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Activated oxygen species in the oxidation of glutathione A kinetic study

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Abstract

The generation of the superoxide ion in the oxidation of glutathione (GSH) by molecular oxygen has been demonstrated in the absence and in the presence of a catalytic amount of copper ion. An effort has been made to quantify the production of superoxide ion by an NMR method utilising Cu,Zn superoxide dismutase and to correlate it to the reaction mechanism. The oxidation process is first-order with respect to GSH and molecular oxygen, the ratio $\Delta[GSH]/\Delta[O_2]$ being 4.0 ± 0.1. The rate of superoxide ion generation is independent of the GSH concentration and increases linearly with the O_2 concentration. The addition of Cu^{2+} increases the rate of GSH oxidation and of O_2 consumption. Also in this case the oxidation process is first-order with respect to GSH and O_2, while a saturation effect with respect to Cu^{2+} is observed. A kinetic scheme involving the formation of a short-living charge-transfer complex between GSH and oxygen is proposed to explain the experimental data.

Keywords: Glutathione oxidation; Activated oxygen species; Superoxide generation; Charge-transfer complexes

1. Introduction

Organic compounds containing SH groups (RSH) have received considerable interest in the past years because of their protective action from radiation damage [1]. This protection was demonstrated to be due to their ability of transferring the hydrogen atom of the SH group to reactive organic radicals with formation of less reactive thiyl radicals [2]. These latter radicals could be generated also in the reaction between RSH and oxidising agents, in particular molecular oxygen or hydrogen peroxide. To this regard, many studies have been carried out on the oxidation of thiols and on the role of heavy metal ions to promote it [3–5]. The involvement of reactive oxygen intermediates such as the superoxide ion have been hypothesised and in some cases positive evidence has been obtained [6–9]. In particular Misra [7] has shown the involvement of superoxide in the oxidation of dithioerythrol and Saez et al. [9] have reported that autoxidising cysteine produces thiyl

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radicals. Among biological sulphydryl compounds, glutathione (GSH) deserves particular interest since it is one of the most common biological reducing agents [10]. Recently, the role of glutathione as scavenger of radicals in the cell has been re-examined taking into consideration the hypothesis that superoxide ion acts as an intracellular radical sink, since some radicals, generated in cells, transfer their unpaired electron to oxygen, via reduced glutathione, to give superoxide ion [11]. Thermodynamic appraisal of this hypothesis was obtained by the calculation of the energetics of the radical reactions occurring via glutathione [12]. However glutathione itself can generate active oxygen species and, up to now, no effort has been done to quantify the production of these species and to correlate them to the reaction mechanism.

The present paper deals with the oxidation mechanism of GSH in the presence and in the absence of copper ion. The rate of formation of the superoxide ion was provided by means of a method utilising ^{19}F NMR spectrometry. The rate constants of some elementary reactions were estimated and reported.

2. Experimental

All chemicals were of the purest grade available. Bidistilled water was used for the solutions. The concentration of heavy metal ions was kept below 100 nM by passing the solution through Chelex 100 [13], just before use. Solutions of known oxygen concentration were obtained by adding calculated amounts of a borate solution, equilibrated with oxygen at room temperature, to deareated buffer solution. The final oxygen concentration was controlled by polarography at the dropping mercury electrode (DME). The deoxygenation of solutions, when necessary, was performed under vacuum (residual pressure $\leq 10^{-5}$ Torr) by freezing–thawing cycles.

Cu,Zn superoxide dismutase (Cu,Zn SOD) was purified according to McCord and Fridovich [14]. The Cu,Zn SOD concentration was measured by the polarographic method of catalytic currents [15]. Catalase was purified from Cu,Zn SOD by passage through a Sephadex G-75 column equilibrated with 0.05 M phosphate, pH 7.3, as already described [16].

The polarographic measurements were performed by an Amel 466 apparatus, using a three-electrode cell equipped with a saturated calomel electrode (SCE) as reference. A Perkin-Elmer Lambda 17 instrument was utilised for spectrophotometry measurements. The NMR longitudinal relaxation times were measured using the inversion recovery pulse sequence [17]. The NMR spectrometer used consisted of a Bruker magnet, a home-built pulse generator system and a detection apparatus operating at 16 MHz for ^{19}F. The rate of generation of the superoxide ion was measured as described previously [18].

X band electron spin resonance (ESR) spectra were acquired by a Bruker ER 200D instrument, utilising a flat cell.

A small Warburg apparatus was used to follow the oxygen consumption.

Perspex or polyethylene vials and bottles were used for the fluoride solutions. All the measurements were carried out at 23°C, in 0.1 M borate, pH 9.2, containing 10 nM catalase. The solutions for the superoxide determination also contained 0.5 M KF and 1 μM Cu,Zn SOD.

3. Results

3.1. Polarographic and spectrophotometric characterisation of the oxidation products of GSH

In Fig. 1 a typical example of the oxidation and reduction waves of glutathione, observed at a DME,

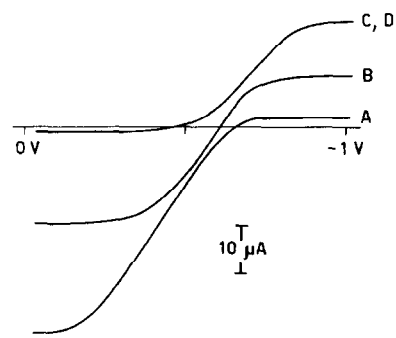


Fig. 1. Polarograms of glutathione and of its oxidation products. The experiments were carried out in 0.1 M borate buffer, pH 9.2, containing 50 mM KCl, 0.24 mM O_2 in the presence of 10 nM catalase. (A) 3.2 mM GSH, initial condition; (B) 3.2 mM GSH after 6 h; (C) 3.2 mM GSH after 72 h; (D) 1.6 mM GSSG.

is reported. From this figure it appears that the redox couple GSH–GSSG is a quasi-reversible system at the DME. The polarograms B and C in Fig. 1 were obtained when a GSH solution was left to oxidise for 6 h (curve B) and 72 h (curve C) under continuous stirring to ensure equilibration with atmospheric oxygen. To rule out the possible reaction between GSH and H_2O_2, which is produced in the oxidation of GSH itself, 10 nM catalase was added to the solution. It appeared that wave C and the GSSG wave were exactly the same (see Fig. 1).

From the UV spectra of GSH and GSSG in 0.1 M borate, pH 9.2, we calculated at 230 nm:

$$\Delta\varepsilon = \varepsilon_{GSH} - 1/2\,\varepsilon_{GSSG} = 1800 \pm 60\,M^{-1}cm^{-1}$$

where ε_{GSH} and ε_{GSSG} are the molar absorptivities of GSH and GSSG, respectively. This $\Delta\varepsilon$ value is in good agreement with that calculated from the change of absorbance at 230 nm due to complete oxidation of GSH to GSSG under conditions similar to those of Fig. 1, curve C. These results indicate that, under our experimental conditions, GSSG is the main product of the GSH oxidation by molecular oxygen. This is in accordance with Sampath and Caughey [8], who showed by NMR that GSH is converted to GSSG in air-saturated solutions.

3.2. Oxidation of GSH by molecular oxygen. Effect of metal ion impurities

The oxidation of GSH was followed by UV spectroscopy at 230 nm under the following conditions:

(i) $[GSH] \gg [O_2]$. In this case the reaction was carried out in a closed cuvette, completely filled with the solution, until the total consumption of oxygen;

(ii) $[GSH] > [O_2]$ = constant. In this case the solution was allowed to equilibrate with oxygen in a vessel where a large contact surface between the liquid and the gas phase was continuously renewed by stirring. Under condition (i) the decrease of the absorbance, due to the oxidation of GSH, was first-order with respect to O_2. From the total decrease of the absorbance, a ratio $\Delta[GSH]/[O_2]_0 = 4.00 \pm 0.12$ was calculated, where $\Delta[GSH]$ is the glutathione oxidised and $[O_2]_0$ is the initial concentration of molecular oxygen in the solution. Under condition (ii), the initial rate of GSH oxidation and of O_2 consumption were measured by the decrease of the

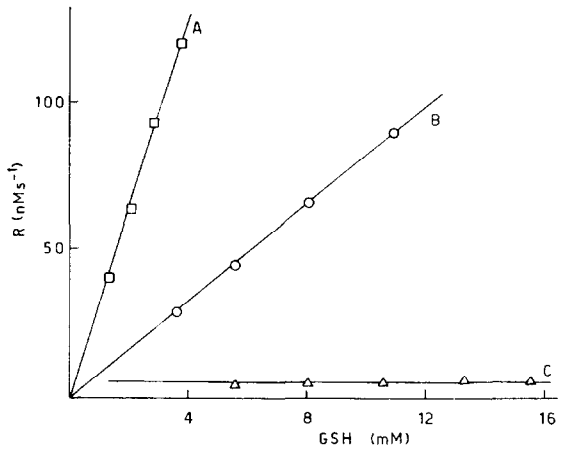


Fig. 2. Rates of GSH oxidation, O_2 consumption and superoxide ion generation as a function of GSH concentration. The GSH oxidation (curve A) was followed by the decrease of the absorbance at 230 nm. A Warburg apparatus was utilised to measure the O_2 consumption (curve B). The superoxide formation (curve C) was measured by ^{19}F NMR. The experiments were carried out in 0.1 M borate buffer, pH 9.2, containing 0.5 M KF, 0.14 mM O_2 in the presence of 10 nM catalase and 1 μM Cu,Zn SOD.

absorbance at 230 nm and by the Warburg apparatus, respectively. Since a lag time of many seconds was observed, the initial rates were calculated from the linear part of the curve, after the lag time. The oxidation process was first-order with respect to GSH and O_2, in the concentration range 1.5–15.0 mM GSH and 0.03–1.00 mM O_2, respectively. In Fig. 2 we reported the initial rate of GSH and O_2 consumption, calculated from the absorbance at 230 nm and from the P_{O_2}, as a function of GSH concentration (curve A and B, respectively). A linear dependence of the initial rate on GSH concentration was observed and the ratio of the slopes of the two straight lines gave $\Delta[GSH]/\Delta[O_2] = 3.92 + 0.11$. According to these results the overall rate law is:

$$\frac{d[GSSG]}{dt} = -\frac{1}{2}\frac{d[GSH]}{dt} = -\frac{1}{4}\frac{d[O_2]}{dt} = k_{ox}[GSH][O_2] \quad (1)$$

where k_{ox} is a second-order kinetic rate constant.

Since the presence of a trace of heavy metal ions catalyses the oxidation of GSH [3,4], the GSH oxidation rate has been measured in the presence of various components of the reaction medium and after purification of the buffer from heavy metal ions. The

Table 1
Effect of metal ion impurities and of Cu,Zn SOD on the GSH oxidation rate

Chelex [a]	EDTA (mM)	Cu,Zn SOD (μM)	k_{ox} ($M^{-1}s^{-1}$) [b]
–	–	–	0.19
–	0.20	–	0.19
–	0.20	0.2	0.21
–	0.20	2.0	0.20
+	–	–	0.09
+	–	1.5	0.11
+	–	–	0.11 [c]

The glutathione oxidation was carried out in 0.1 M borate, pH 9.2, in the presence of 10 nM catalase, under pseudo first-order conditions. The GSH and O_2 concentrations were 3.6 mM, and 0.14 mM, respectively.
[a] When indicated, the buffer solution was passed through a Chelex column.
[b] k_{ox} ($M^{-1}s^{-1}$) was calculated from the pseudo first-order kinetic constant.
[c] 1 m*M* GSSG was present in the reaction system.

results obtained are shown in Table 1. From this table it appears that the addition of EDTA has no significant effect on the oxidation rate, while the purification of the solution by Chelex halves the GSH oxidation rate. From these experiments ($n = 6$) we calculated $\Delta[GSH]/\Delta[O_2] = 4.08 \pm 0.13$. No change of oxidation rate was observed when Cu,Zn SOD was added into the reaction system in the concentration range 0.2–2 μM. A good reproducibility of the oxidation rates was achieved only when the solutions were purified by Chelex just before use.

3.3. Generation of superoxide in the oxidation of GSH

Under anaerobic conditions we found that the Cu^{2+} contained in the native Cu,Zn SOD is completely reduced to Cu^{+} by 2 mM GSH. The rate of this process, as measured by the paramagnetic contribution of the enzyme to the fluoride relaxation rate [18], was very low (the half-life time of the Cu^{2+} contained into the active site of 1 μM Cu,Zn SOD, was about 4 h in 0.1 M borate, pH 9.2, containing 0.5 M KF and 2 mM GSH). The addition of molecular oxygen to this system dramatically increased the rate of reduction of superoxide dismutase. However, in the presence of oxygen, the reduction of the Cu^{2+} contained in the enzyme occurs until half of these ions are reduced (that is $[Cu^{2+}] \approx [Cu^{+}]$), as shown by the paramagnetic contribution to the $^{19}F^{-}$ relaxation rate. This behaviour indicates the generation of superoxide ion and its reaction with Cu,Zn SOD [18]. Because of the equal rate constants of Cu^{2+} reduction by superoxide and Cu^{+} oxidation by the next [19], the rate of superoxide generation can be calculated from the rate of partial reduction of the native Cu,Zn SOD, as detected by ^{19}F NMR [18,20]. Preliminary measurements of the rate of GSH disappearance and of oxygen consumption were performed in the presence and in the absence of 0.5 M KF. No significant change in the oxidation rates was found, thus ruling out a possible perturbation by fluoride. From these results it appears that the one-electron reduction of the enzymatic Cu^{2+} by GSH is a slow reaction which goes to completion in the absence of oxygen. On the contrary under aerobic conditions GSH is oxidised by oxygen with generation of superoxide which, in turn, mediates the partial reduction of the Cu^{2+} of the enzyme.

The rate of superoxide ion generation (R_0) was found independent of the GSH concentration in the range 1.5–15 mM GSH (see curve C of Fig. 2). On the contrary, experiments performed at various concentrations of molecular oxygen showed that the rate of superoxide ion generation increases linearly with the O_2 concentration, being the slope of the straight line $1.5 \cdot 10^{-5}$ s^{-1} ($r = 0.98$).

3.4. GSH oxidation in the presence of copper ion

The addition of copper ions, as $Cu(NO_3)_2$ solution, to 0.2–8.9 mM GSH, increased the rates of GSH oxidation and of oxygen consumption. Also in the presence of copper ion, the oxidation process was first-order with respect to GSH and the ratio between the oxidised GSH and the consumed O_2, $\Delta[GSH]/\Delta[O_2]$, was 4.01 ± 0.15 (see Fig. 3), as found in the absence of copper ion. Furthermore when the added Cu^{2+} was ≥ 5 μM, we observed the disappearance of the lag time. Experiments, performed at $[GSH] \gg [O_2] \gg [Cu^{2+}]$ and $[GSH] \approx [O_2] \gg [Cu^{2+}]$ (where $[Cu^{2+}]$ indicates the concentration of the copper added to the solution as $Cu(NO_3)_2$), showed that the process is first-order

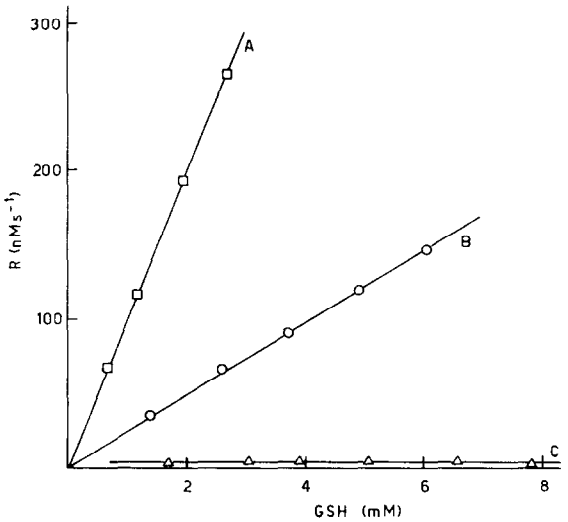


Fig. 3. Rates of GSH oxidation, O_2 consumption and superoxide generation as a function of GSH concentration, in the presence of added Cu^{2+}. Cu^{2+} (1.0 μM) was added as a solution of $Cu(NO_3)_2$ at pH 1. For figure legend and other experimental conditions, see Fig. 2.

with respect to molecular oxygen. In the presence of added Cu^{2+} the kinetic rate law of GSH disappearance is:

$$-\frac{d[GSH]}{dt} = f[GSH][O_2] \quad (2)$$

where f, the apparent second-order rate constant, is a function of the added Cu^{2+} concentration.

Measurements performed by varying the concentration of the added Cu^{2+} in the range 0.9–4.5 μM, indicated a saturation effect with respect to the copper ion (Fig. 4, curve A). In fact, the plot of $1/f$ versus $1/[Cu^{2+}]$ is a straight line (Fig. 4, curve B), being:

$$f(M^{-1}s^{-1}) = 0.10 + \frac{0.45[Cu^{2+}]}{(3 \cdot 10^{-7} + [Cu^{2+}])} \quad (3)$$

From this equation, $f = 0.10\ M^{-1}\ s^{-1}$ is obtained when $[Cu^{2+}] < 10^{-8}$ M. This limiting value of f is close to the value of the second-order rate constant

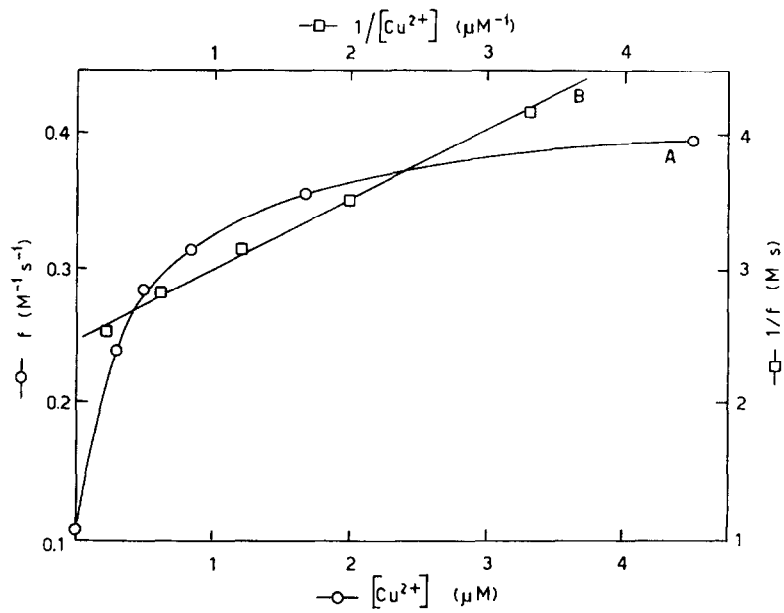


Fig. 4. Pseudo second-order kinetic rate constants (f) of the GSH oxidation as a function of the concentration of the added Cu^{2+}. f values were obtained from the ratio $k'/[GSH]$ were k' is the apparent first-order kinetic rate constant measured at $[GSH] \gg [O_2]$. (A) f versus $[Cu^{2+}]$; (B) $1/f$ versus $1/[Cu^{2+}]$, where $[Cu^{2+}]$ is the concentration of the copper ion added into the solution. The experiments were performed under conditions similar to those reported in Fig. 3; the GSH concentration was 3.3 mM.

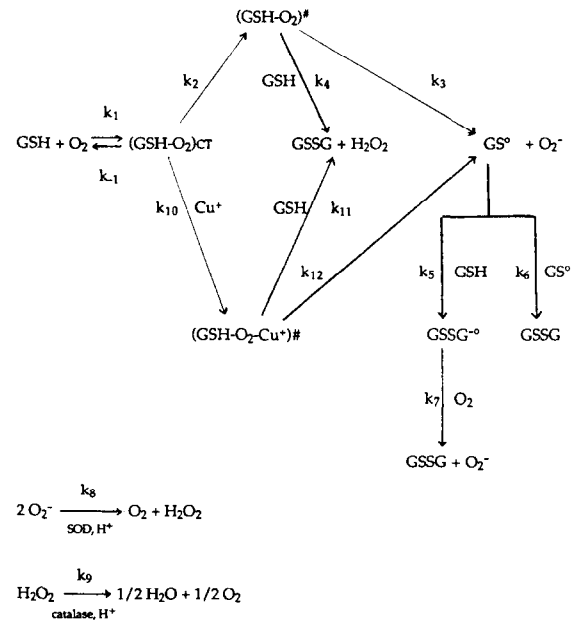
we measured for the GSH oxidation in Chelex-purified solutions (see Table 1). The superoxide generation rate in the presence of 1.0 μM $Cu(NO_3)_2$, measured in the presence of 1 μM Cu,Zn SOD and 0.5 M fluoride, was independent of the GSH concentration for [GSH] in the range 2–16 mM, see also curve C of Fig. 3, while R_0 increased from 5.0 to 9.6 nM s^{-1} raising the Cu^{2+} concentration from 0.3 to 4.5 μM.

3.5. ESR spectra of the copper ion in the presence of GSH

Room temperature ESR spectra of the Cu^{2+}, added to the reaction medium as $Cu(NO_3)_2$, were acquired to detect the oxidation state of the copper ion during the GSH oxidation. The addition of 2 mM GSH to 0.2 mM Cu^{2+} in 0.1 M borate, pH 9.2, containing 10 nM catalase led to the disappearance of the Cu^{2+} spectrum, both in the absence and in the presence of molecular oxygen in the range 0.25–1.25 mM. The disappearance of the Cu^{2+} spectrum suggests that the copper is present as Cu^{+} under our experimental conditions.

4. Discussion

The appearance of the polarographic wave of GSSG, which is stoichiometrically related to the GSH oxidised, and the ratio $\Delta[GSH]/\Delta[O_2] = 4$ within the experimental error, demonstrate that GSSG is the major end product of the oxidation of GSH in solutions containing catalase, both in the presence and in the absence of added Cu^{2+}. It should be noted that a ratio $\Delta[GSH]/\Delta[O_2]$ different from 4 is expected for oxidation products such as GSOH, GSOOH. These products have been found in the presence of H_2O_2 [21–23], which is generated during the oxidation of GSH. The generation of H_2O_2 occurs directly through the two-electron reduction of molecular oxygen or indirectly through the one-electron reduction of oxygen to superoxide ion followed by its fast dismutation into H_2O_2 and O_2. Under our experimental conditions, the accumulation of H_2O_2 was avoided by the presence of catalase, which converts hydrogen peroxide into H_2O and O_2. On the basis of our experimental results and of the most important reactions described in the literature for thiol/disulphide systems [24] and O_2/O_2^- or O_2/H_2O_2 redox couples [25], we propose the following reaction scheme:



(4)

where SOD is Cu,Zn SOD, $(GSH–O_2)_{CT}$ is a charge-transfer complex, $(GSH–O_2)^{\#}$ and $(GSH–O_2–Cu^{+})^{\#}$ are activated complexes and $k_5 \approx 10^9$ M^{-1} s^{-1}, $k_6 = 3.5 \cdot 10^9$ M^{-1} s^{-1}, $k_7 = 1.6 \cdot 10^8$ M^{-1} s^{-1}, $k_8 = 2.3 \cdot 10^9$ M^{-1} s^{-1}, $k_9 = 1.8 \cdot 10^7$ M^{-1} s^{-1} [15,16,24,26].

The formation of the short-living charge-transfer complex $(GSH–O_2)_{CT}$ is supported by the finding of Karmilov et al. [27]. These authors demonstrated that many organic compounds, in experiments carried out at high oxygen pressure, form short-living charge-transfer complexes in which molecular oxygen is the electron acceptor. In the absence of copper ion, the activated complex, $(GSH–O_2)^{\#}$, generated from the charge-transfer complex $(GSH–O_2)_{CT}$, may decompose into superoxide ion or may react with GSH leading to GSSG [see reactions 3 and 4 of Eq. (4)].

Although the formation of thioperoxyl radical, GSOO$^{\cdot}$, has been invoked [21,26], it was neglected in Eq. (4) on the basis of the ratio $\Delta[GSH]/\Delta[O_2] = 4$ we have measured and of the relatively low effi-

ciency of the interaction of molecular oxygen with thiyl radicals leading to the formation of GSOO$^{\cdot}$ radicals [22]. Also the reaction between superoxide ion and GSH was negligible, because of the presence of Cu,Zn SOD in the reaction system. Applying the steady-state hypothesis to the short-living intermediates $(GSH–O_2)^{\#}$, $GS^{\cdot}$, $GSSG^{-\cdot}$ and O_2^- and assuming that k_1, $k_{-1} \gg k_2$, that is $(GSH–O_2)_{CT}$ is in equilibrium with reagents as proposed by Karmilov et al. [27], we obtain:

$$\left[(GSH-O_2)^{\#}\right] = \frac{K_1 k_2 [GSH][O_2]}{\{k_3 + k_4[GSH]\}} \quad (5)$$

where $K_1 = k_1/k_{-1}$.Assuming $k_4[GSH] \gg k_3$, Eq. (5) becomes:

$$\left[(GSH-O_2)^{\#}\right] = \left(\frac{K_1 k_2}{k_4}\right)[O_2] \quad (6)$$

and the glutathione oxidation rate law is:

$$\frac{d[GSSG]}{dt} = K_1 k_2 [GSH][O_2] + k_7[GSSG^{-\cdot}][O_2] + k_6[GS^{\cdot}]^2 \quad (7)$$

Introducing the steady-state concentrations for $GS^{\cdot}$ and $GSSG^{-\cdot}$, according to the hypotheses reported above, the rate expression reduces to:

$$\frac{d[GSSG]}{dt} = K_1 k_2 [GSH][O_2] \quad (8)$$

which is consistent with the empirical rate equation we have found: $k_{ox} = K_1 k_2$.

On the basis of Eq. (4), the superoxide ion generation rate is:

$$R_0 = k_3\left[(GSH-O_2)^{\#}\right] + k_7[GSSG^{-\cdot}][O_2] \quad (9)$$

Introducing the steady-state hypothesis for $(GSH–O_2)^{\#}$ and $GSSG^{-\cdot}$, we obtain:

$$R_0 = 2\left(\frac{K_1 k_2 k_3}{k_4}\right)[O_2] \quad (10)$$

which is consistent with our experimental findings. From our experimental data it appears that only a fraction of O_2 is reduced to O_2^- through one-electron transfer, and this fraction is independent of the glutathione concentration. Moreover, in contrast to the oxidation of ascorbate [28], the superoxide ion is not directly involved in the oxidation of GSH, since the oxidation rate is independent of the presence of superoxide dismutase.

On the basis of the proposed mechanism, we estimated the values of some kinetic constants. In fact, from the time course of the GSH oxidation, as followed by UV spectroscopy (data not shown), and by comparing Eq. (8) to Eq. (1), in solutions containing a low concentration of metal ions (< 100 nM), we obtain: $K_1 k_2 = k_{ox} = 0.10\ M^{-1}s^{-1}$. It can be noticed that the same value of $K_1 k_2$ was calculated from the dependence of glutathione oxidation rate both on oxygen concentration and on GSH concentration. Moreover, introducing the $K_1 k_2$ value into Eq. (10) and from the R_0 dependence on the O_2 concentration, we obtained $k_3/k_4 \approx 10^{-4}$ M. These data support our hypothesis that $k_4[GSH] \gg k_3$ (under our experimental conditions).

The addition of the copper ion leads to an increase of the GSH oxidation rate, while no change in the order of the reaction with respect to GSH and O_2 was observed. The dependence of the oxidation rate of GSH on the concentration of the added Cu^{2+} is unusual, since Fig. 4 clearly shows a saturation effect with respect to the metal ion.

A catalytic mechanism, involving the alternate reduction and oxidation of the copper ion by GSH and by molecular oxygen, respectively, could be proposed:

$$Cu^{2+} + GSH \rightarrow Cu^{+} + GS^{\cdot}$$

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^-$$

However, this scheme does not satisfy the kinetic data we obtained, such as the dependence on GSH and O_2 concentration in a wide concentration range and the saturation effect with respect to the added copper ion (see Fig. 4). On the contrary, the interaction of the $(GSH–O_2)_{CT}$ charge-transfer complex with the copper ion, which leads to the formation of an activated ternary complex $(GSH–O_2–Cu^{+})^{\#}$, explains the experimental kinetic data. This complex, similarly to $(GSH–O_2)^{\#}$, can react with GSH, with the formation of GSSG, or can decompose generating the superoxide ion [see reactions 11 and 12 of Eq. (4), respectively].

According to Eq. (4), on the basis of hypotheses similar to those leading to Eqs. (5)–(10), that is

$k_{11}[GSH] \gg k_{12}$ and introducing the steady-state condition for the short-living species, the rates of oxidation of GSH and of O_2^- production in the presence of Cu^{2+}, are:

$$\frac{d[GSSG]}{dt} = \left(\frac{k_2}{k_{-1}} + \frac{k_{10}[Cu^{2+}]}{k_{-1} + k_{10}[Cu^{2+}]} \right) k_1[GSH][O_2] \quad (11)$$

and

$$R_0 = \frac{2k_1k_3[GSH][O_2]}{k_{-1} + k_{10}[Cu^{2+}]} + \left(\frac{k_1k_{10}k_{12}}{k_{11}} \right) \times \frac{[O_2][Cu^{2+}]}{k_{-1} + k_{10}[Cu^{2+}]} \quad (12)$$

respectively. These equations are in agreement with the experimental results reported in Fig. 3. Furthermore R_0 should increase with the Cu^{2+} concentration, as we have found.

According to the kinetic treatment reported by Laidler and Bunting (1973) [29], the formation of a single-intermediate, which in our case is the charge-transfer complex, explains the lag phase we have observed. In fact, according to Eq. (4) and to the single-intermediate treatment [29], the formation of GSSG is characterised by an induction period $\tau = 1/(k_4[GSH]_0)$. In the presence of Cu^{2+} the pathway leading to GSSG through a $(GSH–O_2–Cu^{2+})$ complex becomes competitive. If $[Cu^{2+}] < 5\ \mu M$, the competition between the two pathways depends on the ratio $k_2/(k_{10}[Cu^{2+}])$ and the induction period decreases as $[Cu^{2+}]$ increases. At $[Cu^{2+}] > 5\ \mu M$ the pathway occurring through the $(GSH–O_2–Cu^{2+})$ complex dominates, that means $k_2 \ll k_{10} \cdot 5 \cdot 10^{-6}$.

Under anaerobic conditions both aqueous Cu^{2+} and the enzyme Cu^{2+} are fully reduced to Cu^+ by an excess of GSH, as shown by ESR and $^{19}F^-$ relaxation rate. In either cases the radical $GS^{\cdot}$ should be generated. On thermodynamic basis these reactions are not favourite, since the $\Delta G^{0\prime}$ is of the order of $+17$ kcal mol^{-1} and $+11$ kcal mol^{-1} in the case of aqueous Cu^{2+} [30,12] and of the enzyme Cu^{2+} [31,12], respectively. However this is an example of a reaction characterised by $\Delta G^{0\prime} > 0$, which is driven by remotion of one of the reaction products. In fact, $GS^{\cdot}$ disappears by fast disproportionation to GSH and GSSG or by reaction with GSH with the formation of $GSSG^{-\cdot}$. If oxygen is present $GSSG^{-\cdot}$ is removed by an irreversible reaction with the formation of superoxide ion, according to the radical sink hypothesis [12]. On the basis of the very high value of k_7, the half life time of $GS^{\cdot}$ under physiological conditions was estimated of the order of a few μs [32].

In conclusion, the oxidation of GSH in the presence and in the absence of the added copper ion appears to be a rather complicated process involving the formation of complexes between GSH, molecular oxygen and the copper ion. In particular the formation of activated species between glutathione, molecular oxygen and Cu^{2+} ion, appears possible on the basis of our experimental data. The decomposition of these complexes is the source of the superoxide ion, being the two-electron, rather than the one-electron transfer, the main oxidation pathway of glutathione.

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