# One-electron oxidation of ergothioneine and analogues investigated by pulse radiolysis: redox reaction involving ergothioneine and vitamin C

Klaus-Dieter ASMUS*, René V. BENSASSON†‖, Jean-Luc BERNIER‡, Raymond HOUSSIN‡ and Edward J. LAND§

*Radiation Laboratory and Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556, U.S.A., †Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, 43, rue Cuvier, 75231 Paris Cedex 05, France, ‡Institute de Chimie Pharmaceutique, rue du Professor Laguesse, 59045 Lille Cedex, France, and §CRC Department of Biophysical Chemistry, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester M20 9BX, U.K.

Redox reactions of endogenous and exogenous sulphur-containing compounds are involved in protection against oxidative damage arising from the incidence and/or treatment of many diseases, including cancer. We have investigated, via pulse radiolysis, the one-electron oxidation of ergothioneine, a molecule with antioxidant properties which is detected at millimolar concentrations in certain tissues and fluids subject to oxidative stress, including erythrocytes and plasma. The spectrum of the transient species, assigned to the product of one-electron oxidation, observed after reaction of ergothioneine with the oxidizing radicals $OH^{\bullet}$, $N_3^{\bullet}$ and $CCl_3O_2^{\bullet}$ has a maximum absorption at 520 nm and is very similar to that obtained by oxidation of analogous molecules such as 2-mercaptoimidazole, 1-methyl-2-mercaptoimidazole, *S*-methyl- and *S*,*N*-dimethyl-ergothioneine. In the presence of vitamin C, the oxidized form of ergothioneine is repaired by a rapid reduction ($k = 6.3 \times 10^8$ $M^{-1} \cdot s^{-1}$) producing ascorbyl radicals. This co-operative interaction between ergothionine and ascorbate, similar to that previously observed between vitamin E and ascorbate, may contribute to essential biological redox protection.

## INTRODUCTION

Free radicals derived from endogenous and exogenous sulphur-containing compounds are involved in a number of important biological processes [1]. The redox reactions of these compounds have been studied profitably by radiation-chemical methods, including pulse radiolysis [2–6]. These redox reactions are implicated in the radiotherapy and chemotherapy of cancer [7]. They are considered to be involved in the protection of living systems subjected to ionizing radiation or other sources of free-radical damage. The underlying processes most often constitute a true repair function, i.e. they lead to regeneration of the original compounds. In other cases, however, the deactivation of the radical species may result in different molecular products, constituting a misrepair. The fate of the thiyl radicals produced by these reactions is controversial, and such reactions are now not generally considered to be harmless [4,6–8].

Thiol or thione functions can be associated with another moiety, the imidazole ring [9], leading to mercaptoimidazole derivatives [10,11], such as ovothiol or ergothioneine (Figure 1), which exert chemoprotection against oxidative stress and carcinogenesis [11–14]. Dietary ergothioneine, a compound of plant origin, is assimilated and conserved by mammals. It has been detected at high concentrations (millimolar levels) in mammalian erythrocytes, seminal fluid, liver, kidney and lens [15,16]. In aqueous solution, ergothioneine has predominantly a thione rather than a tautomeric thiol structure (see Figure 1). It is considered to be a natural chemoprotector against oxidation [12], including lipid peroxidation [17,18]. It deactivates singlet oxygen with a rather high rate constant [19] of $k_{\Delta} = 2.3 \times 10^7$ $M^{-1} \cdot s^{-1}$, in the range of that of other imidazole derivatives such as histidine [20] and triphenylimidazoles [9] ($k_{\Delta} = 10^7$–$10^8$ $M^{-1} \cdot s^{-1}$) and higher than the rate constant observed for simple thiols, including glutathione [19]. Ergothioneine diminishes the mutagenicity of cumene and t-butylhydroperoxides in *Salmonella* bacteria [21].

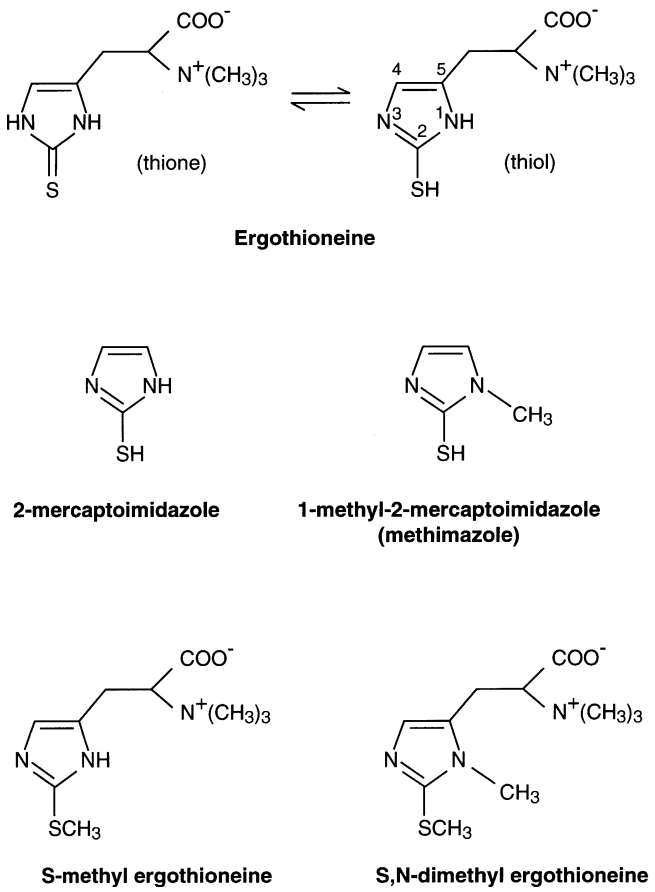


**Figure 1 Structures of the mercaptoimidazoles studied**

Abbreviation used: FAB-MS, fast-atom bombardment MS.

‖ To whom correspondence should be addressed.

A chemoprotective role of ergothioneine is likely to involve single-electron reactions. For example, Hochstein and collaborators [22,23] consider that the oxidation of myoglobin into ferrylmyoglobin ($Mb^{IV}$) is a critical event in tissue damage associated with cardiac ischaemic/reperfusion states and that ergothioneine is able to reduce $Mb^{IV}$ to $Mb^{III}$. Ergothioneine may play this protective role by preventing the accumulation of hypervalent states of Mb.

In order to elucidate the chemoprotective role of ergothioneine, we have now investigated by pulse radiolysis [3] the one-electron oxidation of ergothioneine and a group of related mercaptoimidazoles (see Figure 1), including 2-mercaptoimidazole and 1-methyl-2-mercaptoimidazole (methimazole), both of which can exist in a thiol and thione form, and *S*-methyl- and *S*,*N*-dimethylergothioneine, which cannot. We then go on to study a redox reaction of the one-electron-oxidized ergothioneine with vitamin C, an established major water-soluble intracellular antioxidant.

## MATERIALS AND METHODS

Ergothioneine was either purchased as the L- form from Sigma (Saint Quentin, Fallavier, France) or synthesized as a racemic mixture by Bioxytech® (Oxis International S.A., Bonnevil sur Marne, France). Identical results were obtained with both forms. 2-Mercaptoimidazole was purchased from Aldrich (Gillingham, Dorset, U.K.) and 1-methyl-2-mercaptoimidazole was from Sigma (Poole, Dorset, U.K.).

The structures of the two methylated derivatives of ergothioneine are shown in Figure 1. The *S*-methyl derivative was prepared by reaction of 1 equivalent of dimethyl sulphate with 1 equivalent of L-ergothioneine salt in the presence of 1 equivalent of 0.1 M aq. NaOH solution, over 70 h at 20 °C. The *S*,*N*-dimethyl derivative was obtained in an analagous manner with 3 equivalents of dimethyl sulphate. The two products were isolated and purified by repeated evaporation of their ethanolic solutions and by elimination of NaCl on a Sep-Pak $C_{18}$ column. Their structures were assigned by $^1$H NMR and confirmed by fast-atom bombardment MS (FAB-MS). *S*-Methylergothioneine: $^1$H NMR $\delta$ ($^2H_2O$) 2.5 (s, 3H, $SCH_3$), 3.3 (s, 9H, $N^+CH_3$), 3.9; MS ($FAB^+$): $M^+H = 244$. *S*,*N*-Dimethylergothioneine: $^1$H NMR $\delta$ ($^2H_2O$) 2.6 (s, 3H, $SCH_3$), 3.3 (s, 9H, $N^+CH_3$), 3.9 (s, 3H,$N_\pi CH_3$); MS ($FAB^+$): $M^+H = 258$.

The pulse radiolysis experiments were carried out in the laboratory of E. J. L. with a Vickers linear accelerator as previously described [24,25] using 10–100 ns pulses of 9–12 MeV electrons. The transient spectra of the solute (S) oxidized species were studied by kinetic absorption spectrophotometry, the oxidizing radicals $OH^\bullet$ and $N_3^\bullet$ being produced via the following reactions [26]:

$$H_2O \rightsquigarrow e^-_{aq} + OH^\bullet$$

$$e^-_{aq} + N_2O + H_2O \rightarrow OH^\bullet + N_2 + OH^-$$

$$OH^\bullet + N_3^- \rightarrow OH^- + N_3^\bullet$$

These may subsequently be followed by:

$$OH^\bullet + S \rightarrow OH^- + S^{\bullet+}$$

or:

$$N_3^\bullet + S \rightarrow N_3^- + S^{\bullet+}$$

The trichloromethyl peroxyl oxidizing radical, $CCl_3O_2^\bullet$, was prepared by pulse radiolysis of aerated aqueous solutions containing 2 M propan-2-ol, 1 M acetone and 0.01 M carbon tetrachloride buffered to pH 7 with phosphate, via the following reactions [27]:

$$OH^\bullet + (CH_3)_2CHOH \rightarrow H_2O + (CH_3)_2{}^\bullet COH$$

$$e^-_{aq} + (CH_3)_2CO \rightarrow (CH_3)CO^\bullet\text{-} \overset{H^+}{\rightleftharpoons} (CH_3)_2{}^\bullet COH$$

$$(CH_3)_2{}^\bullet COH + CCl_4 \rightarrow (CH_3)_2CO + CCl_3^\bullet + H^+ + Cl^-$$

$$CCl_3^\bullet + O_2 \rightarrow CCl_3O_2^\bullet$$

Absorbed doses were determined from transient $(SCN)_2^{\bullet-}$ formation in air-saturated aqueous 0.01 M KSCN solutions, using $G = 0.30\ \mu mol \cdot Gy^{-1}$ and $\epsilon_{500} = 7100\ dm^3 \cdot mol^{-1} \cdot cm^{-1}$ [28].

## RESULTS AND DISCUSSION

### Reactions with $OH^\bullet$

Oxidation of ergothioneine by $OH^\bullet$ ($k = 1.2 \times 10^{10}\ M^{-1} \cdot s^{-1}$) [19] was carried out by pulse radiolysis of a nitrous oxide-saturated 0.1 mM solution of ergothioneine in water buffered to pH 7. The initial transient spectrum obtained displayed main peaks at 520 and 290 nm, together with a small peak at 340 nm (Figure 2a). Both the main peaks subsequently decayed over hundreds of microseconds by a process following second-order kinetics ($2k/\epsilon_{520} = 3.1 \times 10^5\ cm \cdot s^{-1}$), leaving a species absorbing in the range 280–360 nm, where the stable product of chemical oxidation of ergothioneine, considered to be the disulphide, also absorbs [29]. Air, rather than $N_2O$, saturation did not affect the transient decay rate constant at 520 nm, although the peak height was halved. The initial transient absorption obtained from $OH^\bullet$ + ergothioneine (Figure 2a) was similar to that found previously by Willson and co-workers [30] on reacting $OH^\bullet$ with methimazole (see Figure 1 for structure). The latter, which was also formed by oxidation with $I_2^{\bullet-}$, was assigned to the corresponding radical cation formed by one-electron oxidation. A similar assignment seems likely here.

The effect the side-chain of ergothioneine has on the spectra shown in Figure 2(a) was tested by reacting $OH^\bullet$ with 2-mercaptoimidazole ($k = 1.2 \times 10^{10}\ M^{-1} \cdot s^{-1}$). The similarity of the spectra obtained (Figures 2a and 2b) suggests that the side-chain of ergothioneine has little influence on such spectra. Again, the main initial transient species decayed by second-order kinetics ($2k/\epsilon_{520} = 6.3 \times 10^5\ cm \cdot s^{-1}$) into a species absorbing relatively weakly in the 280–360 nm region.

The effect of placing a methyl group on the S atom of ergothioneine, thus preventing thione–thiol tautomerism and deprotonation of the SH group, was studied by pulse radiolysis of a solution of *S*-methylergothioneine (Figure 1). As can be seen from Figures 2(a) and 2(c), very little change in the transient spectrum was observed, indicating that the one-electron-oxidized ergothioneine radical exists in the thiol form (with two double bonds in the the ring at the 2,3 and 4,5 positions), rather than the thione form (with only one 4,5 double bond), and that the replacement of the hydrogen of the SH group by $CH_3$ has little effect. The fact that both the initial radical species with maxima at 520 nm from ergothioneine and *S*-methylergothioneine lead to products with weak absorption at 280–360 nm precludes the 520 nm transient species from being S-centred thiyl-type radicals, which lead to disulphides. Consequently, we suggest that these longer-lived products result from the decay of differently structured radicals, possibly N-centred. Rate constants of $1.8 \times 10^{10}\ M^{-1} \cdot s^{-1}$ and $2k/\epsilon_{520} = 2.9 \times 10^5\ cm \cdot s^{-1}$ were obtained respectively for $OH^\bullet$ + S-methylergothioneine and the second-order decay of the initial product.

As well as *S*-methylation, methylation of the nitrogen in the 1-position in the imidazole ring (Figure 1) again had only a minor

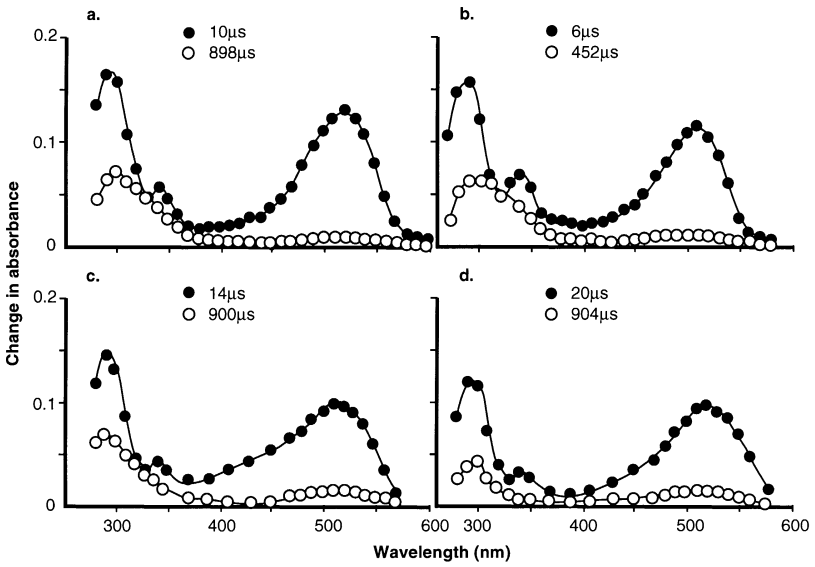


**Figure 2 Products of the reaction of OH• radicals with mercaptoimidazoles**

Changes in absorption spectra after pulse radiolysis of aqueous (**a**) ergothioneine (0.1 mM), (**b**) 2-mercaptoimidazole (0.1 mM), (**c**) *S*-methylergothioneine (0.035 mM) and (**d**) *S*,*N*-dimethylergothioneine (0.035 mM). All solutions also contained 0.1 M phosphate buffer, pH 7.0, and were flushed with nitrous oxide. The dose was 20 Gy.

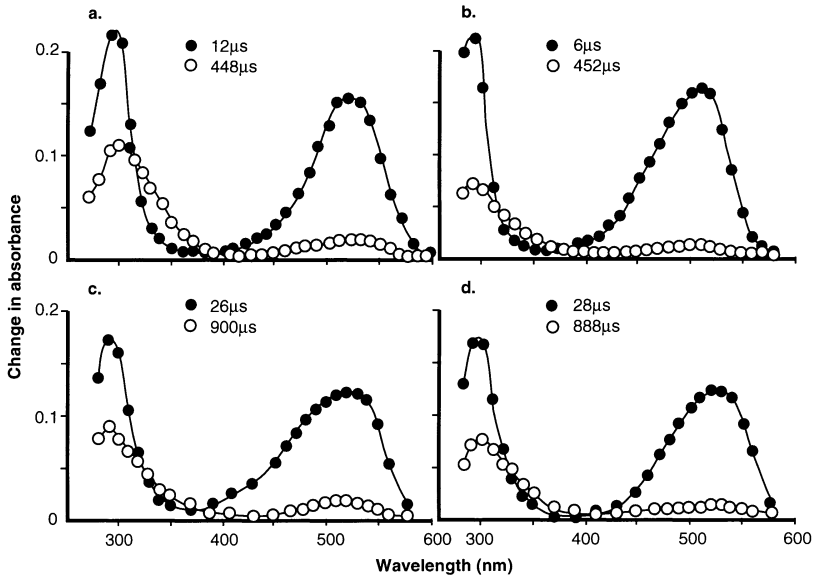


**Figure 3 Products of the reaction of $N_3^•$ radicals with mercaptoimidazoles**

Details as in Figure 2, except that all solutions also contained 0.05 M $NaN_3$.

effect on the spectrum of the initial product of OH• attack (Figure 2d). This suggests that in the radical this imidazole nitrogen cannot be involved in C = N double bond formation. The corresponding rate constants for formation (OH• + *S*,*N*-dimethylergothioneine) and bimolecular decay of the main initial transient intermediate were $1.5 \times 10^{10}\ M^{-1} \cdot s^{-1}$ and $2k/\epsilon_{520} = 2.7 \times 10^5\ cm \cdot s^{-1}$ respectively.

## Reactions with $N_3^•$

Since the OH• radical often undergoes addition reactions as well as one-electron oxidations, the reactions of $N_3^•$, normally a simple one-electron oxidant [31,32], with ergothioneine and analogues were investigated. Figure 3(a) shows the maximum change in absorption immediately after pulse radiolysis of 0.1 mM ergothioneine in $N_2O$-saturated aqueous 0.05 M $NaN_3$, buffered to pH 7 with phosphate. Comparison of this spectrum with that given in Figure 2(a) shows that a very similar transient absorption is formed, albeit without the small peak at 340 nm and in slightly higher concentrations, commensurate with a 100% electron-transfer process for $N_3^•$, rather than mainly electron transfer together with a small amount (~ 15%) of

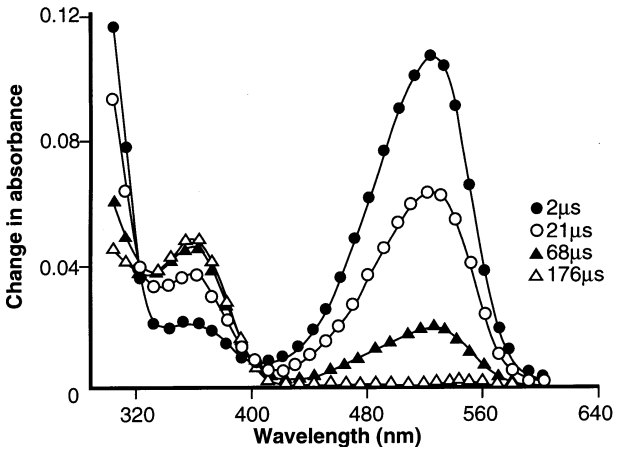


**Figure 4 Reaction of one-electron-oxidized ergothioneine with vitamin C to give the ascorbyl radical**

Changes in the absorption spectrum after pulse radiolysis of aqueous 0.1 mM ergothioneine and 0.3 mM ascorbic acid are shown. The solution also contained 0.05 mM $NaN_3$ and 0.01 mM phosphate buffer, pH 7.1, and was flushed with nitrous oxide. The dose was 13 Gy.

addition on reaction with $OH^{\bullet}$. This $OH^{\bullet}$ addition is probably the cause of the small transient peaks observed at 340 nm (see Figure 2). The build-up at 520 nm yielded a rate constant for $N_3^{\bullet}$ + ergothioneine of $8.2 \times 10^9$ $M^{-1} \cdot s^{-1}$. This absorption in turn decayed by a process following second-order kinetics, with $2k/\epsilon_{520} = 2.7 \times 10^5$ $cm \cdot s^{-1}$. If it is assumed that $N_3^{\bullet}$ does in fact react with ergothioneine exclusively by electron transfer, and that the $G$ value for $N_3^{\bullet}$ production in $N_2O$-saturated aqueous 0.05 M $NaN_3$ is 0.58 $\mu M \cdot G^{-1}$, then the molar absorption coefficient for the one-electron-oxidized ergothioneine radical at 520 nm is 7400 $M^{-1} \cdot cm^{-1}$. This then leads to a second-order rate constant for decay of the cation of $2k = 2.0 \times 10^9$ $M^{-1} \cdot s^{-1}$.

As with $OH^{\bullet}$, the transient intermediates formed on reaction of $N_3^{\bullet}$ with 2-mercaptoimidazole (Figure 3b), *S*-methylergothioneine (Figure 3c) and *S*,*N*-dimethylergothioneine (Figure 3d) were studied. Very similar initial transient absorption spectra were obtained in comparison with $OH^{\bullet}$ attack, each being assigned to exclusive one-electron oxidation by $N_3^{\bullet}$. From the corresponding oscillograms of the build-up observed at 520 nm, rate constants of $8.4 \times 10^9$, $8.7 \times 10^9$ and $5.8 \times 10^9$ $M^{-1} \cdot s^{-1}$ respectively were obtained for $N_3^{\bullet}$ with 2-mercaptoimidazole, *S*-methylergothioneine and *S*,*N*-dimethylergothioneine. Furthermore, based on the same assumption as used for ergothioneine itself with regard to the $G$ values for the production of $N_3^{\bullet}$ in $N_2O$-saturated aqueous 0.05 M $NaN_3$, molar absorption coefficients at 520 nm for the one-electron-oxidized radicals of 2-mercaptoimidazole, *S*-methylergothioneine and *S*,*N*-dimethylergothioneine of 7600, 5900 and 5900 $M^{-1} \cdot cm^{-1}$ respectively were obtained. These in turn led to second-order rate constants for decay of these cations of $2k = 2.3 \times 10^9$, $1.5 \times 10^9$ and $1.5 \times 10^9$ $M^{-1} \cdot s^{-1}$ respectively.

Although no stable product analysis was performed, it is clear that, after the decay of the 520 nm species, longer-lived products were detected in the region 280–360 nm for ergothioneine and analogues after both $OH^{\bullet}$ and $N_3^{\bullet}$ attack. Since disulphide formation is unlikely to result from oxidation of *S*-methyl- and *S*,*N*-dimethyl-ergothioneine, it may be that different types of products are formed with the different ergothioneines. For example, dimers could result from the combination of radicals at the C-4 positions. Positive identification of the products could lead to their use as measures of oxidative stress *in vivo*.

## Reaction with $CCl_3O_2^{\bullet}$

The trichloromethylperoxyl radical, involved in carbon tetrachloride hepatoxicity [33], has a reduction potential ($E_7$) of $\geqslant$ 1.1 V, but $<$ 1.3 V from its lack of reactivity with deoxyadenosine 5′-monophosphate [34]. The corresponding $E_7$ values for $OH^{\bullet}$ and $N_3^{\bullet}$ are 1.90 and 1.33 V respectively [35].

The initial transient absorption spectrum after reaction of $CCl_3O_2^{\bullet}$ with ergothioneine was very similar to that observed after $N_3^{\bullet}$ attack (see Figure 3a). No evidence was obtained for an intermediate ergothioneine–$CCl_3O_2^{\bullet}$ adduct corresponding to those found when carotenoids react with $CCl_3O_2^{\bullet}$ [36], which ultimately lead to carotene radical cations [37]. The rate constant for the reaction of $CCl_3O_2^{\bullet}$ + ergothioneine was determined to be $1.2 \times 10^9$ $M^{-1} \cdot s^{-1}$, surprisingly double the estimate previously obtained by Jovanovic and Simic ($6.3 \times 10^8$ $M^{-1} \cdot s^{-1}$ [38]).

**Table 1 Kinetic and absorption properties at pH 7 of ergothioneine and its analogues, and of derived radicals**

| | Parent compounds | | | One-electron-oxidized radicals | | | |
|---|---|---|---|---|---|---|---|
| | Rate constant $k$ ($M^{-1} \cdot s^{-1}$) for reaction with: | | | Absorption maximum (nm) | Molar absorption coefficient ($M^{-1} \cdot cm^{-1}$) | Bimolecular decay rate constant $2k$ ($M^{-1} \cdot s^{-1}$) | Rate constant $k$ ($M^{-1} \cdot s^{-1}$) for reaction with ascorbate |
| | $OH^{\bullet}$ | $N_3^{\bullet}$ | $CCl_3O_2^{\bullet}$ | | | | |
| Ergothioneine | $1.2 \times 10^{10}$ | $8.2 \times 10^9$ | $1.2 \times 10^9$<br>$6.3 \times 10^{8*}$ | 520 | $7.4 \times 10^3$ | $2.0 \times 10^9$ | $6.7 \times 10^7$ |
| *S*-Methylergothioneine | $1.8 \times 10^{10}$ | $8.7 \times 10^9$ | – | 520 | $5.9 \times 10^3$ | $1.5 \times 10^9$ | – |
| *S*,*N*-Dimethylergothioneine | $1.5 \times 10^{10}$ | $5.8 \times 10^9$ | – | 520 | $5.9 \times 10^3$ | $1.5 \times 10^9$ | – |
| 2-Mercaptoimidazole | $1.2 \times 10^{10}$ | $8.4 \times 10^9$ | – | 520 | $7.6 \times 10^3$ | $2.3 \times 10^9$ | $6.0 \times 10^7$ |
| 1-Methyl-2-mercaptoimidazole (methimazole) | $1.36 \times 10^{10}$† | $8.6 \times 10^9$ | $6.5 \times 10^{8*}$ | 510† | $6.1 \times 10^3$ | $3.9 \times 10^9$ | $5.3 \times 10^7$ |

* From [38].
† From [30].

Interestingly, these authors report a rate constant of $1.5 \times 10^8\ M^{-1} \cdot s^{-1}$ for the reaction of the guanyl radical ($E_7 = 1.04$ V [39], generated from deoxyguanosine monophosphate) with ergothioneine. They also measured a rate constant for the reaction of guanyl radicals with glutathione, considered to be one of the most important biological reductants, of $1.0 \times 10^7\ M^{-1} \cdot s^{-1}$, i.e. over an order of magnitude lower than that observed with ergothioneine. The above results are consistent with ergothioneine being a very good reductant, and suggest an upper limit of $\sim 1$ for its reduction potential, just below the reduction potential of the guanyl radical (1.04 V) [39].

### Redox reactions of oxidized ergothioneine

We have investigated the reaction of the one-electron-oxidized form of ergothioneine with the common antioxidant vitamin C. The $N_2O$-saturated aqueous 0.05 M $NaN_3$ solution used to study this reaction contained 1 mM ergothioneine and 0.3 mM ascorbic acid. Taking account of these concentrations and of the previously measured [40] rate constant for the reaction of $N_3^{\bullet}$ with ascorbic acid ($2.9 \times 10^9\ M^{-1} \cdot s^{-1}$), and the rate constant for the reaction of $N_3^{\bullet}$ with ergothioneine measured herein ($8.2 \times 10^9\ M^{-1} \cdot s^{-1}$), about 90 % of the azide radicals react initially with ergothioneine. As shown in Figure 4, these ergothioneine radicals ($\lambda_{max}$ at 520 nm) then decay, under the present conditions, by a process following pseudo-first-order kinetics to produce the ascorbyl radical ($\lambda_{max}$ at 360 nm). Clear isobestic points at 320 and 400 nm are apparent. A bimolecular rate constant of $6.7 \times 10^7\ M^{-1} \cdot s^{-1}$ was obtained for the repair of the ergothioneine radical by ascorbate. The corresponding rate constants for repair of the 2-mercaptoimidazole and methimazole radicals by ascorbate were $6.0 \times 10^7$ and $5.3 \times 10^7\ M^{-1} \cdot s^{-1}$ respectively. In a similar approach, the repair of the phenoxyl radical derived from vitamin E, or from Trolox, by ascorbate, leading to the formation of an ascorbyl radical, has been directly observed via pulse radiolysis experiments [27,41]. Our results indicate an additional co-operative interaction between ascorbate and ergothioneine, which can participate in essential biological redox protection.

### Concluding remarks

All the physicochemical properties of ergothioneine and its derivatives, and their one-electron-oxidized forms, determined during the course of these studies are summarized in Table 1, together with the few data measured in earlier work. These new data will facilitate further investigations of the redox properties of ergothioneine, an important chemoprotector present at up to millimolar levels in humans, in particular, interactions with other antioxidants besides ascorbate.

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