Kinetics of Nitroxide Reaction with Iron(II)

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Abstract: Like superoxide dismutase (SOD), nitroxide stable radicals can catalyze the dismutation of superoxide radicals and provide protection against oxidative stress. The SOD-mimic activity of nitroxides prompted the study of their biological effects; however, accumulated evidence suggested that the reactions of nitroxide with redox-active transition metals play an important role in its antioxidative activity. The present study concentrated on the kinetics of the reaction of iron(II) coordinated to various ligands with piperidinyl and pyrrolidinyl nitroxides [nitroxide + H^+ + Fe(II) $\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$ hydroxylamine + Fe(III)]. The effects of pH, temperature, buffer, and ligands on the reaction kinetics were examined. UV−Vis absorption spectroscopy and electron paramagnetic resonance spectrometry were used to follow the change in the concentrations of iron and the nitroxide, respectively. The results show that under physiological conditions: (a) equilibrium 1 is shifted to the right; (b) OH^- catalyzes the oxidation of iron and shifts the equilibrium further to the right; (c) phosphate, even at the submillimolar range, greatly catalyzes the reaction; and (d) piperidinyl nitroxides are more effective than pyrrolidinyl derivatives in oxidizing iron. The results imply that nitroxides rapidly and effectively oxidize iron, and most likely copper too, in the pool of chelatable redox-active metals in cells and tissues. This oxidation prevents the Fenton chemistry, suppresses the oxidative injury, and explains the narrow time-window anti-oxidative activity of nitroxides observed using cellular, animal, and organ experimental models.

Introduction

Transition metals and reactive oxygen- (and nitrogen-) derived species play a major role in mediating biological damage. Therefore, chelating agents, anti-oxidative enzymes, and low molecular weight anti-oxidants are generally employed to protect against oxidative stress. An alternative strategy of protection employs nitroxide antioxidants. Nitroxides are widely used as biophysical probes to study structure and dynamics of cellular components,[1] to assess perfusion, oxygenation, and tissue viability,[2−4] and to serve for three-dimensional EPR (electron paramagnetic resonance) imaging[5] as well as contrast agents for magnetic resonance imaging.[6] Nitroxides in cells and tissues undergo rapid one-electron enzymatic reduction to yield their respective sterically hindered cyclic hydroxylamines.[7] Also, several cellular components such as ascorbate, though not NAD(P)H, can directly reduce nitroxides.[7,8] Sulfhydryl compounds, which were reported to reduce nitroxides, have been later found to do it only through the mediation of metal−ion impurities.[8] On the other hand, iron(II) or Cu(I) within the tissue pool of chelatable metals can directly reduce nitroxides through reaction 1:

$$\text{nitroxide} + H^+ + \text{Fe(II)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{hydroxylamine} + \text{Fe(III)} \quad (1)$$

More than a decade ago, Rosen et al. reported that superoxide can oxidize the hydroxylamine of 2,2,5-trimethyl, 5-ethyl oxazolidine-*N*-oxyl and employed this reaction to detect superoxide.[9] The reoxidation of hydroxylamine to nitroxide has been studied also for piperidinyl derivatives,[10−11] but considering the reaction rate constant and the steady-state concentration of superoxide within cells, this oxidation in tissues would be relatively slow.[11] Later, nitroxide reduction by $O_2^{\bullet-}$ to its respective hydroxylamine has also been demonstrated.[12] This finding supports a proposed mechanism which accounts for the catalytic removal of $O_2^{\bullet-}$ by the redox couple nitroxide/hydroxylamine.[12] More recently, accumulated evidence suggested an additional reaction pathway, which underlies the removal of $O_2^{\bullet-}$ by piperidinyl nitroxides. According to this mechanism, the dismutation of $O_2^{\bullet-}$ radicals takes place through

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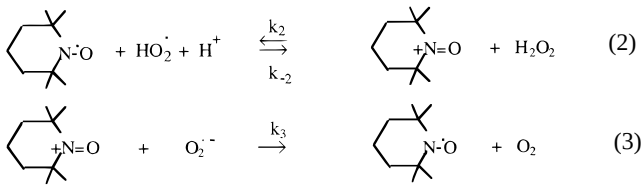
their reaction with an alternative redox couple, namely, the nitroxide/oxo-ammonium cation,[13−14] as presented below for 2,2,6,6-tetramethyl-piperidinoxyl (TPO).



The capability of cell-permeable nitroxides to act as SOD-mimics prompted an extensive investigation of their potential anti-oxidative activity.[15] Indeed, five- and six-membered ring nitroxides have been found to protect diverse biological systems from oxidative damage induced by different insults,[15−21] while demonstrating only little adverse effects.[22] Apparently, such protective effects could have been attributed to the removal of both extra- and intracellular $O_2^{\bullet-}$.

However, nitroxides have been found to protect various biological systems also from superoxide-independent injury. Therefore, additional mechanisms underlying the cytoprotective activity of nitroxides have been considered, including a detoxification of intracellular radicals, such as, alkyl, alkoxyl, and peroxyalkyl radicals,[16,23] and oxidation of adventitious redox-active metal ions, thus preventing them from undergoing Fenton reaction that elicits $^{\bullet}$OH-induced damage. Since this reaction involves reduced metal ions, the cytoprotective effect of nitroxides against injury induced by H_2O_2 and $O_2^{\bullet-}$ is most likely associated with the oxidation of reduced metals.[24]

The present work centers on the kinetics of nitroxide reaction with reduced iron in an attempt to correlate their reactivity toward metals with their anti-oxidative activity. Since most of the nitroxide radicals, administered to a tissue or a whole animal are reduced within several minutes, reaction 1 should be relatively fast in order to play any significant role in protection from oxidative stress. The present results demonstrate the reversibility of the reaction and its dependence on temperature, pH, phosphate concentration, and type of metal ligand.

Materials and Methods

The nitroxides 4-OH-2,2,6,6-tetramethyl-piperidinoxyl (TPL), 4-amino-2,2,6,6-tetramethyl-piperidinoxyl (TPA), 2,2,6,6-tetramethyl-piperidinoxyl (TPO), 3-carbamoyl-2,2,5,5,-tetramethylpyrrolidinoxyl (carbamoyl-proxyl), and 3-carboxy-2,2,5,5-tetramethylpyrrolidinoxyl (carboxy-proxyl) were obtained from Aldrich. Adenosine 5-diphosphate (ADP), 3-[*N*-morpholino]propanesulfonic acid (MOPS), bovine serum albumin (BSA), nitrilotriacetic acid (NTA), catalase, ethylenediamine tetraacetic acid (EDTA), and 2-[*N*-morpholino]-ethanesulfonic acid (MES) were obtained from Sigma. Ferrous sulfate and ferric chloride were obtained from BDH chemicals. Ferrous ammonium sulfate was obtained from Williams. Ferric ammonium sulfate and sodium citrate were obtained from Baker Chemical Co. All chemicals were used without further purification. Solutions were prepared with water that was passed through a Milli-Q water purification system.

Hydroxylamine Preparation. The reduced form of TPL, 4-OH-2,2,6,6,-tetramethyl-*N*-hydroxypiperidine (TPL-H), was prepared by catalytic reduction using H_2 bubbling over Pt powder or by bubbling HCl gas through an ethanolic solution of the respective nitroxide until a complete disappearance of the yellow color, followed by drying. The completion of the reduction was checked using EPR spectroscopy (99.8%). TPL-H was kept solid until used.

Iron Solution. Solutions of ferrous ammonium sulfate dissolved with BSA or other ligands in deionized water, continuously flushed with N_2 gas, were freshly prepared before each experiment to minimize auto-oxidation of iron(II). In several experiments the iron solution was not deaerated but the concentration of iron(II) was 100-fold greater than that of oxygen; thus, any oxidation of iron(II) would have been negligible.

Spectrophotometry. The formation of iron(III) was followed spectrophotometrically at 340 or 350 nm, using a dual beam Uvikon-860, Kontron (Switzerland) spectrophotometer. Unless otherwise stated, the pH was adjusted using 20−50 mM MOPS. A sufficient excess of bovine serum albumin (BSA) or other ligand was always added in order to coordinate the iron, maintain it in solution, and minimize its hydrolysis and precipitation. Pseudo-first-order conditions were achieved by keeping [TPL]/[iron(II)] $\geq$ 10. The change of optical density with time was monitored, and $\Delta OD = OD_\infty - OD_t$ was calculated where OD_∞ achieved was time-invariable, indicating that the reaction has ended. The observed pseudo-first-order rate constant (k_{obs}) was evaluated from the exponential dependence of ΔOD on time. The experiment was repeated with various nitroxide concentrations, and the second-order rate constant was determined from the dependence of k_{obs} on [nitroxide]. The standard error of the mean did not exceed 10% of the evaluated values.

EPR Spectroscopy. The oxidation of iron(II) was also studied by following the spin loss of the nitroxide. Pseudo-first-order reaction conditions were kept by maintaining a sufficiently large excess of iron over the nitroxide. During the experiments, the solutions were flushed with N_2 gas, and samples were drawn into a gas permeable Teflon capillary of 0.81 mm inner diameter and of 0.38 mm wall thickness. The capillary was folded twice, inserted into a quarts tube open at both ends, and placed within the EPR cavity. Alternatively, the samples were introduced through an injection port into a capillary permanently fitted inside a quartz tube held within the EPR cavity and flushed with N_2. At the end of each experiment the solution was removed by suction, and the capillary was washed with water followed by a flow of dry gas. The EPR spectrometer (JEOL X-band JES−RE3X) was operated at 3362 G center field, 100 kHz modulation frequency, 1 G modulation amplitude, and 4 mW microwave power.

Results

The Reaction of Nitroxide with Iron. Iron ions in the chelatable pool of redox-active metals in plasma or cells are not present unbound but rather coordinated to biological components. Because serum albumin is a major component of plasma, the oxidation kinetics of iron(II) by nitroxide in the presence of BSA were studied. The reduced form of nitroxide, the hydroxylamine, can be reoxidized by oxygen, H_2O_2, and superoxide,[10−11] yet these reactions are relatively slow.[11] Since oxygen can also oxidize iron(II) ions directly, it was removed from the reaction system in order to distinguish the oxidation of iron by nitroxide from that by oxygen. In a typical experiment, iron(II) was mixed with a 1:10 excess of nitroxide in MOPS buffer containing BSA at 30 °C, and the reaction was

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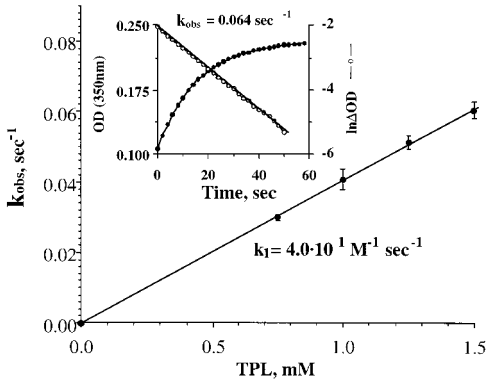

Figure 1. Iron(II) oxidation by nitroxide is first-order with respect to both reactants. The reaction of iron(II) with TPL in the presence of 25 μg/mL BSA in 20 mM MOPS buffer pH 7 at 30 °C was followed spectrophotometrically by monitoring at 350 nm the appearance of iron-(III). Time dependence of ΔOD and of $\ln\{OD_\infty - OD_t\}$ observed using 1.5 mM TPL is demonstrated in the inset. The pseudo-first-order rate constant, k_{obs}, was evaluated, the reaction was repeated at various concentrations of TPL, maintaining [TPL]/[iron] $\geq$ 10, and the bimolecular rate constant k_1 was evaluated.

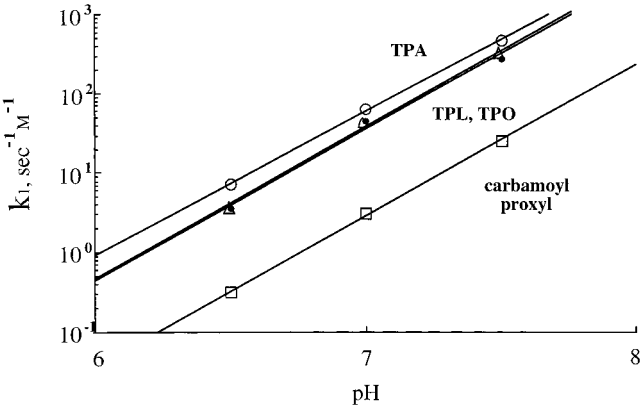

Figure 2. The bimolecular rate constant for the oxidation of iron(II) by nitroxide linearly depends on [OH$^-$]. Values of k_1 for the reaction of iron(II) in the presence of 25 μg of BSA/mL in 50 mM MOPS at 30 °C with TPL (solid circles), TPO (triangles), TPA (open circles), and carbamoyl-proxyl (squares), at several pH values, were determined, as detailed in Figure 1.

followed spectrophotometrically at 350 nm. Under such experimental conditions the reaction obeyed a first-order kinetics with respect to [iron(II)], and the pseudo-first-order rate constant, k_{obs}, was determined. The reaction was repeated using various nitroxide concentrations, and values of k_{obs} were calculated and plotted vs [nitroxide], as seen in Figure 1 for TPL at pH 7 and 30 °C. From the linear graph, the second-order rate constant, k_1, was calculated.

Effect of Temperature, pH, and BSA. The oxidation of iron-(II) by TPL was repeated at several pH values and at various temperatures and concentrations of BSA. Values of k_1 at several temperatures were determined, and $\ln\{k_1\}$ was plotted vs $1/T$ (data not shown). The activation energy was evaluated as 37.3 and 46.6 kJ·mol^{-1} for the reaction at pH 6 and 7, respectively. The pH dependence of the rate of iron(II) oxidation by TPL is displayed in Figure 2 and demonstrates that k_1 increases as the pH increases.

Since BSA binds iron nonspecifically in several sites, the kinetics of iron(II) reaction with TPL in the presence of various concentrations of BSA were examined. The results presented in Tables 1 and 2 show that k_1 slightly increases as the protein concentration increases.

Table 1. The Effect of pH on the Rate of Fe(II) Oxidation by Pyrrolidinyl nitroxides[a]

nitroxide \ pH	pH 6.5	pH 7.0	pH 7.5
carboxy-proxyl	12.9	13.1	22.4
carbamoyl-proxyl	49.0	54.6	79.7

[a] The second-order rate constant k_1 (M^{-1} s^{-1}) of Fe(II) oxidation in the presence of 1 mM ADP in 20 mM MOPS at 30 °C by nitroxides was determined at several pH values, as detailed in the legend for Figure 1. The standard error of the mean did not exceed 10%.

Table 2. The Effect of BSA and Phosphate on the Rate of Fe(II) Oxidation by TPL[a]

phosphate, mM	0	0	0	0	0.1	0.5	1
BSA, mg/mL	0	0.01	0.5	1	25	25	25
k_1, M^{-1} s^{-1}	0.63	0.68	0.80	1.1	1.0	8.7	19.5

[a] The second-order rate constant k_1 (M^{-1} s^{-1}) of Fe(II) oxidation in 20 mM MOPS, pH 6, at 20 °C by TPL was determined at several concentrations of either BSA or phosphate, as detailed in the legend for Figure 1. The standard error of the mean did not exceed 10%.

Effect of Phosphate. When MOPS was replaced by a phosphate buffer, the reaction of TPL with iron(II) in the presence of BSA was too fast to follow using conventional spectrophotometry. Therefore, the reaction was repeated using MOPS buffer supplemented with low concentrations of phosphate. At [phosphate] < 1.5 mM the reaction kinetics could be conventionally studied. Table 2 presents also the dependence of k_1 on [phosphate] at pH 6 and 20 °C, indicating that k_1 linearly increases with the increase of [phosphate].

Other Nitroxides. The kinetics of iron(II) oxidation by other two piperidinyl derivatives (TPO and TPA) as well as by pyrrolidine derivative (carbamoyl-proxyl) were studied, and the results are included in Figure 2. While their respective values of k_1 differ, their pH dependence appears to be the same.

Other Ligands. ADP is another physiological ligand which can bind iron in vivo. The oxidation of iron(II) in the presence of 1 mM ADP by the piperidinyl derivatives, TPL, TPO, and TPA, was also studied. However, the reaction was over immediately upon mixing and, therefore, too fast to follow by conventional spectrometry. Conversely, the reduction of the pyrrolidinyl nitroxides, carbamoyl proxyl and carboxy-proxyl, by iron(II) in the presence of ADP was slower. Hence, the kinetics of the reaction could be studied and the values of k_1 have been determined and are presented in Table 1.

To study the reaction of citrate-iron(II), we used sufficiently high concentrations of citrate in order to maintain the iron in a true solution. Otherwise, iron(III) would have progressively aggregated at neutral pH to form high molecular weight polymers.[25] Upon adding TPL, the oxidation of iron(II)-citrate was completed instantaneously and could not be followed by conventional spectrophotometry or by EPR spectrometry. Similar results were obtained using EDTA-Fe(II) at 1:1 metal/ligand ratio (data not shown).

It seemed relevant to study also the effect of NTA because this synthetic chelator is commonly used for loading iron in laboratory animals. NTA binds iron strongly enough to maintain it in solution but not too avidly to prevent it from coordination to cellular sites. For experiments using NTA, which forms a stable 2:1 complex with iron, an excess of the ligand was used. The oxidation of iron(II) by TPL, even at pH 6, was too fast, and the reaction could not be studied using conventional spectrophotometry.

The Equilibrium Constant. Following the spin loss of TPL upon reaction with iron(II) in the presence of NTA showed that

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Table 3. The pH Dependence on the Equilibrium Constant, K'_{eq}, of Fe(II) Reaction with TPL[a]

	MES[b]		phosphate[b]		
pH	5.7	6.4	5.7	6.4	7.1
K'_{eq}	1.07×10^2	7.1×10^1	2.2×10^4	9.2×10^3	3.7×10^3

[a] TPL was mixed under anoxia with an excess of iron(II) and NTA in 0.1 M phosphate or 0.1 M MES buffers at 25 °C. The reaction mixture was injected under anoxia into a capillary mounted in the EPR cavity, and the intensity of the EPR signal of the nitroxide was measured and compared with the respective signal monitored before the addition of iron. The apparent equilibrium constant $K'_{eq} = [H^+]K_{eq} = [TPL\text{-}H][Fe(III)]/\{[TPL][Fe(II)]\}$ was determined by calculating the equilibrium concentration of the various species taking into consideration their initial values and the changes in the total volume. The experiments were repeated using different initial [Fe(II)] and [Fe(III)] at several pH values.

the EPR signal did not decay completely even with a large excess of iron(II), indicating that an equilibrium state was achieved. For kinetic experiments EPR spectrometry suffers from a long "dead time" of about 30 s, which is required for the position of the reaction mixture in the EPR cavity. When we attempted to follow, using EPR spectrometry, the reverse direction of reaction 1 by mixing TPL-H with iron(III) in the presence of NTA, a time-invariant TPL signal was established immediately, indicating that an equilibrium was reached before the start of the measurement. On the other hand, it allows the detection of low residual concentration of nitroxide also at the submicromolar range and is useful for the determination of the reaction equilibrium constant. TPL was mixed under anoxia with an excess of iron(II) in the presence of NTA. The reaction mixture was injected under anoxia into the capillary mounted in the EPR cavity, and the final intensity of the EPR signal of the nitroxide was measured and compared with the respective signal monitored before the addition of iron. The value of K'_{eq}

$$K'_{eq} = [H^+]K_{eq} = [TPL\text{-}H][Fe(III)]/\{[TPL][Fe(II)]\} \quad (4)$$

was determined by calculating the equilibrium concentrations of the various species, while taking into consideration the initial values and changes in the total volume. The experiments were repeated using various initial concentrations of iron(II) and iron(III) at several pH values. The values estimated for K'_{eq} are presented in Table 3, indicating the large dependence on pH.

Discussion

The normal levels of redox-active, available iron and copper, which are not a structural moiety of a protein, are minute and consist only a small fraction of the total metal content in cells and tissues. However, this is the fraction which is responsible for the mediation and potentiation of oxidative damage. According to a simplistic model of site-specific biological injury, iron and/or copper ions reversibly coordinate to biological components (L), some of them essential:[26−27]

$$L + Fe(III) \rightleftarrows L\text{-}Fe(III) \quad (5)$$

The bound metal can be reduced by superoxide radicals or other cellular reductants:

$$L + Fe(III) + O_2^{\bullet -} \rightleftarrows L\text{-}Fe(II) + O_2 \quad (6)$$

and then form, through the Fenton reaction, a peroxo complex

$$L\text{-}Fe(II) + H_2O_2 \rightarrow L\text{-}Fe(II) - H_2O_2 \quad (7)$$

which yields in turn a hypervalent metal (such as ferryl)

$$L\text{-}Fe(II) - H_2O_2 \rightarrow L\text{-}Fe(IV) + 2OH^- \quad (8)$$

and/or hydroxyl radicals

$$L\text{-}Fe(II) - H_2O_2 \rightarrow L\text{-}Fe(III) + {}^{\bullet}OH + OH^- \quad (9)$$

The metal not only mediates a continuos generation of reactive species at the same site but also dictates the location of their attack near or at the site of metal binding. A common strategy of protection uses chelating agents which avidly bind the chelatable metals (denoted also "catalytic" metals) and affect their redox potential, mode, and site of binding and accessibility. Consequently, chelation provides protection but, in principle, might also exert a pro-oxidative effect. An alternative pathway is offered by nitroxides, which block the Fenton reaction by oxidizing the metals rather than by sequestrating them.

The interaction of iron(II) in acidic pH with nitroxides has been previously investigated.[28] In the present study, the reaction of nitroxides with ferrous ions at physiological conditions was found to be first-order with respect to both the iron and the nitroxide. The reduction of TPL, in the majority of cases, must be carried out in the presence of both an electron and a proton donor. Consequently, this reduction takes place faster at low pH when the reducing agents possess fairly labile hydrogen, activated by means of a catalyst (see TPL-H preparation in Methods). Conversely, with an increase of the pH, the reduction of TPL by iron(II) is catalyzed by strong bases such as OH^- or phosphate, as seen in Figure 2 and Table 3.

The present results show not only that equilibrium 1 is shifted strongly to the right under physiological conditions but also that it is established rapidly and in most cases almost instantaneously. The generality of this conclusion is evident since the oxidation of iron(II) by nitroxides was tested using several ligands. It implies that nitroxides rapidly and effectively oxidize iron, and presumably copper too,[29] in the chelatable pool of redox-active metals in cells and tissues. This oxidation pre-empts the Fenton chemistry, inhibits oxidative injury, and explains the narrow time window of the anti-oxidative activity of nitroxides observed with many animal and organ experimental models. Thus, nitroxides would be able to rapidly and effectively detoxify chelatable reduced iron, and probably copper, before being reduced when administered to the body. Moreover, the residual small fraction of nitroxide radical persisting in the tissue after a steady-state distribution of nitroxide/hydroxylamine is established might be enough to maintain the iron in the chelatable pool in its oxidized form.

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