Amino Acids (1997) 13: 355–367

Amino
Acids

Novel findings on the copper catalysed oxidation of cysteine*

L. Pecci[1], **G. Montefoschi**[1], **G. Musci**[2], and **D. Cavallini**[1]

[1] Dipartimento di Scienze Biochimiche "A. Rossi Fanelli" and Centro di Biologia Molecolare del CNR, Università di Roma "La Sapienza", Roma, and
[2] Dipartimento di Chimica Organica e Biologica, Università di Messina, Messina, Italy

Accepted June 23, 1997

Summary. The oxidation of cysteine (RSH) has been studied by using O_2, ferricytochrome c (Cyt c) and nitro blue tetrazolium (NBT) as electron acceptors. The addition of 200 μM Cu^{II} to a solution of 2 mM cysteine, pH 7.4, produces an absorbance with a peak at 260 nm and a shoulder at 300 nm. Generation of a cuprous bis-cysteine complex (RS^-Cu^I-SR) is responsible for this absorbance. In the absence of O_2 the absorbance is stable for long time while in the presence of air it vanishes slowly only when the cysteine excess is consumed. The neocuproine assay and the EPR analysis show that the metal remains reduced in the course of the oxidation of cysteine returning to the oxidised form at the end of reaction when all RSH has been oxidised to RSSR. Addition of Cu^{II} enhances the reduction rate of Cyt c and of NBT by cysteine also under anaerobiosis indicating the occurrence of a direct reduction of the acceptor by the complex. It is concluded that the cuprous bis-cysteine complex (RS^-Cu^I-SR) is the catalytic species involved in the oxidation of cysteine. The novel finding of the stability of the complex together with the metal remaining in the reduced form during the oxidation suggest sulfur as the electron donor in the place of the metal ion.

Keywords: Amino acids – Cysteine – Copper catalysis – Cuprous complex

Abbreviations: RSH: cysteine; RS^-: cysteine in the thiolate form; $RS^\bullet$: thiyl radical of cysteine; RSSR: cystine; Cyt c: cytochrome c; SOD: superoxide dismutase; NBT: nitro blue tetrazolium; NBF: nitro blue formazan; DTNB: 5,5′-dithiobis-2-nitrobenzoic acid; DTPA: diethylenetriaminepentaacetic acid

Introduction

The oxidation of cysteine (RSH), glutathione and other biological thiols, catalysed by transition metal ions (essentially Cu^{II} and Fe^{III}), has been the

* Dedicated to Prof. A. Ballio on the occasion of his 75th birthday.

subject of extensive investigation in the past. Even though transition metals are present in biological material in highest percentage firmly bound in proteins or in well defined organic structures, the free metal ion catalysis of thiol oxidation stimulated interest as a representative model of biological oxidation and as a producer of sulfur, carbon and oxygen free radicals. Taking cysteine as the main representative of biological thiols, it has been shown (Cavallini et al., 1968, 1969) that the oxidation catalysed by Cu^{II} in 0.1 N NaOH proceeds with two molecules of cysteine in a complex characterised by the absorbance at 330 nm unchanged in the course of the oxidation. Another relevant point was the finding that in alkaline conditions the metal remains complexed in the cupric form, being reduced very slowly when O_2 is removed. At that time cysteine oxidation was studied in 0.1 N NaOH where the 330 chromophore is stable and where luminol could be used to excite a flash of light illustrating some details of the final reaction. In the present note we reinvestigate the copper catalysed oxidation of cysteine at physiological pH 7.4. The relevant point that in the course of this catalysis the metal remains complexed in the reduced form appears a novel finding worthy to be emphasized.

Materials and methods

SOD, catalase, cytochrome c (Cyt c) and nitro blue tetrazolium (NBT) were from Sigma Co. All other reagents were of the best commercial source and used without further purification. Deionised water was used throughout. Sulfhydryl groups were determined with Ellman's reagent (DTNB) as described (Riddles et al., 1983). Modification of the procedure according to Suzuki et al. (1990) gave the same results. Reduction of cystine was carried out by the Reduce-Imm. reducing kit (Pierce), used according the producer instructions. Cuprous chloride was dissolved in N_2-saturated acetonitrile immediately before use. As a general rule, the copper solution was always added last to the experiment. Cu^{I} was measured spectrophotometrically at 454 nm (ε= 7,950 M^{-1} cm^{-1}) by the neocuproine assay (Smith et al., 1952) performed in 0.1 M acetate buffer, pH 4. Spectrophotometric analyses were carried out with UVIKON 940 (Kontron) equipped with a thermostated cell compartment and with a magnetic stirrer. O_2 consumption was monitored using a Clark-type oxygen electrode (Gilson oxygraph).

Low-temperature X-band EPR spectra were recorded on a Varian E9 spectrometer equipped with a Stelar temperature controller and interfaced with a Stelar Prometheus Data Acquisition system for analysis and handling of the data. Paramagnetic copper content was calculated with 1.81 mM Cu^{II}-EDTA as standard.

To secure constant aerobiosis, the reacting mixtures were stirred magnetically at the desired temperature all along the experiments. Anaerobiosis was obtained by bubbling the reacting solutions with extra-pure nitrogen for at least 10 min, maintaining the solutions under the flush of water-saturated N_2 during the experiment.

Results

Formation of the cuprous complex

As illustrated in Fig. 1, the addition of ten molar excess of cysteine to 200 μM $CuCl_2$ in phosphate buffer pH 7.4 produces the absorbance with a peak at 260 nm and a shoulder at 300 nm. The same absorbance is obtained when the solutions have been previously flushed with N_2 for 10 min or when $CuCl_2$ is

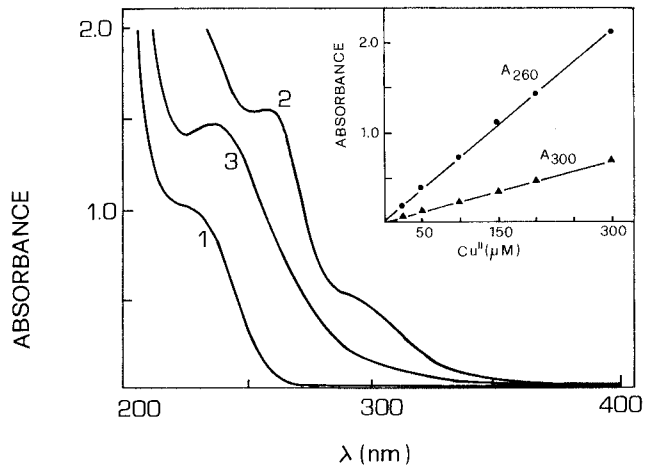


Fig. 1. Absorption spectra of a solution of 2 mM cysteine in 50 mM phosphate buffer, pH 7.4, before (curve 1) and after the addition of 200 μM $CuCl_2$ (curve 2: as soon as $CuCl_2$ is added; curve 3: after 20 min at room temperature under stirring). Insert: absorbance at 260 and 300 nm as a function of $CuCl_2$ concentration. Cysteine concentration 2 mM

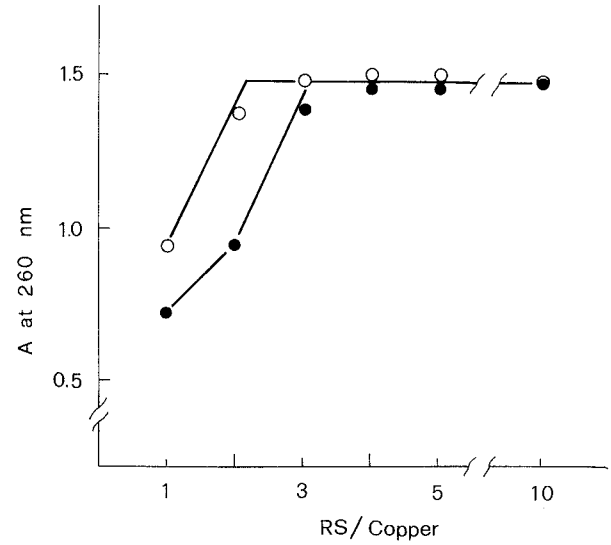


Fig. 2. Absorbance changes at 260 nm as a function of the cysteine to copper ratio. 200 μM $CuCl_2$ (full circles) or CuCl (open circles) was added, under anaerobic conditions, to different cysteine solutions (0.2–2 mM) in 50 mM phosphate buffer, pH 7.4

replaced by CuCl. Under aerobic conditions the compound responsible of the 260 nm absorbance decays after a defined length of time (Fig. 1, curve 3). In the presence of cysteine excess the 260, 300 nm absorbance is proportional to the $CuCl_2$ added (Fig. 1 insert). By reacting under N_2 different concentrations of cysteine (0.2–1 mM) with a fixed amount of $CuCl_2$ (200 μM), the maximal absorbance at 260 nm is reached when the ratio cysteine/Cu^{II} is 3 (Fig. 2).

When the same assay is done with reduced copper the same absorbance is obtained with a ratio of 2 (Fig. 2). Because in both the experiments the neocuproine assay indicates that all the copper is in the reduced form it is concluded that in the case of $CuCl_2$ one mole of cysteine has been used to reduce the metal and that the complex of two cysteine with a reduced copper ($RS^-Cu^I{}^-SR$) is responsible of the 260, 300 nm absorbance. Sulfhydryls titration with DTNB indicates that of the two cysteine bound to Cu^I in the complex, one is not reactive with the reagent (not shown). Figure 3 shows that the 260 nm absorbance remains fully intact in the course of the oxidation of the thiol and that it starts to decrease when approximately 80 per cent of cysteine has been consumed. The neocuproine assay shows that the metal remains in the reduced form in the course of the oxidation, decreasing slowly and not completely at the end of the oxidative reaction. Fig. 3 (insert) shows also that when the same experiment is done under anaerobic conditions, the 260, 300 nm absorbance is unchanged and copper remains in the reduced state as long as the anaerobiosis is maintained. All the data illustrated above are consistent with the bis-cysteine cuprous complex being the intermediate species involved in the catalytic oxidation of cysteine and with its continuous regeneration during the catalysis. In accord with this interpretation, the time length preceding the decay of the complex (followed by the absorbance at 260 nm) is strictly related to the cysteine excess (Fig. 4).

Reaction products

Thiols can be oxidized to form disulfide, sulfinate and sulfonate (Wefer et al., 1983; Misra, 1974; Saez et al., 1982; Harmann et al., 1984), therefore the products of cysteine oxidation in the conditions of Fig. 1 were determined after 20 min reaction time. More than 95% of the oxidized thiol was recovered by reduction with an immobilized thiol reductant, indicating that cysteine had been oxidized mainly to the disulfide. This agrees with the spectral curve recorded at the end of oxidation (Fig. 1, curve 3) which is similar to that obtained by adding 1 mM cystine with 200 μM $CuCl_2$ (not shown). The formation of cysteine sulfinic acid and cysteic acid, determined by the amino acid analyzer, were together in the amount of only 2% of the initial cysteine (not shown). H_2O_2 has been detected as a product of the copper catalysed oxidation of thiols (Harmann et al., 1984). The amount of H_2O_2 produced has been calculated by measuring O_2 in the absence and in the presence of catalase. As seen in Fig. 5, when catalase is added at the end of cysteine oxidation, O_2 is released accounting for the H_2O_2 produced. When the oxidation is run in the presence of catalase, added at zero time, the consumption of O_2 appears halved because of the regeneration of O_2 from the produced H_2O_2. From these results the formation of 0.32 mol of H_2O_2 per mol of RSH oxidised could be calculated.

As seen in Fig. 3, copper is partially present in its cuprous form also at the end of oxidation. This result is explained with the presence of the accumulated cystine, known to be reduced by Cu^I (Rossouw et al., 1935) producing an

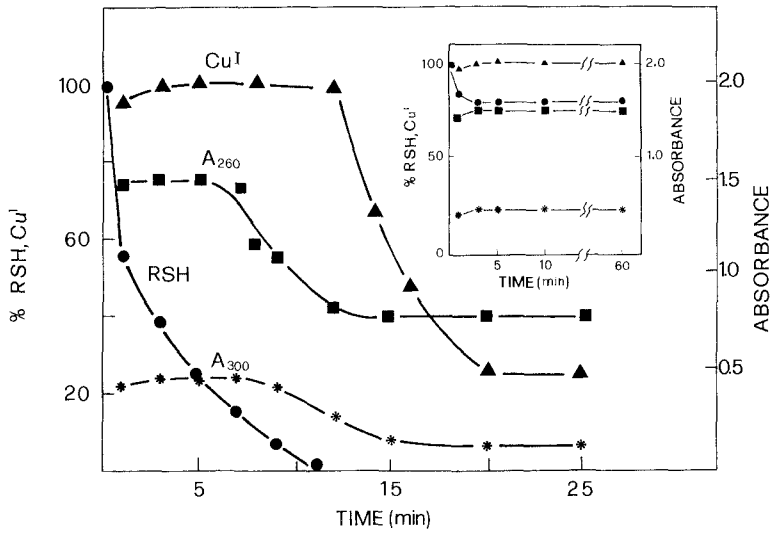


Fig. 3. Time course of cysteine oxidation. The reacting mixture contained 2 mM cysteine and 200 μM $CuCl_2$ in 50 mM phosphate buffer, pH 7.4. The reaction was started by the addition of $CuCl_2$ and was allowed to occur at room temperature under stirring. At appropriate time intervals, aliquots were withdrawn and analysed for the absorbance at 260 (■) and 300 nm (*), for the disappearance of sulfhydryl groups (●) and for reduction of copper (▲). Insert: the same experiment under anaerobic conditions

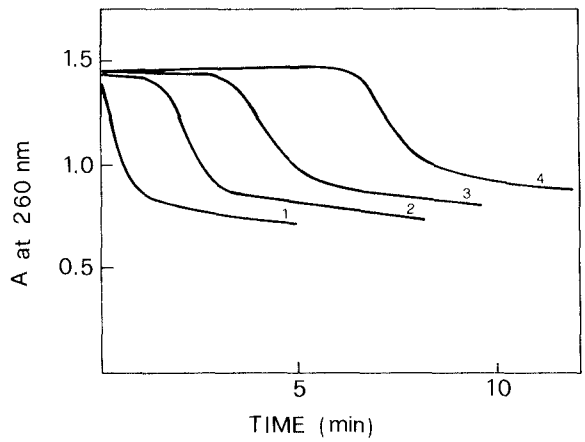


Fig. 4. Time course of the absorbance at 260 nm as a function of the cysteine to copper ratio. The reacting mixtures contained 200 μM $CuCl_2$ and cysteine in 50 mM phosphate buffer, pH 7.4, at room temperature under stirring. Cysteine concentration: 0.6 mM (line 1), 1 mM (line 2), 1.4 mM (line 3) and 2 mM (line 4). Copper addition to start the reaction

equilibrium (Kolthof et al., 1951), slowing down the terminal oxidation of Cu^{I}. In accord with this, we found that when a solution of 400 μM cystine in 50 mM phosphate buffer, pH 7.4, is added with 200 μM CuCl, the amount of copper in the reduced form is still 25% of the original one after 20 min incubation

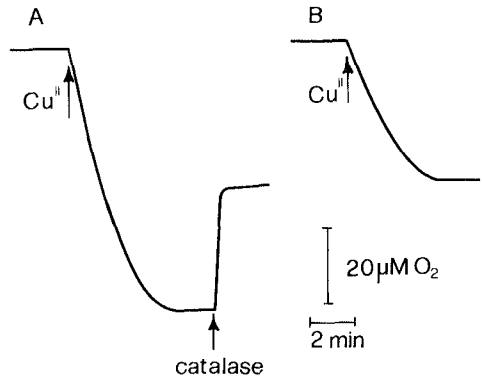


Fig. 5. Oxygen consumption time profile of 200μM cysteine in 50mM phosphate buffer, pH 7.4, at 30°C, following the addition of 2μM $CuCl_2$ (**A**) with subsequent addition of 50μg/ml catalase. **B** The same as **A** with catalase added at zero time

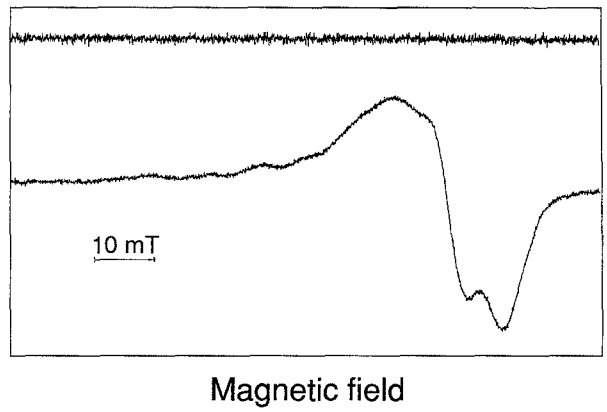


Fig. 6. EPR spectra of a frozen solution of 1mM cysteine and 200μM $CuCl_2$ in 50mM phosphate buffer, pH 7.4, incubated for 1min (upper curve) and for 20min (lower curve) at room temperature under stirring). Experimental settings: microwave power, 20mW, modulation amplitude, 1mT, frequency, 9.075Ghz, temperature, 100K. Spectra are averages of 9 scans

(neocuproine assay). A solution of CuCl in the same buffer autoxidises completely to Cu^{II} (assay with neocuproine negative) in less than 5min.

EPR analysis

The reduced form of the copper in the complex, indicated by the neocuproine assay, is confirmed by the EPR analysis. As seen in Fig. 6, no signal from paramagnetic cupric copper is observed upon addition of cysteine to $CuCl_2$. On the other hand, at the end of oxidation, when the thiol is no more

present, a signal with an intensity accounting for 75% of that expected for fully oxidized copper appears. Computerized analysis of the spectrum lineshape suggests that the signal arises from two different species, with magnetic parameters ($A_{//} = 18.5$ mT, $g_{//} = 2.25$ and $A_{//} = 15.1$ mT, $g_{//} = 2.30$) consistent with 2N-20 and 1N-30 metal coordinations, respectively (Peisach et al., 1974). It is likely that the signals belong to different complexes of Cu^{II} with cystine.

Reduction of Cyt c

Cyt c is known to be reduced by thiols (Saez et al., 1982; Butler et al., 1982). The reaction occurs in the presence of air and is thought to be mediated by the $O_2^{\bullet -}$ produced by trace metal catalysed oxidation of the thiol by O_2. Table 1 collects the results obtained under our experimental conditions. Cyt c is reduced by cysteine also in the absence of O_2 indicating the occurrence of a reduction independent of the $O_2^{\bullet -}$ intermediacy. The importance of contaminating metals is evident by the inhibitory effect of DTPA and of CN^- (line 2, 3) on the reduction rate and also of neocuproine (line 4), the latter suggesting that the main contaminant is presumably copper ion. The last conclusion is emphasised by the strong activation obtained by the addition of 2μM Cu^{II} either in aerobiosis and in anaerobiosis (line 6). Enhanced reduction of Cyt c by added Cu^{II} under anaerobiosis indicates the occurrence of direct reduction of Cyt c by the cuprous bis-cysteine complex. The favourable effect of the addition of SOD (line 5) has been explained (Saez et al., 1982) by the increased H_2O_2 production as a result of superoxide dismutation. However in

Table 1. Reduction of ferricytochrome c by cysteine. Effect of Cu^{II} and other factors. Control system: cysteine 1 mM, Cyt c 44μM, 50 mM phosphate buffer, pH 7.4 at 25°C. Cysteine added last. Values in ΔA_{550}/min. Average of at least 3 values of separate experiments with S.D. less than 10 per cent

Additions	Aerobiosis	Anaerobiosis
1 Control	0.110	0.090
2 DTPA 20 mM	0.025	0.025
3 CN^- 10 mM	0.026	
4 Neocuproine 0.3 mM	0.035	0.035
5 SOD 50μg/ml	0.170	
6 $CuCl_2$ 2μM	0.387	0.196
7 $CuCl_2$ 2μM + SOD 50μg/ml	0.374	
8 H_2O_2 0.25 mM	0.109	
9 $CuCl_2$ 2μM + H_2O_2 0.25 mM	0.566	
10 Mannitol 10 mM	0.104	
11 $CuCl_2$ 2μM + H_2O_2 0.25 mM + Mannitol 10 mM	0.570	

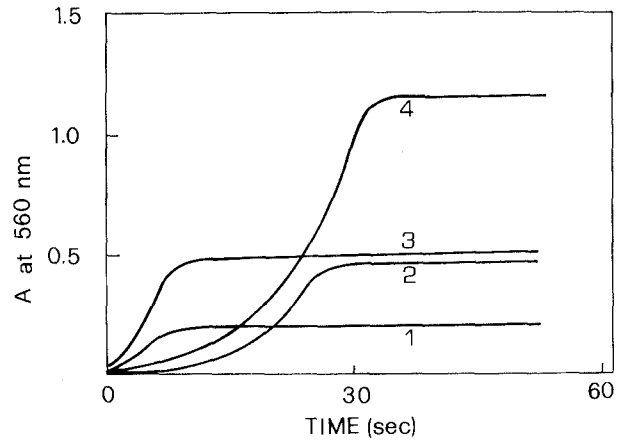


Fig. 7. Reduction of NBT by the cysteine-copper complex. Reaction mixture contained 500μM NBT in 50mM phosphate buffer, pH 7.4, at 25°C under stirring. Additional components and conditions were: 150μM cysteine plus 50μM $CuCl_2$ (line 1); 250μM cysteine plus 50μM $CuCl_2$ (line 2); 150μM cysteine plus 50μM $CuCl_2$ under anaerobic conditions (line 3); 250μM cysteine plus 50μM $CuCl_2$ under anaerobic conditions (line 4). In all cases $CuCl_2$ was added last

our conditions H_2O_2 is found to have an evident stimulatory effect only when added together with Cu^{II} (line 9). Addition of mannitol, a known hydroxyl radical scavenger, has no effect on the reduction rate (line 10, 11) indicating that this radical has not an essential role as an inter mediate.

Reduction of NBT

When NBT is reacted with cysteine (up to 1 mM) in the conditions reported in Fig. 7, no reduction of the dye to the 560nm absorbing formazan (NBF) takes place. The addition of $CuCl_2$ produces the reduction of NBT after an initial lag phase. The unreactive phase is prolonged and the production of NBF is increased by increasing the cysteine concentration. In the absence of O_2 (solutions flushed with N_2 for 10min) the extent of NBT reduction is higher than in aerobiosis (Fig. 7). The reported data are consistent with the assumption of a direct reduction of NBT by the cuprous bis-cysteine complex and with the occurrence, in the presence of O_2, of reactions competing with NBT reduction. Using the molar extinction of $1.6 \times 10^4 M^{-1} cm^{-1}$ for NBT reduction at pH 7.4 (Bielski et al., 1980) we calculated that under anaerobic conditions about 0.34mol of NBF per mol of cysteine is produced at the end of the reaction. This value is close to that expected (0.5mol/mol RSH) considering the complete univalent reduction of NBT by cysteine.

Discussion

The course of the Cu^{II} catalysed oxidation of cysteine in phosphate buffer pH 7.4 may be divided in three parts.

(A) Reductive chelation and formation of the complex

The first reaction upon the addition of Cu^{II} with cysteine is characterised by the production of the 260,300 nm chromophore assigned to the complex of two cysteine with one reduced Cu. This reaction has been termed reductive chelation (Vortisch et al., 1976), and we maintain such appropriate definition. A two five-membered rings structure united by a copper ion has been proposed for such complex (Cavallini et al., 1968; Vortisch et al., 1976; Davis et al., 1983). In the absence of supporting evidence for this structure we prefer to use the simplified formulation $RS^-Cu^I{}^-SR$ for the bis-cysteine cuprous complex, without suggesting any particular geometry. Reductive chelation (production of the 260 nm absorbance) occurs also in the absence of O_2 and the following simplified reactions are appropriate:

$$2\ RS^- + 2\ Cu^{II} \rightarrow 2\ RS^{\bullet}\ Cu^I \quad (1)$$

$$2\ RS^{\bullet}\ Cu^I + 4\ RS^- \rightarrow RSSR + 2\ RS^-Cu^I{}^-SR \quad (2)$$

$$6\ RS^- + 2\ Cu^{II} \rightarrow RSSR + 2\ RS^-Cu^I{}^-SR \quad (3)$$

(B) The intermediate path of cysteine oxidation

This follows the reductive chelation and is done by the transfer of electrons to anyone of the acceptors studied (O_2, Cyt c, NBT). Cyt c and NBT are reduced by the complex also in the absence of O_2 indicating the occurrence of a direct reduction independent of the $O_2^{\bullet -}$ intermediacy (Table. 1, Fig. 7). A minor part of the Cyt c reduction is possibly due to a non-metal dependent reduction as shown by the incomplete inhibition in the presence of DTPA, CN^- and neocuproine (Table 1, lines 2–4) and also as reported for other thiols (Dikalov et al., 1996). Even though thiyls and $O_2^{\bullet -}$ radicals are known to be produced in the course of metal catalysed oxidation of thiols (Misra, 1974; Saez et al., 1982; Duprè et al., 1975) it is not clear where the site of the redox cycle is allocated. The data reported here indicate that the species involved in the redox shuttle is the cysteine-copper complex with the thiolate sulfur functioning as the electron donor to the acceptor tested, producing a transiently bound thiyl radical, and with the metal remaining in the reduced form. The following formulations are therefore proposed:

$$RS^-Cu^I{}^-SR + O_2 \rightarrow RS^-Cu^I{}^{\bullet}SR + O_2^{\bullet -} \quad (4)$$

$$RS^-Cu^I{}^-SR + \text{Cyt c} \rightarrow RS^-Cu^I{}^{\bullet}SR + \text{Cyt c}_{red} \quad (5)$$

$$RS^-Cu^I{}^-SR + \text{NBT} \rightarrow RS^-Cu^I{}^{\bullet}SR + 1/2\ \text{NBF} \quad (6)$$

In the presence of air, Cyt c and NBT are reduced also by $O_2^{\bullet -}$ coming from the simultaneous reaction of the complex with O_2 (4) according to :

$$O_2^{\bullet -} + \text{Cyt c} \rightarrow O_2 + \text{Cyt c}_{red} \quad (7)$$

($k = 2.6 \times 10^5\ M^{-1}\ s^{-1}$; Butler et al., 1982)

$$O_2^{\bullet -} + NBT \rightarrow O_2 + 1/2\ NBF \quad (8)$$
$$(k = 6 \times 10^4\ M^{-1}\,sec^{-1};\ Bielski\ et\ al., 1977)$$

The superoxide radical also reacts with RSH:

$$2\ RSH + 2\ O_2^{\bullet -} \rightarrow RSSR + 2\ H_2O_2 \quad (9)$$

Reaction (9) is an oversimplification of a complex, not yet totally understood, series of reactions (Winterbourn et al., 1994), running with a rate constant of $2 \times 10^5 M^{-1}\ sec^{-1}$ (reported for glutathione, Dikalov et al., 1996). The lower extent of NBT reduction in the presence of O_2 compared to that in anaerobiosis is explained by the simultaneous occurrence of the reaction of the complex with O_2 (4) and with NBT (6) and by the competition of reaction (9) with (8). The spontaneous dismutation of $O_2^{\bullet -}$ ($k = 2 \times 10^5 M^{-1}\ sec^{-1}$; Bielski et al., 1977):

$$2\ O_2^{\bullet -} + 2\ H^+ \rightarrow O_2 + H_2O_2 \quad (10)$$

is an additional competing reaction.

Reactions (4–6) generate thiyl radical ($RS^{\bullet}$) which is reported to have low affinity for complexed copper (Graf et al., 1964). and is expected to be replaced by a new RS^- from the excess thus regenerating the cuprous bis-cysteine complex:

$$RS^-Cu^I\ ^{\bullet}SR + RS^- \rightarrow RS^-Cu^I\ ^-SR + RS^{\bullet} \quad (11)$$

The fate of the thiyl radical is to dimerize with rate constant of $10^{10} M^{-1}\ sec^{-1}$ (Butler et al., 1982):

$$2\ RS^{\bullet} \rightarrow RSSR \quad (12)$$

or interact with a thiolate anion with $k = 1.2 \times 10^9 M^{-1}\ sec^{-1}$ (Hoffman et al., 1972):

$$RS^{\bullet} + RS^- \rightarrow RSSR^{\bullet -} \quad (13)$$

or interact with O_2 with $k = 8.1 \times 10^9 M^{-1}\ sec^{-1}$ (Zhao et al., 1994):

$$RS^{\bullet} + O_2 \rightarrow RSOO^{\bullet} \quad (14)$$

The electron transfer from $RSSR^{\bullet -}$ to O_2 with $k = 5 \times 10^9 M^{-1}\ sec^{-1}$, reported for glutathione (Micic et al., 1978):

$$RSSR^{\bullet -} + O_2 \rightarrow RSSR + O_2^{\bullet -} \quad (15)$$

is an additional source of $O_2^{\bullet -}$. $RSSR^{\bullet -}$ is a strong reductant and is expected to reduce Cyt c and NBT with production of RSSR. The reversible reaction (14) generates the peroxysulfenyl radical $RSOO^{\bullet}$ as an intermediate to yield sulfinate and sulfonate as final stable products (Davis et al., 1983; Ross et al., 1985). Under our experimental conditions, reaction (14) should occur at low level because cysteine sulfinic acid and cysteic acid were detected as minor products.

(C) The final decay of the complex

In the oxidation system, with O_2 as single electron acceptor, the complex decays slowly only at the end when no more free cysteine is present (Fig. 3 and 4). Thiyls in this case should be removed according to:

$$RS^-Cu^I \cdot SR \rightarrow 1/2\ RSSR + RS^-Cu^I \quad (16)$$

thus making free the cuprous mercaptide which undergoes the final oxidation:

$$RS^-Cu^I + O_2 + 2\ H^+ \rightarrow 1/2\ RSSR + H_2O_2 + Cu^{II} \quad (17)$$

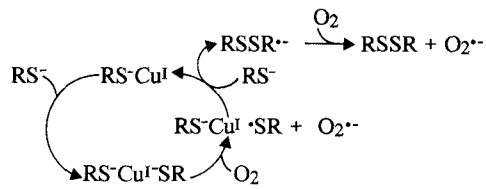
In the experimental conditions of the present work, H_2O_2 produced in reaction (17) together with that accumulated in reaction (9) and (10) appears as final product in the amount of 0.32 mol per mol of cysteine oxidised (Fig. 5). Considering the overall process of cysteine oxidation as:

$$2\ RSH + O_2 \xrightarrow{Cu} RSSR + H_2O_2 \quad (18)$$

a ratio of 0.5 mol H_2O_2/mol RSH oxidised should be obtained. The difference from the expected stoichiometry suggests the involvement of H_2O_2 in other reactions. Among the various possible reactions the one between the cuprous mercaptide and H_2O_2 (Hanna et al., 1992; Spear et al., 1995) is of interest because could account for the formation of the hydroxyl radical which has been reported as a final product of RSH oxidation (Saez et al., 1982).

Concluding remarks

As a conclusion it appears that in the oxidation of cysteine the metal plays the role of producing the reactive complex more than the element primarely involved in the redox cycle. In the present work it is shown that the copper remains in the reduced form bound with two cysteine all along the oxidation of the RSH excess while in the previous work, done in 0.1 N NaOH, it was found to remain in the oxidised form (Cavallini et al., 1968, 1969). Scheme 1 is therefore proposed to illustrate the central main reaction of the copper catalysed oxidation of cysteine at pH 7.4. Presented in this way the cuprous mercaptide (RS^-Cu^I) assumes the role of the formal catalyst, able to bind the substrate-like second RS^- molecule, and the bis-cysteine complex ($RS^-Cu^I{-}SR$) exhibits the resemblance of a Michaelis-Menten complex with the substrate. In the formulations reported above the oxidation of the complex has been presented to occur at the level of sulfur. This is in accord with



Scheme 1. Main reactions of the intermediate path of copper catalysed cysteine oxidation

the finding of the metal remaining in the reduced form in the course of the oxidation and is in accord with other reports where sulfur is proposed as the electron donor in the case of the oxidation of copper complexes with penicillamine (Younes et al., 1977) and with the cysteine-rich metallothioneins (Deters et al., 1994).

Acknowledgments

This work was supported in part by grants from MURST and by Sigma Tau Foundation.

References

Bielski BHJ, Richter HW (1977) A study of the superoxide radical chemistry by stopped-flow radiolysis and radiation induced oxygen consumption. J Am Chem Soc 99: 3019–3023

Bielski BHJ, Allen AO (1977) Mechanism of the disproportionation of superoxide radicals. J Phys Chem 81: 1048–1050

Bielski BHJ, Shiue GG, Bajuk S (1980) Reduction of nitro blue tetrazolium by CO_2^- and O_2^- radicals. J Phys Chem 84: 830–833

Butler J, Koppenol WH, Margoliash E (1982) Kinetics and mechanism of the reduction of ferricytochrome c by the superoxide anion. J Biol Chem 257: 10747–10750

Cavallini D, De Marco C, Duprè S (1968) Luminol chemiluminescence studies of the oxidation of cysteine and other thiols to disulfides. Arch Biochem Biophys 124: 18–26

Cavallini D, De Marco C, Duprè S, Rotilio G (1969) The copper catalysed oxidation of cysteine to cystine. Arch Biochem Biophys 130: 354–361

Davis FJ, Gilbert BC, Norman ROC (1983) Electron spin resonance studies. Characterization of copper(II) complexes in the oxidation of D-penicillamine, L-cysteine and related sulfur-containig compounds. J Chem Soc Perkin Trans 2: 1763–1771

Deters D, Jurgen HJ, Weser U (1994) Transient thiyl radicals in yeast copper(I) thionein. Biochim Biophys Acta 1208: 344–347

Dikalov S, Khramtsov V, Zimmer G (1983) Determination of rate constants of the reactions of thiols with the superoxide radical by electron paramagnetic resonance: critical remarks on spectrophotometric approaches. Arch Biochem Biophys 326: 207–218

Duprè S, Federici G, Santoro L, Rossi Fanelli MR, Cavallini D (1975) The involvement of superoxide anions in the autoxidation of various cofactors of cysteamine oxygenase. Mol Cell Biochem 9: 149–155

Graf L, Fallab S (1964) Zum Reaktionmechanismus von Oxidasen. Experientia 20: 46–47

Hanna PM, Mason RP (1992) Direct evidence for inhibition of free radicals formation from Cu(I) and hydrogen peroxide by glutathione and other potential ligands using the EPR spin-trapping technique. Arch Biochem Biophys 295: 205–213

Harmann LS, Mottley C, Mason RP (1984) Free radical metabolites of L-cysteine oxidation. J Biol Chem 259: 5606–5611

Hoffman MZ, Hayon E (1972) One-electron reduction of the disulfide linkage in aqueous solution. Formation, protonation and decay kinetics of the RSSR radical. J Am Chem Soc 94: 7950–7957

Kolthoff JM, Stricks W (1951) Polarographic investigations of reactions in aqueous solutions containing copper and cysteine (cystine). J Am Chem Soc 73: 1728–1733

Micic OJ, Nenadovic MT, Carapellucci PA (1978) Solvent participation in reactions. 2. Reactions of cystamine radical. J Am Chem Soc 100: 2209–2212

Misra HP (1974) Generation of superoxide free radical during the autoxidation of thiols. J Biol Chem 249: 2151–2155

Peisach J, Blumber WE (1974) Structural implications derived from the analysis of electron paramagnetic resonance spectra of natural and artificial copper proteins. Arch Biochem Biophys 165: 691–708

Riddles PW, Blakeley RL, Zerner B (1983) Reassesment of Ellman's reagent. Meth Enzymol 91: 49–55

Rossouw SD, Wilken-Jorden TJ (1935) Studies on the origin of sulfur in wool. Biochem J 29: 219–224

Saez G, Thornalley PJ, Hill HAO, Hems R, Bannister JV (1982) The production of free radicals during the autoxidation of cysteine and their effect on isolated rat hepatocytes. Biochim Biophys Acta 719: 24–31

Schafer K, Bonifacic M, Bahneman D, Asmus K-D (1978) Addition of oxygen to organic sulfur radicals. J Phys Chem 82: 2777–2780

Smith GF, McCurdy (1952) 2,9-dimethyl-1,10-phenanthroline. New specific spectrophotometric determination of copper. Anal Chem 24: 371–373

Spear N, Aust SD (1995) Hydroxylation of deoxyguanosine in DNA by copper and thiols. Arch Biochem Biophys 317: 142–148

Suzuki Y, Lyall V, Biber TUL, Ford GD (1990) A modified technique for the measurement of sulfhydryl groups oxidized by reactive oxygen intermediates. Free Rad Biol Med 9: 479–484

Vortisch V, Kroneck P, Hemmerich P (1976) Model studies on the coordination of copper in enzymes. IV. Structure and stability of cuprous complexes with sulfur-containing ligands. J Am Chem Soc 98: 2821–2826

Wefers H, Sies H (1983) Oxidation of glutathione by the superoxide radical to the disulfide and the sulfonate yielding singlet oxygen. Eur J Biochem 137: 29–36

Winterbourn CC, Metodiewa D (1994) The reaction of superoxide with reduced glutathione. Arch Biochem Biophys 314: 284–290

Younes M, Weser U (1977) Superoxide dismutase activity of copper-penicillamine: possible involvement of Cu(I) stabilized sulfur radical. Biochem Biophys Res Comm 78: 1247–1253

Zhao R, Lind J, Merenyi G, Eriksen TR (1994) Kinetics of one-electron oxidation of thiols and hydrogen abstraction by thiyl radicals from α-amino C-H bonds. J Am Chem Soc 116: 12010–12015

Authors' address: Prof. L. Pecci, Dipartimento di Scienze Biochimiche "A. Rossi Fanelli", Università di Roma "La Sapienza", Piazzale A. Moro 5, I-00185 Roma, Italy.

Received February 2, 1997