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Kinetics and Mechanism of the Reaction of a Nitroxide Radical (Tempol) With a Phenolic Antioxidant

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In the absence of redox-active transition metal ions, the removal of Tempol by Trolox occurs by a simple bimolecular reaction that, most probably, involves a hydrogen transfer from phenol to nitroxide. The specific rate constant of the process is small ($0.1\,M^{-1}\,s^{-1}$). Metals can catalyze the process, as evidenced by the decrease in rate observed in the presence of diethylenetriaminepentaacetic acid (DTPA). Furthermore, addition of Fe(II) (20 μM ferrous sulfate and 40 μM EDTA) produces a noticeable increase in the rate of Tempol consumption.

Keywords: Tempol; Trolox; Kinetic; Metals effect; Hydrogen transfer

INTRODUCTION

Nitroxide radicals are one of the most extensively employed families of stable free radicals. Due to their special characteristics, they are widely employed as spin probes,[1] as final products of spin trap experiments,[2] and as antioxidants in several experimental models.[3,4] The measurement of nitroxide radicals in spin trap experiments relies on the stability of these radicals. An understanding of the reactivity and stability of these radicals is then fundamental to allow a quantitative evaluation of free radicals in biological systems.[5–7] In spite of this, there are few kinetic studies of the reactions of nitroxide radicals with non-radical substrates and of the effect of metals upon these reactions.

Kinetic data on the reaction of nitroxide radicals with ascorbic acid[5,8,9] and hydroxylamines[10,11] have been obtained. Also, there are reports of a fast reaction between nitroxide radicals and ubiquinol-9[6] and partial reports on the capacity of nitroxide radicals to mediate redox processes with metal ions.[12,13] The stability of nitroxide radicals is due to rather unfavorable redox processes and/or the weakness of the O–H bond in hydroxylamines.[14] Potential substrates are then strong reductants, strong oxidants, and/or compounds bearing relatively weak hydrogen linkages. Among these compounds stand phenols and thiols. Reaction of these compounds with nitroxide radicals in the existing reports are conflicting. Lissi *et al.* have reported that, in organic solvents, phenols are unreactive towards nitroxide radicals and that the kinetics of the reaction of thiols is complex, with evidences of metal catalysis.[11] Similar results have been reported by Filkenstein *et al.*[15] On the other hand, there are reports of a rather fast reaction of Tempo in aqueous media with phenolic and thiol antioxidants.[16] In particular, it has been reported a nitroxide has a half life of 1 h when the Trolox

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concentration is 20 mM. Since the observed reactions could be due to catalysis by contaminant transition metal ions (i.e. Fe or Cu ions),[15] we have performed a detailed kinetic study of the reaction of the water-soluble nitroxide radical, Tempol with Trolox. This type of reaction could be a source of potentially harmful phenoxyl radicals, and could prove a plausible mechanism to explain nitroxide promoted cytotoxicity.[17]

MATERIALS AND METHODS

Tempol and Trolox were Sigma products employed as received. Some experiments were carried out employing a Trolox sample that has been purified twice by recrystallization by acidification of a concentrated solution prepared at high pH. The purity of the samples was assessed by high performance liquid chromatography (HPLC) in a Inertsil ODS-2 C18 column employing methanol/phosphate buffer, pH 7.0 (330/70, V/V) mobile phase, and a diode arrange absorption detector. Tempol-derived hydroxylamine was prepared by reduction of the nitroxide with ammonium chloride/Zn.[18]

A stock solution of Tempol was prepared in 0.1 M phosphate buffer (pH 7.0). The concentration was evaluated spectrophotometrically ($\varepsilon = 1440\,M^{-1}\,cm^{-1}$ at 240 nm).[19] An aliquot of this solution was mixed with buffer to the working concentration in the micromolar range. All solutions were prepared with water pretreated (overnight) with Chellex 100 in order to minimize the concentration of contaminant metal ion. Furthermore diethylenetriaminepentaacetic acid (DTPA) (0.1 mM) was added to some solutions.

Tempol concentration was monitored by the intensity of the low field peak of its EPR signal, measured at 3426 G. Measurements were carried out at room temperature (23 ± 1°C) in a Bruker ER 200 D-SRC spectrometer. The intensity of the signal was directly related to the spin concentration. A concentration calibration was performed by double integration of the whole spectra prior to the addition of the reactive and at the end of the experiment. In some experiments, the kinetics of the process was followed monitoring Trolox concentration by HPLC.

RESULTS

Addition of Trolox (10 mM) readily reduces the intensity of the ESR signal from Tempol (10 μM) without any change in its shape. Tempol consumption as a function of the reaction time, obtained in the absence and in the presence of added DTPA, is shown in Figure 1. The data shows that the chelating agent reduces the reaction rate. All kinetic measurements were then carried out in water pretreated with Chellex 100 and in the presence of 0.1 mM DTPA.

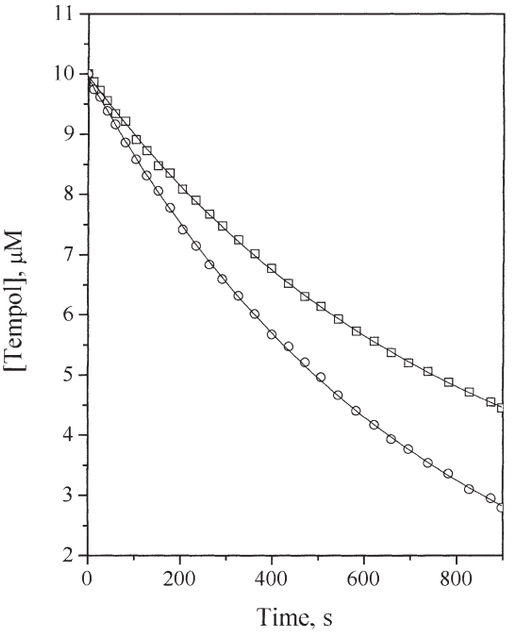

FIGURE 1 Effect of DTPA (0.1 mM) addition on the rate of Tempol (10 μM) consumption promoted by 10 mM Trolox addition, pH = 7.0. Experiments carried out in a buffer solution that has not been pretreated with Chellex 100. (○) Control experiment without DTPA; (□) experiment in presence of DTPA.

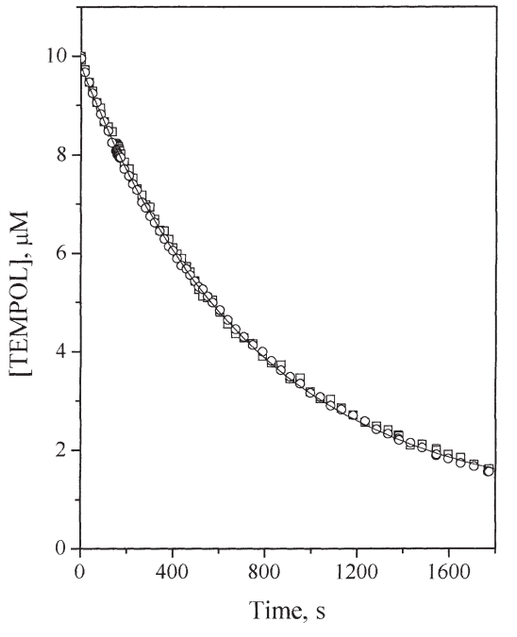

FIGURE 2 Effect of oxygen removal on the rate of Tempol consumption. (○) Experiment in presence of air; (□) experiment in nitrogen-purged solution. The fit corresponds to a monoexponential decay.

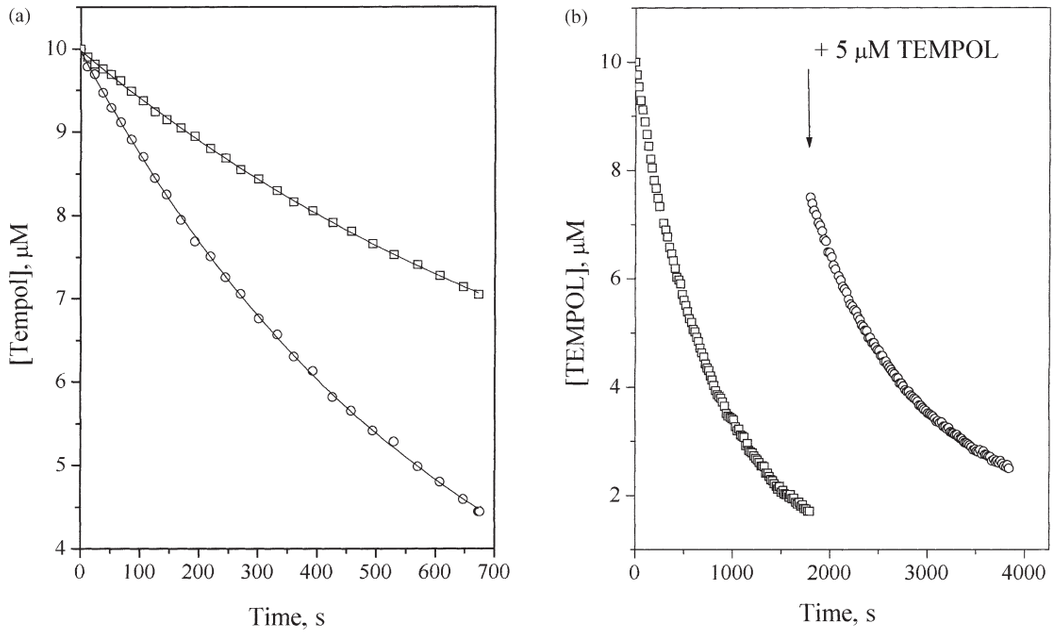

FIGURE 3 (A) Effect of 4-hydroxy-2,2,6,6-tetramethyl-1-piperinylhydroxylamine (20 μM) on the reaction of Tempol (10 μM) with Trolox (10 mM). (○) Control without the hydroxylamine; (□) experiment in presence of the hydroxylamine. (B) Effect of the addition of fresh 5 μM Tempol after its almost total consumption by 10 mM Trolox.

The kinetic data obtained in excess Trolox, can be fitted to a monoexponential decay (see Figure 2). The measured Tempol consumption is independent of the oxygen concentration. Although it can be fitted to a monoexponential decay, the data analysis revealed a small residual signal (ca. 10%). A better fit of the data shown in Fig. 2 is given by

$$[\text{Tempol}](\mu\text{M}) = 0.91 + e^{0.0014t} \qquad (1)$$

with t in seconds, indicating that the reaction shows some reversibility that could be ascribed to the accumulation of the hydroxylamine. Indeed, an initial addition of hydroxylamine (20 μM) reduces the rate of the reaction (Figure 3A). Similarly, addition of a new Tempol aliquot after its almost total consumption renders a slower kinetics and a higher residual (Figure 3B). While the initial consumption takes place with a rate constant of $1.25 \times 10^{-3}\,s^{-1}$, and renders 0.74 μM as extrapolation to infinite time, the decay of a second Tempol aliquot occurs with a constant of $0.96 \times 10^{-3}\,s^{-1}$, and leaves 1.7 μM Tempol when extrapolated to infinite time.

The reaction between Tempol and Trolox was further analyzed by its rate dependence on reagent concentrations. The dependence of the initial rate with Trolox concentration at fixed Tempol (10 μM) is shown in Figure 4, while the dependence of the initial rate with Tempol at fixed Trolox (10 mM) is shown in Figure 5. The slopes obtained were 0.94 ± 0.14 and 0.95 ± 0.06, respectively. Under all employed experimental conditions, the initial rate at pH 7.0 can then be expressed by

$$-\frac{d}{dt}[\text{Nitroxide}] = k[\text{Trolox}][\text{Tempol}] \qquad (2)$$

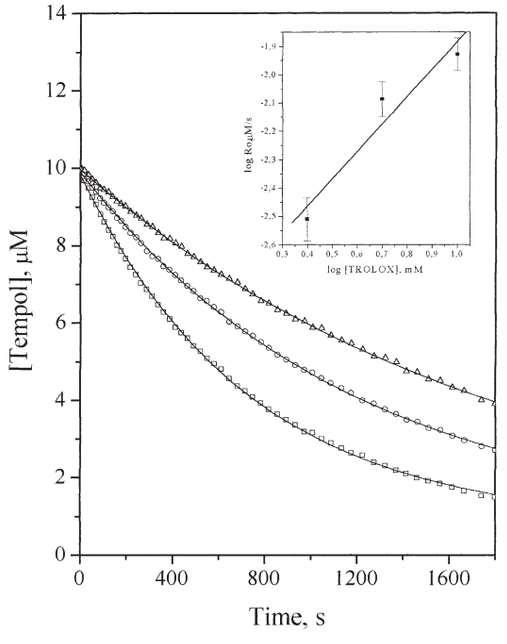

FIGURE 4 Dependence of the pseudo unimolecular rate constant as a function of Trolox concentration, measured at fixed (10 μM) Tempol concentration. (Δ) 2.5 mM Trolox; (○) 5 mM Trolox; (□) 10 mM Trolox. Inset shows the dependence of the log of the initial rate plotted as a function of the log of Trolox concentration.

with

$$k = 0.1\,\mathrm{M^{-1}\,s^{-1}}$$

This rate equation, as well as the lack of oxygen effect, is compatible with a reaction taking place by a simple bimolecular step, such as

$$\mathrm{RNO^{\cdot}} + \mathrm{PhOH} \rightarrow \mathrm{RNOH} + \mathrm{PhO^{\cdot}} \qquad (3)$$

followed by secondary reactions involving the phenoxyl radicals. The predominant formation of the corresponding hydroxylamine was confirmed by its oxidation to the parent nitroxide elicited by the addition of potassium ferricyanide at the end of the experiment. Addition of an excess of this oxidant regenerates ca. 90% of the initial nitroxide signal. This shows that the main fate of phenoxyl radicals are their complex self reactions,[20] and not a cross reaction involving a phenoxyl and a nitroxide radical. However, an initial small contribution of a reversible cross reaction, such as that proposed for phenoxyl radicals and nitroxides[21] cannot be completely disregarded.

In order to discard a significant consumption of the nitroxide by a small impurity present in the Trolox solution, experiments were performed employing a purified Trolox sample and nearly stoichiometric concentrations of both reactants (Trolox, 2 mM; Tempol, 5 mM). Trolox consumption was followed by HPLC. The rate constant, extrapolated to low reaction times, was similar to that obtained employing 10^3 times lower Tempol concentrations.

The bimolecular reaction between the nitroxide and the phenol can correspond to a hydrogen abstraction (such as that depicted by Reaction (3)) or can be due to an electron transfer such as

$$\mathrm{RNO^{\cdot}} + \mathrm{PhO^{-}} \rightarrow \mathrm{RNO^{-}} + \mathrm{PhO^{\cdot}} \qquad (4)$$

followed by protonation of the nitroxide anion to the corresponding hydroxylamines. If reaction (4) was the dominant reaction path, it can be expected that, at pHs considerably below the phenol p*K*a (11.8), the rate would be inversely proportional to the proton concentration. The effect of pH on the initial rate is shown in Figure 6. These data show a very minor dependence of the initial reaction rate with the pH (order in protons ca. −0.05), in contradiction with the order expected (−1.0) for a process such as that depicted by Reaction (4). This would suggest that the main reaction path corresponds to a hydrogen abstraction, as that represented by reaction (3).

Another possibility is a reaction between a small amount of the protonated nitroxide and the phenolate:

$$\mathrm{RNOH^{\cdot +}} + \mathrm{PhO^{-}} \rightarrow \mathrm{RNOH} + \mathrm{PhO^{\cdot}} \qquad (5)$$

The rate of this process should also be nearly independent of pH. The specific rate constant of Reaction (5) is related to the experimentally

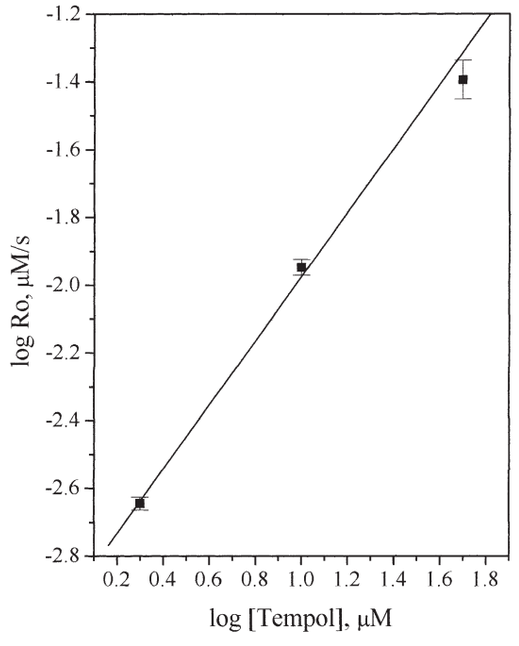

FIGURE 5 Dependence of the initial rate of Tempol consumption, measured at fixed (10 mM) Trolox concentration, with the initial Tempol concentration. The data are plotted as log Rate vs. log [Tempol].

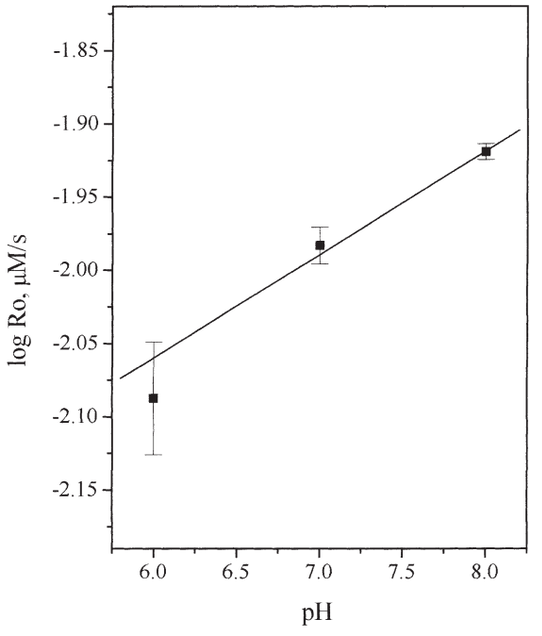

FIGURE 6 Dependence of the initial rate of Tempol consumption, measured at fixed Tempol (10 μM) and Trolox (10 mM) concentrations, with pH.

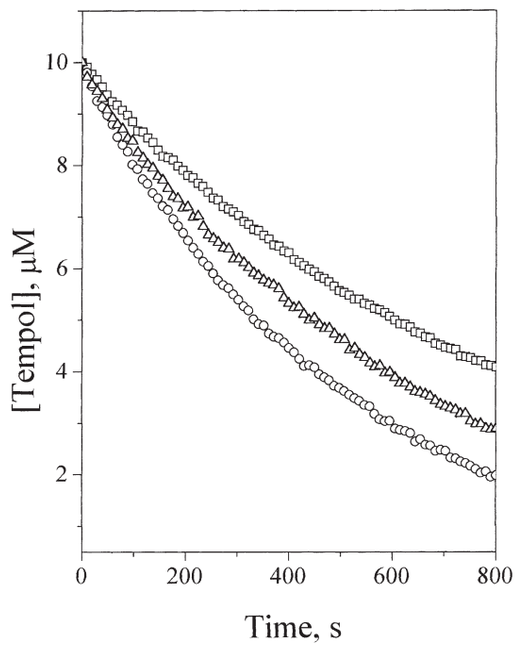

FIGURE 7 Effect of Fe(II) addition on the consumption of Tempol elicited by Trolox (10 mM) addition. All solutions were prepared in water pretreated with Chellex 100. (□) Without added Fe; (Δ) in presence of Fe(II) (20 μM ferrous sulfate and 40 μM EDTA); (○) in presence of Fe(II) (40 μM ferrous sulfate and 80 μM EDTA); no consumption was observed in the absence of Trolox irrespective of the added Fe.

determined second order rate constant (k) by

$$\log k_5 = \log k + |\Delta pK_{\text{Trolox}}| + |\Delta pK_{\text{Tempol}}| \qquad (6)$$

where $|\Delta pK|$ corresponds to the absolute value of the difference between the pK considered and the working pH. Taking 11.8 and 0 as the pKa values of Trolox and the hydroxylamine,[19] the Reaction (6) leads to

$$\log k_5 = \log k + 11.8$$

and hence

$$\log k_5 \approx 10.8\,(\text{M}^{-1}\,\text{s}^{-1})$$

This estimate does not completely disregard the occurrence of Reaction (5), but this process can explain the present data only if the reaction is in the diffusion-controlled limit.

Our results indicated that, in the absence of redox-active transition metal ions (solvent treated with Chellex 100 and in the presence of DTPA), the removal of Tempol by Trolox occurs by a simple bimolecular reaction that, most probably, involves the protonated phenol and the nitroxide. Metals can catalyze this process, as evidenced by the decrease in rate observed in the presence of DTPA (Figure 1). Furthermore, addition of Fe(II) (20 μM ferrous sulfate and 40 μM EDTA) produces a noticeable increase in the rate of Tempol consumption (Figure 7). This could be explained in terms of redox reactions of the nitroxide radicals.[22] The rate constant obtained in the absence of redox-active transition metal ions is small ($0.1\,\text{M}^{-1}\,\text{s}^{-1}$). This would imply that, in the absence of catalyzers, removal of nitroxide radicals by phenolic antioxidants would not be competitive to other processes, such as their removal by ascorbic acid.[5,8]

Acknowledgments

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