,[11,12] as described under Results.

Assay of Benzoate Hydroxylation

Benzoate hydroxylation[13] was monitored by incubating solutions (2.0 ml) containing 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 2.0 mM Na-benzoate and additions, for the time indicated under Results, at 30°C. After incubation, fluorescence was measured using an Aminco-Bowman or an SLM Aminco 8000 spectrofluorometer, set at 310 nm (excitation) and 405 nm (emission).

Fluorescence Measurements

The fluorescence of modified GR Tyr residues and the intrinsic fluorescence of GR Trp residues, were used to monitor the changes resulting from oxidative damage. Measurements were performed in an SLM Aminco 8000 spectrofluorometer. Samples containing 50 mM H_2PO_4–K_2HPO_4, pH 7.4, 0.5 μM GR (subunit), 25 μM $CuSO_4$ and/or 3.0 mM H_2O_2 and additions as indicated under Results; total volume, 2.0 ml, in a 1.0 cm-path cuvette. Samples were excited at 325 nm (dityrosine) or 295 nm (Trp) and emission was measured at 410 nm (dityrosine) or 345 nm (Trp)[14,15] with a 5.0 nm bandpass. Measurements were performed at the times indicated under Results. The modification of Tyr residues was estimated by comparing the fluorescence of modified GR, with that of free Tyr (13.5 μM) supplemented with Cu(II)/H_2O_2 (25 μM/3.0 mM), in the presence or absence of GR.

Expression of Results

GR specific activity is expressed in μmol NADPH oxidized/min per mg protein. For the sake of brevity, in most cases relative activity values (100% for the control sample) are presented. Protection (*P*) against inhibitors was calculated from the equation $P(\%) = 100(i(\%) - ip(\%)/i(\%)$ where *P*, *i* and *ip* are the protector relative activity, the inhibition of GR activity by the free-radical generator, and GR inhibition by the same system plus protector, respectively. Negative P values represent enhancement of GR inactivation. Average values for duplicate measurements deviated from the experimental values by less than 5%. When more than two measurements were performed, the values presented are average ± S.D. Protection values lower than 10% were considered non-significant, because of the propa-

gation of experimental error to the calculated P values. Statistical analysis was performed using Student's t-test for paired values.

RESULTS

Effects of Fenton and Related Systems

Incubation of GR with the Fenton systems assayed caused significant diminution of the enzyme NADPH-GSSG reductase activity. Figure 1 shows the time-course of GR inactivation by Cu(II)/H_2O_2 and Cu(II)/Asc. It is to be seen that the effect of the former system was relatively slow and monophasic, the 50% activity decay being reached after 22 min of incubation. The Cu(II)/Asc effect involved an initial fast inactivation phase, during the first five min of incubation, followed by a slow decrease of activity during the subsequent 5–30 min period. At variance with these results, treatment of GR with Cu(II)/H_2O_2 increased the enzyme diaphorase activity (Table I).

Figure 2 shows the effect of Cu(II) concentration on GR inactivation by the Cu(II)/H_2O_2 system. The inactivation values (I) plotted against

TABLE I Effect of the Cu(II)/H_2O_2 system on GR diaphorase activity

Additions	Diaphorase activity (μmol DCI/min• mg protein)
None	0.70 ± 0.11
Cu(II)/H_2O_2	6.69 ± 0.57*
Cu(II)	5.21 ± 0.16*
H_2O_2	0.88 ± 0.17

GR inactivation medium contained 50 mM KH_2PO_4–K_2HPO_4 pH 7.4, 0.25 μM GR, 25 μM $CuSO_4$ and 3.0 mM H_2O_2, as indicated above. Incubation time, 30 min. Diaphorase activity was measured as described under Materials and Methods. Values represent the average ± S.D. (n = 3). * $P < 0.001$ against control sample.

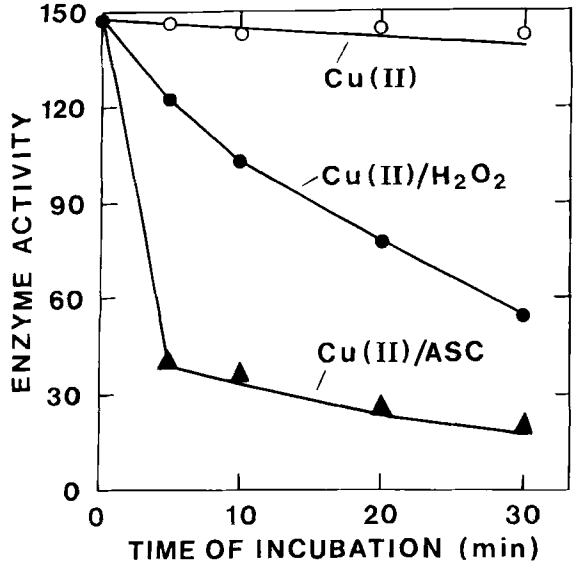

FIGURE 1 Effect of incubation time on GR inactivation by Cu(II)/H_2O_2 or Cu(II)/Asc. The inactivation medium contained 50 mM KH_2PO_4–K_2HPO_4, 0.25 μM GR, 25 μM $CuSO_4$, and 3.0 mM H_2O_2 (or 0.5 mM Asc), as indicated in the figure. Time of incubation was as indicated in the abscissa. Other experimental conditions were as described under Materials and Methods. Enzyme activity values are expressed in μmol NADPH oxidized/min per mg of protein.

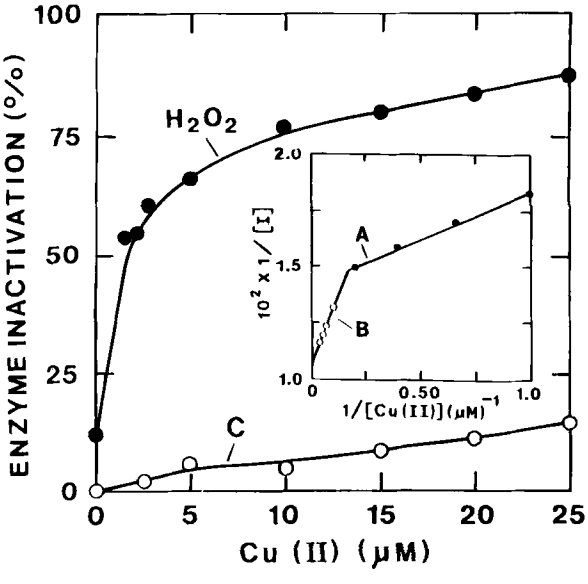

FIGURE 2 Effect of Cu(II) concentration on GR inactivation by Cu(II)/H_2O_2. Inactivation medium was as in Figure 1 legend, except $CuSO_4$, the concentration of which was as indicated on the abscissa. H_2O_2, assay samples; C, control samples (H_2O_2 omitted). Incubation time, 60 min. Other experimental conditions were described under Materials and Methods. Inset: double-reciprocal plot of GR inactivation (I (%)) against Cu(II) concentration values. Slope value ± S.D.: A, 0.42 ± 0.05; B, 2.50 ± 0.18.

Cu(II) concentration yielded a hyperbolic curve, the half-maximum value corresponding to about 1.7 μM Cu(II). The double reciprocal plot of the same values yielded two straight lines (A and B) with different slopes and intercepts (Figure 2, inset), thus showing a complex kinetics.

Figure 3 shows the effect of H_2O_2 concentration on Cu(II)/H_2O_2 action. No saturation kinetics was observed and a double reciprocal plot of the same values yielded a straight line (not shown) that intersected the ordinate-axis at the origin.

Table II shows that copper-chelators[16,17] prevented GR inactivation by Cu(II)/H_2O_2 when chelators were added to GR before the oxygen radical generating system. EDTA and DETAPAC counteracted Cu(II)/H_2O_2 action to a significant degree, especially at the 250 μM concentration. At a concentration equivalent to Cu(II) concentration (25 μM), EDTA and DETAPAC were still effective antioxidants. When chelators were added after GR incubation with Cu(II)/H_2O_2, the enzyme activity was not restored (Table II). The effect of L-histidine was examined in relation to its action on HO$^\bullet$ production by Cu(II)/H_2O_2. The results in Table III show that at L-histidine/Cu(II) molar ratios of 0.5 or 1.0, L-histidine enhanced both GR inactivation and HO$^\bullet$ production, but at greater molar ratios, the opposite effects were observed.

Modification of GR Tyr residues by Cu(II)/H_2O_2 treatment was determined by the appearance of dityrosine fluorescence.[14] Figure 4 shows that fluorescence increased as a function of incubation time. A similar increase was observed with the free L-tyrosine, although in that case a slow final decay of fluorescence was noted. At variance with these results, the Cu(II)/H_2O_2 treat-

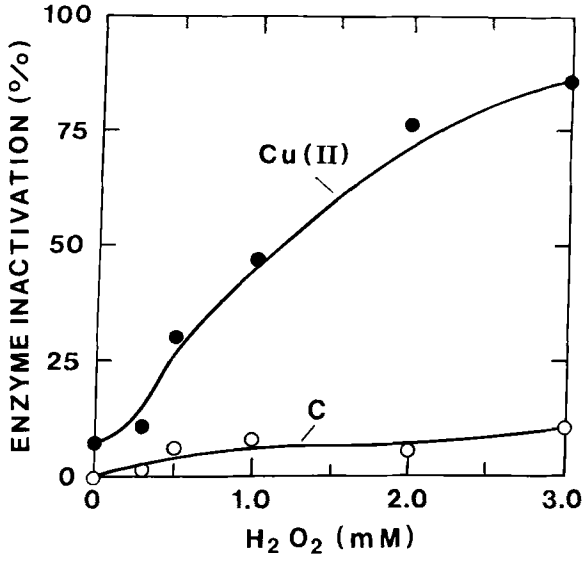

FIGURE 3 Effect of H_2O_2 concentration on GR inactivation by Cu(II)/H_2O_2. Inactivation medium was as in Figure 1 legend, except H_2O_2, the concentration of which is indicated on the abscissa. Cu(II), assay samples; C, control samples ($CuSO_4$ omitted). Incubation time, 60 min. Other experimental conditions were as described under Materials and Methods.

TABLE II Effect of Cu(II) chelators on GR inactivation by Cu(II)/H_2O_2

Chelator (μM)	GR protection (%)	
	Exp. A	Exp. B
EDTA (25)	70	0
EDTA (250)	90	0
DETAPAC (25)	59	1
DETAPAC (250)	88	3

GR inactivation medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 0.25 μM GR, 25 μM $CuSO_4$, 3.0 mM H_2O_2 (standard medium), and chelator as indicated above; incubation time, 30 min. The chelator was added before (exp. A) or after (B) incubation of GR with the Cu(II)/H_2O_2 system. Protection values (%) were calculated as described under Materials and Methods. GR inactivation by Cu(II)/H_2O_2, 69%. Values represent the average of duplicate measurements.

TABLE III Effect of L-histidine on GR inactivation and hydroxyl radical production by Cu(II)/H_2O_2

L-Histidine (μM)	GR inactivation (%)	HO$^\bullet$ production (Fluorescence units)
None	71 ± 2.1	35 ± 0.8
12.5	89 ± 2.1* (–25)	49 ± 2.1*
25	93 ± 2.1* (–31)	52 ± 1.7*
50	61 ± 3.7* (14)	47 ± 1.6*
100	32 ± 4.6* (55)	14 ± 0.5*
250	23 ± 1.0 (67)	5 ± 0.8*

Exp. A: GR inactivation medium as in Table II legend (standard medium); L-histidine, as indicated above. Exp. B: The reaction medium contained 2.0 mM Na benzoate and additions as in Exp. A. Incubation time, 30 min. Other experimental conditions were as described under Materials and Methods. Values represent average ± S.D. (n = 3); in parenthesis, GR protection (P (%)). * $P < 0.01$.

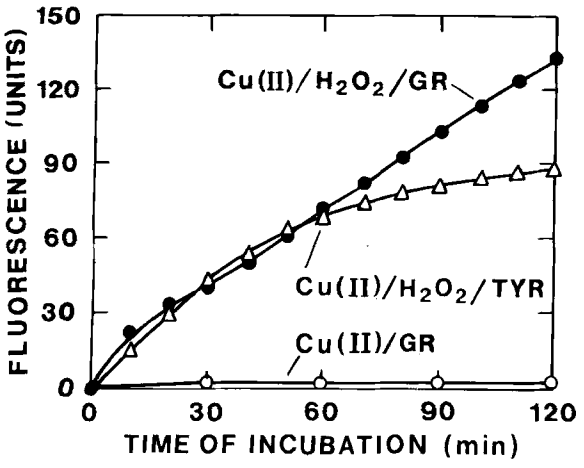

FIGURE 4 Effect of Cu(II)/H_2O_2 on GR tyrosinyl residues. The reaction medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 1.5 μM GR (or 13.5 μM Tyr (TYR)); 25 μM $CuSO_4$ (Cu(II)) and 3.0 mM H_2O_2, as indicated in the figure. Time of incubation was as indicated on the abscissa. Other experimental conditions were described under Materials and Methods.

ment significantly decreased the enzyme Trp fluorescence (Figure 5). Incubation of GR with Cu(II) or H_2O_2 alone scarcely modified GR fluorescence and therefore the observed effects

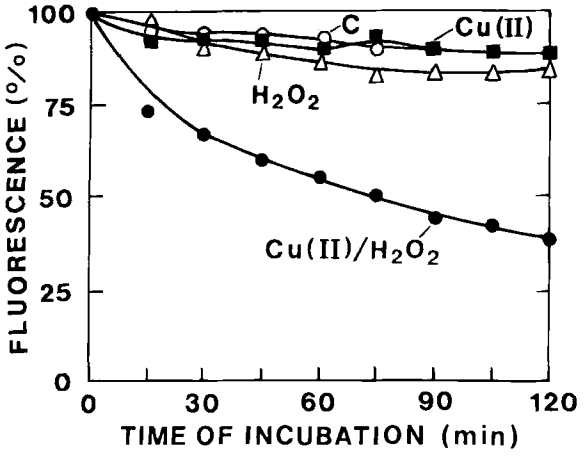

FIGURE 5 Effect of Cu(II)/H_2O_2 on GR tryptophanyl residues. The reaction medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 0.5 μM GR; 25 μM $CuSO_4$ and 3.0 mM H_2O_2; additions as indicated in the figure. Values represent relative fluorescence intensity at the indicated time of incubation, as compared with the initial value (100%). The decrease of Trp fluorescence immediately after adding $CuSO_4$ or Cu(II)/H_2O_2 was subtracted from the subsequent values. Other conditions were as described under Materials and Methods. C, control sample (additions omitted).

implied reactive oxygen species generated by the Cu(II)/H_2O_2 system.

Table IV shows the effect of several antioxidants on GR inactivation by the Cu(II)/H_2O_2 and Cu(II)/Asc systems. It is to be seen that catalase protected GR against both systems. With Cu(II)/H_2O_2, catalase protection was readily understood, considering the role of added H_2O_2 as precursor of HO•. As regards Cu(II)/Asc effect, GR protection by catalase supported an essential role of endogenously generated H_2O_2 in GR inactivation. The specificity of catalase effect was borne out by the lesser action of the denatured enzyme, especially with Cu(II)/Asc. At variance with the latter results, SOD did not prevent GR inactivation but bovine serum albumin protected GR, especially against Cu(II)/H_2O_2. This latter protection would depend on Cu(II) binding by albumin.[18,19] The HO• scavengers ethanol and sodium benzoate protected GR against Cu(II)/H_2O_2 but not against Cu(II)/Asc. The differences noted would depend on where HO• radicals were generated, namely, in the "bulk-phase" (with Cu(II)/H_2O_2) or at GR active site (with Cu(II)/Asc).

Copper ions oxidize NAD(P)H producing HO• radicals and/or other reactive oxygen species.[20,21]

TABLE IV Effect of catalase, SOD and HO• scavengers on GR inactivation by Cu(II)/H_2O_2 and Cu(II)/Asc

Additions	GR inactivation (%)	
	Cu(II)/H_2O_2	Cu(II)/Asc
None	30	75
Catalase native (40 μg/ml)	5 (82)	17 (78)
Catalase denatured (40 μg/ml)	23 (23)	73 (3)
SOD (80 μg/ml)	34 (–13)	67 (11)
Bovine serum albumin (40 μg/ml)	7 (76)	49 (34)
Ethanol (6.0 mM)	22 (26)	71 (5)
Na-benzoate (200 mM)	10 (67)	68 (9)
Mannitol (300 mM)	29 (3)	72 (4)

GR inactivation medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 0.25 μM GR, 25 μM $CuSO_4$ and 3.0 mM H_2O_2 or 0.5 mM ascorbate; additions as indicated above. Incubation time, 10 min. Catalase was denatured by heating at 100°C and was 100% inactive. Other experimental conditions were as described under Materials and Methods. Values represent the average of duplicate measurements; in parenthesis, protection of GR activity (P (%)).

Accordingly, the Cu(II)/NAD(P)H treatment produced GR inactivation (Table V). NADH and NADPH effects were similar and the omission of Cu(II) prevented GR modification to a significant degree. The copper chelators EDTA, DETAPAC and L-histidine prevented GR inactivation by the Cu(II)/NAD(P)H systems and protection was also provided by SOD. Measurement of $HO^{\bullet}$ production using the Cu(II)/NADPH system yielded values significantly greater than with Cu(II) or NADPH alone (Table VI). Interestingly enough, GR was necessary for increasing $HO^{\bullet}$ production above the control levels, thus suggesting the role of a GR-Cu catalytically competent complex.

CAs stimulated GR inactivation by Cu(II)/H_2O_2, in close agreement with CA effects on dihydrolipoamide dehydrogenase.[8] Table VII shows that epinephrine, norepinephrine, dopamine and L-DOPA enhanced several-fold the effect of Cu(II)/H_2O_2. GR inactivation by the Cu(II)/H_2O_2/CA systems was relatively fast, as compared with the sluggish kinetics of GR inactivation by Cu(II)/H_2O_2. Therefore, shorter inactivation periods were necessary in order to obtain the effects described. Omission of H_2O_2 reduced GR inactivation to the small CA specific action whereas omission of CA and H_2O_2 allowed conservation of GR initial activity (Table VII). In close agreement with GR inactivation, CAs enhanced $HO^{\bullet}$ production by the Cu(II)/H_2O_2 system (Table VII).

TABLE V Effect of Cu(II) chelators and SOD on GR inactivation by the Cu(II)/NAD(P)H systems

Cu(II) (μM)	Additions (μM)	GR inactivation (%)
5.0	NADPH (100)	82
	NADPH (50)	75
	NADPH (50) + SOD (40 μg/ml)	55 (27)
	NADH (100)	84
	NADH (50)	74
	NADH (50) + SOD (40 μg/ml)	49 (36)
	None	0
25	NADPH (100)	93
	NADPH (100) + DETAPAC (50)	–3.0 (103)
	NADPH (100) + EDTA (50)	–5.0 (105)
	NADPH (100) + L-histidine(250)	56 (40)
0	NADPH (100)	13
	NADH (100)	15
	NADPH (50)	7

GR inactivation medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 0.25 μM GR, $CuSO_4$ and additions as indicated above; incubation time, 10 min. Other experimental conditions were as described under Materials and Methods. Values represent the average of duplicate measurements; in parenthesis, protection of GR activity (P (%)).

TABLE VI Hydroxyl radical production by the Cu(II)/NADPH system

Addition	$HO^{\bullet}$ production (Fluorescence units)
Cu(II)	0
Cu(II)/GR	1.0 ± 1.0
NADPH	13 ± 0.5*
NADPH + GR	10 ± 0.9*
NADPH + Cu(II)	13 ± 0.6*
NADPH + Cu(II) + GR	33 ± 2.0*

The reaction medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, and 2.0 mM Na benzoate. 0.1 mM NADPH, 25 μM Cu(II) and 0.25 μM GR, as indicated above. Incubation time, 30 min. Other experimental conditions were as described under Materials and Methods. Values represent the average ± S.D. (n = 6). *$P < 0.05$.

TABLE VII Effect of CAs on GR inactivation (A) and hydroxyl radical production (B) by Cu(II)/H_2O_2

CA (25 μM)	Exp. A: GR inactivation (%)		Exp. B: Fluorescence (units)	
	Cu(II)/H_2O_2	Cu(II)	Cu(II)/H_2O_2	Cu(II)
None	18	0	40	0
Epinephrine	83	16	99	33
Norepinephrine	82	10	183	15
Dopamine	83	13	133	10
L-DOPA	78	14	123	67

Exp. A: GR inactivation medium as in Table II legend (standard medium) and CA as indicated above. Incubation time, 5 min. Exp. B: The reaction medium contained 2.0 mM Na benzoate and additions as in Exp. A. Other experimental conditions were as described under Materials and Methods. Incubation time, 1 h. Values represent the average of duplicate measurements.

Effect of Thiol Compounds

Thiol compounds enhanced GR inactivation by the Cu(II)/H_2O_2 system, their action depending on thiol structure and incubation time. Figure 6 shows the effects of GSH and Cys. It is to be seen

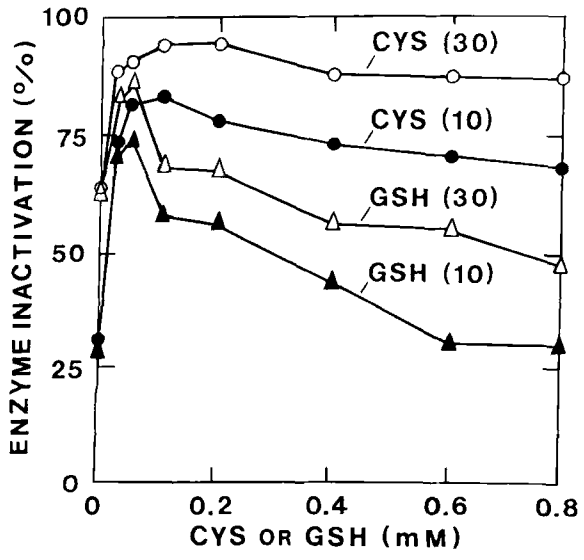

FIGURE 6 Effect of Cys and GSH on GR inactivation by Cu(II)/H_2O_2 (standard medium). The inactivation medium was as indicated in Figure 1 legend (Cu(II)/H_2O_2). Cys and GSH concentrations were as indicated on the abscissa. Incubation time (min) as indicated by the numbers in parenthesis. Other experimental conditions were as described under Materials and Methods. Values represent the average of 6 or more measurements. S.D. values (not shown) were < 5% of the average value.

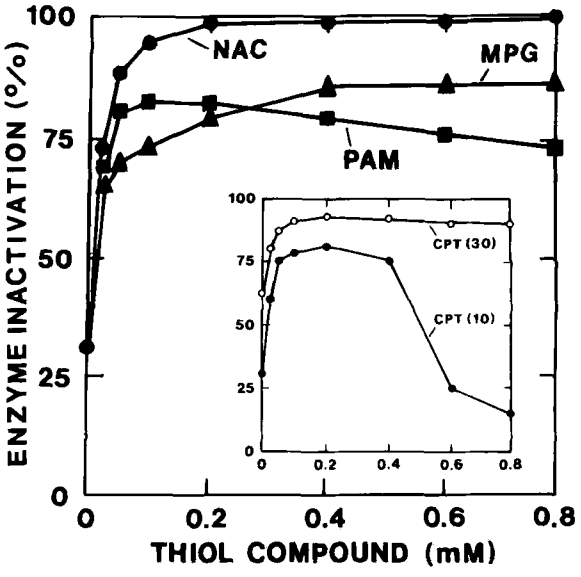

FIGURE 7. Effect of NAC, MPG and PAM on GR inactivation by Cu(II)/H_2O_2. Experimental conditions were as in Figure 6 legend, except thiol compounds. Incubation time, 10 min. Inset: effect of CPT on GR inactivation by Cu(II)/H_2O_2. Experimental conditions as above, except thiol compounds; incubation time as indicated by the number in parenthesis.

that the GSH-supplemented system was more effective than the control one (Cu(II)/H_2O_2), especially at GSH concentrations in the 25–200 μM range. Increasing GSH concentration above the latter values produced relatively less enzyme inactivation and with 800 μM GSH (10 min incubated sample), the Cu(II)/H_2O_2/GSH- and Cu(II)/H_2O_2-treated GR samples were equally affected. More extensive pro-oxidant effects of high GSH concentrations were observed after 30 min incubation. At variance with GSH effects, increase of Cys concentration in the 400–800 μM range did not decrease GR inactivation by the Cu(II)/H_2O_2/Cys system (Figure 6). Figure 7 shows that NAC, MPG and PAM behaved essentially like Cys. The thiol compound CPT (Figure 7, inset) produced (a) enhancement of the Cu(II)/H_2O_2 effect at all CPT concentrations (30 min incubated samples); (b) enhancement of the Cu(II)/H_2O_2 effect at 0.1–0.4 mM CPT concentrations and less inactivation or enzyme protection at 0.6 and 0.8 mM CPT concentrations (10 min incubated samples).

Table VIII shows the capability of the Cu(II)/H_2O_2/RSH systems for generating HO$^\bullet$ radicals. Allowance being made for GSH, HO$^\bullet$ values correlated qualitatively with the modification of GR

TABLE VIII Effect of thiol compounds on hydroxyl radical production by the Cu(II)/H_2O_2/RSH and Cu(II)/RSH systems

RSH (μM)	Fluorescence (units)	
	Cu(II)H_2O_2/RSH	Cu(II)/RSH
None	40 ± 4.8	0
Cys (200)	85 ± 1.7*	5
NAC (100)	140 ± 3.7*	4
PAM (200)	134 ± 1.4*	6
MPG (200)	73 ± 1.3*	0
CPT (100)	133 ± 6.6*	3
GSH (25)	59 ± 1.2	1
GSH (50)	49 ± 1.7	2
GSH (200)	7.0 ± 0.5*	0

Reaction medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 25 μM $CuSO_4$, 3.0 mM H_2O_2, 2.0 mM Na-benzoate and RSH, as indicated above. Incubation time, 60 min (Cu(II)/H_2O_2/RSH) or 30 min (Cu(II)/RSH). Other experimental conditions were as described under Materials and Methods. Cu(II)/H_2O_2/RSH values represent the average ± S.D. (n = 3). * $p < 0.01$. Cu(II)/RSH values represent the average of duplicate measurements.

activity (Figures 6 and 7). GSH (25 and 50 μM) enhanced the production of HO$^\bullet$ radicals to a slight degree but at 200 μM or higher concentrations, GSH inhibited HO$^\bullet$ generation (Table VIII). Omission of H_2O_2 reduced by more than 80% the action of the thiol-containing systems on both HO$^\bullet$ production (Table VIII) and GR inactivation (experimental data not shown). EDTA (50 μM) and DETAPAC (25 μM) prevented by 97 and 59%, respectively, GR inactivation by the Cu(II)/H_2O_2/Cys system and similar results were obtained with the Cu(II)/H_2O_2/PAM system (experimental data not shown).

Dithiol and disulfide compounds were also assayed for their action on GR inactivation by Cu(II)/H_2O_2. Figure 8 shows the effect of DHLA after different incubation times. These results and the data in Table IX indicate that DHLA and DTT enhanced GR inactivation by the Cu(II)/H_2O_2 system, especially after 5 or 10 min incubation. At variance with these observations, GSSG and TS_2 proved to be effective protectors of GR (Table IX). GSSG also protected GR against

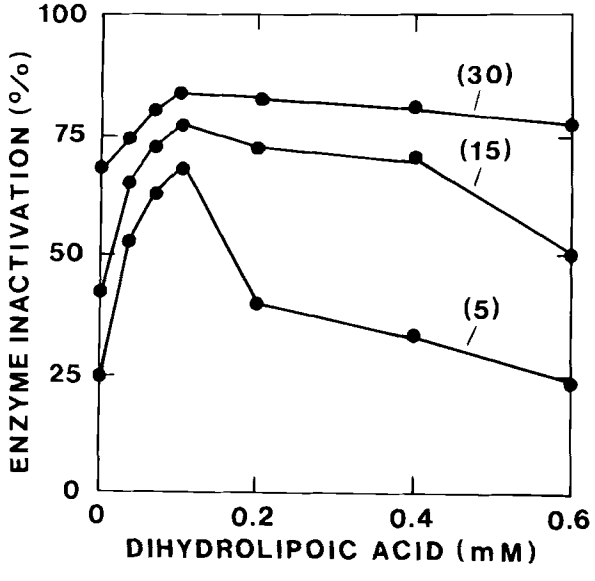

FIGURE 8 Effect of DHLA on GR inactivation by Cu(II)/H_2O_2. Experimental conditions were as described in Figure 6 legend, except thiol compound. DHLA concentration was as indicated on the abscissa and incubation time (min) was as indicated by the numbers in parenthesis.

TABLE IX Effect of dithiol and disulfide compounds on GR inactivation by Cu(II)/H_2O_2

Addition (μM)	GR protection (%)	
	Incubation: 10 min	Incubation: 30 min
DTT (25)	–50	–20
DTT (200)	–57	–32
GSSG (250)	96	81
TS_2 (50)	89	71
TS_2 (100)	100	80

GR inactivation medium as in Table II legend (standard medium) and additions as indicated above. Other experimental conditions were as described under Materials and Methods. GR inactivation by Cu(II)/H_2O_2 (%): 28 and 59, after 10 and 30 min incubation, respectively. Values represent the average of duplicate measurements.

the NADPH/Cu(II) system (Figure 9), in contrast with GSH, which was much less active.

Production of thiyl radicals by the ABTS/HRP/H_2O_2 system (Reaction 2)[22, 23] allowed us to investigate a possible role of these radicals in GR inactivation. ABTS$^{\bullet+}$ inactivated GR as a

$$ABTS^{\bullet+} + RSH \longrightarrow ABTS + RS^\bullet + H^+ \quad (2)$$

function of incubation time and ABTS concentration (Figure 10). Table X shows the effect of thiol compounds, GSSG and TS_2 under the same experimental conditions, but at fixed incubation time and ABTS concentration. It is to be seen that the thiols assayed were GR protectors, especially PAM and CPT, whereas GSSG and trypanothione were not so.

DISCUSSION

In close agreement with the properties of dihydrolipoamide dehydrogenase,[6,7] GR was irreversibly inhibited by several HO$^\bullet$ generating systems, namely, Cu(II)/H_2O_2 (Figures 1–3; Table III), Cu(II)/Asc (Figure 1 and Table IV), Cu(II)/NAD(P)H (Table V and Figure 9); Cu(II)/H_2O_2/CAs (Table VII) and Cu(II)/H_2O_2/RSH (Figures 6–8). Among the Fenton systems assayed, Cu(II)/H_2O_2 was an effective one and its

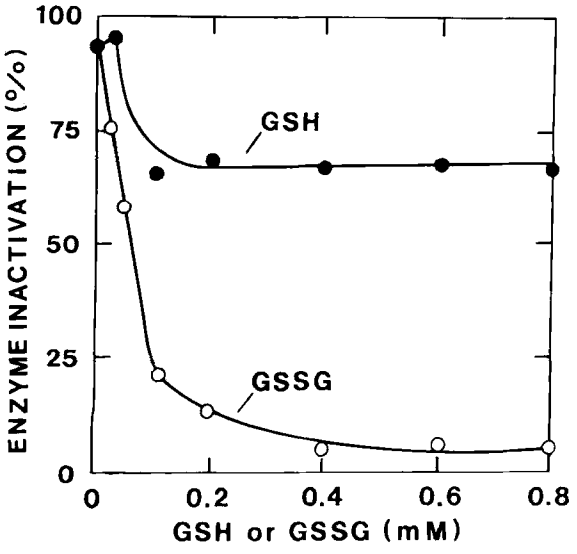

FIGURE 9 Effect of GSH and GSSG on GR inactivation by NADPH/Cu(II). The inactivation medium contained KH_2PO_4–K_2HPO_4, pH 7.4, 0.25 μM GR, 25 μM $CuSO_4$ and 0.1 mM NADPH. GSH or GSSG, as indicated on the abscissa. Incubation time, 10 min. Other experimental conditions were as described under Materials and Methods.

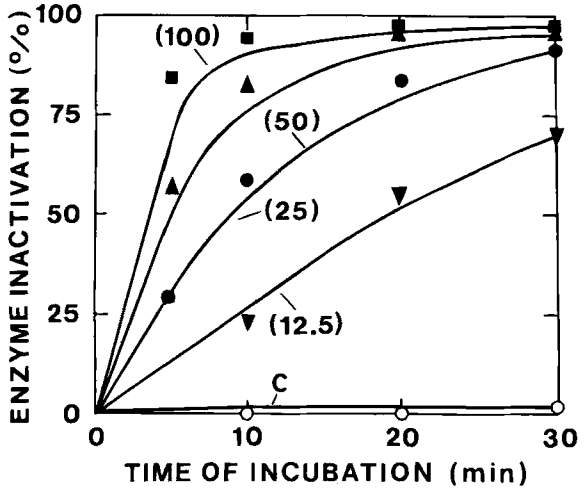

FIGURE 10 GR inactivation by ABTS/HRP/H_2O_2. The inactivation medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 0.25 μM GR, 5.0 U/ml HRP, 100 μM H_2O_2 and ABTS whose concentration was (μM) as indicated by the numbers in parenthesis. Time of incubation was as indicated on the abscissa. Other experimental conditions were as described under Materials and methods. C, control sample, with 100 μM ABTS and 100 μM H_2O_2 (HRP omitted).

TABLE X Effect of thiol compounds on GR inactivation by the ABTS/HRP/H_2O_2 system

Addition (0.2 mM)	GR inactivation (%)	
	Incubation: 10 min	Incubation: 20 min
None	88	92
GSH	12 (86)	14 (85)
Cys	17 (81)	19 (79)
PAM	2 (98)	3 (98)
CPT	0 (100)	0 (100)
GSSG	90 (–2)	95 (–3)
TS_2	90 (–2)	97 (–5)

GR inactivation medium contained 50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 0.25 μM GR, 50 μM ABTS, 100 μM H_2O_2, 5.0 U/ml HRPI; additions and incubation time, as indicated above. Other conditions were as described under Materials and Methods. Values represent the average of duplicate measurements; in parenthesis GR protection (P (%)).

action deserved especial investigation because that system may operate in yeasts,[9] organisms which are sensitive to oxidative stress.[24] The sluggish kinetics of GR inactivation by Cu(II)/H_2O_2 (Figure 1), in all probability reflected the slow rate[25] of Cu(II) reduction by H_2O_2.

Cu(II) was essential for GR inactivation by Cu(II)/H_2O_2 and related systems, as demonstrated by (a) the effect of Cu(II) concentration (Figure 2) and (b) the action of copper chelators (Tables II, III, and V). Cu(II) reduction to Cu(I) (Reaction 3–8) would be the first step of HO• generation by the systems assayed

$$Cu(II) + H_2O_2 \longrightarrow Cu(I) + HO_2^{\bullet} + H^+ \quad (3)$$

$$Cu(II) + HO_2^{\bullet} \longrightarrow Cu(I) + O_2 + H^+ \quad (4)$$

$$Cu(II) + AH^- \longrightarrow CuAH^+ \longrightarrow Cu(I) + A^{\bullet} + H^+ \quad (5)$$

$$Cu(II) + NAD(P)H \longrightarrow Cu(I) + NAD(P)^{\bullet} + H^+ \quad (6)$$

$$Cu(II) + QH_2 \longrightarrow Cu(I) + QH^{\bullet} + H^+ \quad (7)$$

$$Cu(II) + RSH \longrightarrow Cu(I) + RS^{\bullet} + H^+ \quad (8)$$

(in these reactions, AH^-, $A^{\bullet}$, QH_2, $QH^{\bullet}$ and $RS^{\bullet}$ represent the ascorbate anion, the ascorbyl radical, CA *o*-quinol, CA semi-quinone and thiyl rad-

ical respectively). Evidence for the operation of these reactions is given in References.[8,20,21,25–30] The kinetics of Cu(II) effect (Figure 2, inset) suggested at least two Cu-GR interaction sites, with different affinities for Cu(II). His containing peptides complex Cu(II)[19,31,32] and these complexes catalyse the Haber-Weiss reaction (Reaction 9). The Cu-dependent catalysis could involve either exogenous (added) H_2O_2, or active site generated H_2O_2, as the result of Reactions 10 and 11. These

$$Cu(I) + H_2O_2 \longrightarrow Cu(II) + HO^{\bullet} + HO^{-} \quad (9)$$

$$Cu(I) + O_2 \longrightarrow Cu(II) + O_2^{-} \quad (10)$$

$$O_2^{-} + O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad (11)$$

latter reactions would occur with the Cu(II)/NAD(P)H systems, as suggested by the effect of SOD (Table V). With added H_2O_2, the inhibition of endogenous H_2O_2 production by SOD would not be apparent (Table IV). Production of $HO^{\bullet}$ at GR active site would be a "caged" process.[17] The weak action of free radical scavengers on GR inactivation by Cu(II)/Asc (Table IV) is characteristic of "caged" radical production.[17]

The role of H_2O_2 for GR inactivation was supported by (a) the effect of H_2O_2 concentration (Figure 3); (b) the greater effect of the Cu(II)/H_2O_2/CA systems, as compared with the Cu(II)/CAs counterparts (Table VII); (c) the greatest production of $HO^{\bullet}$ radicals by the Cu(II)/H_2O_2/RSH systems, as compared with their Cu(II)/RSH counterparts (Table VIII) and (d) the action of catalase (Table IV). Moreover, with Cu(II)/Asc, production of H_2O_2 by ascorbate oxidation,[16,30] might contribute to $HO^{\bullet}$ generation. Production of superoxide by the Cu(II)/NAD(P)H system[20,21] was supported by the effect of SOD as in Table V. Finally, the role of $HO^{\bullet}$ radicals in GR inactivation was confirmed by the effect of $HO^{\bullet}$ scavengers (Table IV) and the correlation of GR inactivation with $HO^{\bullet}$ production (Tables III, VI, VII, VIII).

GR inactivation by oxygen radicals was a site-specific process that would exclude the enzyme FAD domain, as indicated by the enhancement of GR diaphorase activity (Table I). The selectivity of GR oxidative damage fits in well with the modification of essential amino acid residues at GR active site, such as His467, Cys58 and Tyr114 (References 1 and 34). The pro-oxidant action of low L-histidine concentrations (Table III), as well as the acknowledged[35] sensitivity of His residues to Cu(II)/H_2O_2 attack, support a central role of the putative Cu-His467 complex in GR inactivation. His467 imidazolyl N(3) points to Cys58.[1,36] That orientation would facilitate Cys58 oxidation.[37] Moreover, Cu(II) chelation by His467, would initiate the amino acid oxidation to aspartate,[35] a modification that would interfere with the essential function of His467 during GR catalysis. Tyrosine residues, especially Tyr114, may also be oxidized by oxygen radicals generated by the Cu-complex redox-cycling, as indicated by the production of fluorescent dityrosine (Figure 4) via dimerization of tyrosyl radicals.[14] Trp was affected by the Cu(II)/H_2O_2 treatment (Figure 5), in close agreement with Cu chelation by Trp, and the subsequent amino acid oxidation.[15,38]

At variance with dihydrolipoamide dehydrogenase,[7,8] thiol compounds stimulated GR inactivation and $HO^{\bullet}$ production by the Cu(II)/H_2O_2 system (Figure 6–8; Table VIII and text). Association of Reactions 8 and 10–12, would allow generation of $HO^{\bullet}$ radicals by the Cu(II)/H_2O_2/RSH systems (Table VIII) and, accordingly, in the absence of H_2O_2, the Cu(II)/RSH systems were scarcely effective either as $HO^{\bullet}$ generators (Table VIII) or GR modifiers (experimental data omitted). Results in Figures 6–8 show that the pro-oxidant effect of thiols varied with their structure and incubation time. The biphasic effects of GSH, CPT and DHLA (Figures 6–8) would result from the combination of (a) Cu redox-cycling and subsequent oxygen radical production (Reactions 8–11);[25,39,40] (b) formation of catalytically inactive Cu(I)/RSH complexes;[41–43] (c) scavenging of $HO^{\bullet}$ and superoxide and other radicals (Reaction 12–14).[22,23,44–47] The pro-oxidant effect would

prevail at low, and the antioxidant effect at high thiol/Cu(II) molar ratios.[40] The GSH

$$RSH + O_2^- + H^+ \longrightarrow RS^\bullet + H_2O_2 \quad (12)$$

$$RSH + HO^\bullet \longrightarrow RS^\bullet + H_2O \quad (13)$$

$$RS^\bullet + RS^\bullet \longrightarrow RSSR \quad (14)$$

and DHLA copper complexes should be more stable than other thiol complexes and, accordingly, after 30 min incubation, the less stable ones would cause GR damage.[40] Cu(II) was *essential* for the pro-oxidant action of thiols since in its absence, thiols exerted antioxidant activity (Table X). This antioxidant activity did not support a direct contribution of thiyl radicals to GR inactivation, otherwise thiols would increase GR inactivation by the ABTS/HRP/H_2O_2 system. Disulfide compounds, such as GSSG and TS_2 were effective protectors of GR (Table XI) in all probability because of copper chelation.[48] In fact, in the absence of Cu(II), GSSG and TS_2 disulfide did not protect GR against free-radical damage (Table X).

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