A Pulse Radiolysis Study of Catalytic Superoxide Radical Dismutation by a Manganese(II) Complex with an N-Tripodal Ligand

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This article presents a pulse radiolysis study of the reactivity of a SOD-mimic Mn^{II} complex $[Mn^{II}(IPG)]^+$ **1** with superoxide. It uses an improved set-up with a circulating cell. It provides convergent pieces of evidence that **1** catalyses efficiently the dismutation of superoxide. Three sets of experiments are described: (1) Transient formation and decay of an adduct labeled $\{MnOO\}^6$ were studied. (2) A multi-pulse experiment showed a reproducible behaviour upon successive superoxide pulses. (3) Superoxide decay was monitored at 270 nm to provide an overall kinetic constant. A mechanism is proposed.

The superoxide radical anion ($O_2^{\cdot -}$), produced by the one-electron reduction of dioxygen, has been shown to be a highly toxic species.[1–6] It is known to be involved in oxidative stress.[2,7–9] Superoxide dismutases (SOD) catalyze $O_2^{\cdot -}$ dismutation into dioxygen and hydrogen peroxide and constitute an endogenous protection.[2–5,10] Since superoxide has been shown to play an important role in several diseases, low-molecular-weight SOD mimics have potential use as pharmaceutical agents.[8,9,11] Synzymes[12] can also enlighten the understanding of the natural enzyme catalytic mechanism. A wide range of putative catalysts for superoxide dismutation has been reported.[4,9,13] SOD activity is usually estimated on the basis of indirect activity tests, such as the Mc-Cord Fridovich test.[10] These tests are informative, as they provide a quantification of the activity in conditions close to physiological, but they may lead to errors in the interpretation of results, as previously shown.[9,14,15] According to us and others,[9,14,16] both indirect and direct methods are necessary, as they provide complementary information. In the indirect methods, superoxide is provided at a constant rate and at concentrations close to what is encountered during oxidative stress. This provides a test for the ability of the putative SOD mimic to be useful under physiological situations. However, in most cases it is difficult to distinguish between a catalyst and a scavenger. With direct methods, as superoxide can be used in large excess relative to the putative SOD mimic, unambiguous characterization of the catalyst and the scavenger is accomplished. However, direct methods for the determination of catalytic rate constants, including stopped-flow and pulse radiolysis, are rarely used.[9,13,15–17]

In this work, we provide convergent pieces of evidence by pulse radiolysis that a manganese(II) complex **1**, $[Mn(IPG)(MeOH)]_n(PF_6)_n$ (IPG^- = *N*,*N*-(2-pyridylmethyl[(1-methylimidazol-2-yl)methyl]glycinate, see Figure 1), catalyses the dismutation of superoxide. The crystal structure of complex **1** and its reactivity towards superoxide both in anhydrous DMSO and in aqueous medium have been previously reported.[18,19] It displays a good SOD-like activity, as shown by the McCord–Fridovich test (IC50 = $(2.0 \pm 0.2) \times 10^{-6}$ mol L^{-1} or k_{cat} = $(2.9 \pm 0.3) \times 10^6$ mol^{-1} L s^{-1}).[19,20] In the present article, three sets of experiments are described with different purposes: (a) transient formation and decay of an adduct between Mn and superoxide were studied under conditions for which $[\mathbf{1}]_0 > [O_2^{\cdot -}]_0$. (b) A multi-pulse experiment showed a reproducible behaviour upon successive $O_2^{\cdot -}$ pulses. (c) Superoxide decay was monitored at 270 nm under conditions for which $[\mathbf{1}]_0 < [O_2^{\cdot -}]_0$ to provide an overall kinetic constant.

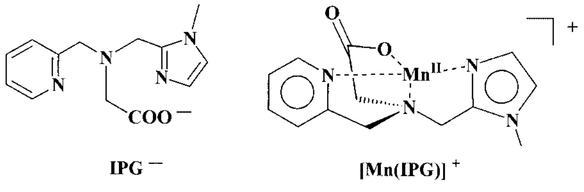

Figure 1. Ligand IPG^- = *N*,*N*-(2-pyridylmethyl[(1-methylimidazol-2-yl)methyl]glycinate and schematic structure for $[Mn(IPG)]^+$.

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The superoxide radical was produced in oxygenated aqueous solutions by pulse radiolysis,[21] and its direct reaction with the putative SOD mimic was monitored by UV/Visible spectrophotometry. High-energy ionizing radiation generates primary radicals ($H^{\cdot}$, e^-_{aq}, $HO^{\cdot}$), that are rapidly and quantitatively converted into either superoxide $O_2^{\cdot-}$ or its protonated form hydroperoxyl $HO_2^{\cdot}$, in the presence of either formate or alcohols.[22,23] Propan-2-ol was chosen as formate might coordinate to manganese. The aqueous solution was driven at a constant rate through the UV/Visible microcell, where it was irradiated for a short time period. The irradiation frequency (1 Hz) was chosen so that between two pulses the solution was totally renewed. Absorbance was averaged over 100 pulses. This technique provides an improvement of the signal-to-noise ratio, relative to the single-pulse experiments generally used.[4,16,17,24]

The reaction of **1** with superoxide was studied over a wide range of time scales (from 10^{-6} to 1 s). Experiments were performed at pH 7.8 (5 mM phosphate buffer) in order to allow comparison with the previous indirect test.[19]

1. Self-Decay of Superoxide

First, it was checked that the self-decay of $O_2^{\cdot-}$ followed a second order rate law, as expected for the auto-dismutation of superoxide. The observed rate constant had a value of 1.3×10^5 mol^{-1} L s^{-1}, consistent with that reported in the literature[23] at pH 7.8 (1.0×10^5 mol^{-1} L s^{-1}).

2. Reaction of 1 with Superoxide under Conditions for which $[\mathbf{1}]_0 > [O_2^{\cdot-}]_0$: Search for an Intermediate

Absorbance was monitored at different wavelengths during the reaction between **1** and $O_2^{\cdot-}$, under conditions where $[\mathbf{1}]_0 > [O_2^{\cdot-}]_0$ [conditions (A): $[\mathbf{1}]_0 = 10^{-5}$, 2.5×10^{-5}, 5×10^{-5}, 10^{-4} and 5×10^{-4} mol L^{-1} and $[O_2^{\cdot-}]_0 = 1.3\times10^{-6}$ mol L^{-1} and conditions (B): $[\mathbf{1}]_0 = 5\ 10^{-4}$ mol L^{-1} and 10^{-3} mol L^{-1} and $[O_2^{\cdot-}]_0 = 7.3\times10^{-5}$ mol L^{-1}]. The difference absorption spectrum of a transient T, recorded 40 µs after the pulse, showed an intense peak at 300 nm and a smaller peak at 600 nm, as shown in Figure 2. Figure 2 inset shows the kinetic trace of transient formation at 350 nm. A rate constant, k_1, for the formation of the transient T was derived, $(2.2\pm0.3)\times10^8$ mol^{-1} L s^{-1}).

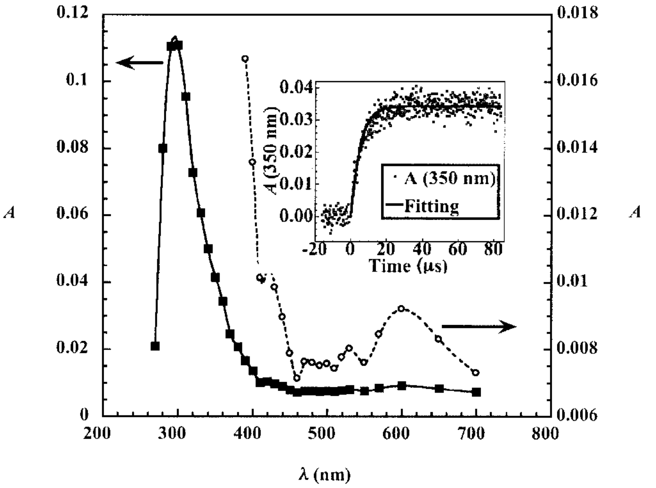


Figure 2. Difference absorption spectrum of the transient T formed during the reaction of **1** with superoxide 40 µs after the pulse. T decays with a half-life of ca. 0.04 s. Squares refer to the left axis and circles to the right axis. [**1**] = 10^{-3} mol L^{-1}; propan-2-ol 0.1 mol L^{-1}; phosphate buffer 5 mmol L^{-1}; irradiation pulse 1 µs; $[O_2^{\cdot-}] = 7.3\times10^{-5}$ mol L^{-1}; averaging over 100 pulses; flux 1 mL min^{-1}; irradiation frequency 1 Hz. Inset: kinetic trace at 350 nm, fitted by $A = A_0[1 - \exp(-k_{app}t)]$.

T can reasonably be ascribed to an adduct between $[Mn(IPG)]^+$ and $O_2^{\cdot-}$. No assignment of the oxidation state of manganese and oxygen (neither Mn^{II} and superoxide nor Mn^{III} and peroxide) is made here. We suggest to label it $\{MnOO\}_n$ (with $n = 6$ here), by analogy to Enemark and Feltham $\{FeNO\}_n$ nomenclature: n represents the number of d electrons from the metal center and the π* single electron from superoxide.[1,25] Such adducts have already been described for reactions involving Mn^{II} complexes and superoxide, by pulse radiolysis[16,23,24,26,27] and stopped-flow[28] studies. Complexes with an oxygenated coordination sphere (sulfate, formate, pyrophosphate,[26] phosphate,[24] aqua[27]) form adducts labeled $[MnO_2]^+$ characterised by an absorption maximum at 260–270 nm. The adduct observed with the complex [Mn^{II}(TPAA)](PF$_6$)$_2$ (TPAA = tris[2-[*N*-(2-pyridylmethyl)amino]ethyl]amine, a heptadentate nitrogen ligand) exhibits absorption maxima at 290 and 310 nm.[16] Although protonated species (MnOOH) have been previously considered,[24,26] we, and others,[24,27] excluded them on the basis of the published pK_a for (MnOOH/MnOO) (2.5 to 5.5).[24,26] Complex **1** shows an N_3O_1 coordination sphere. The absorption maximum for T at 300 nm is thus in the expected range. Furthermore, its formation rate constant is consistent with rate constants reported for similar adducts, ranging from 7.5×10^6 mol^{-1} s L^{-1} to 4.4×10^8 mol^{-1} L s^{-1}.[16,23,24,26–29] In addition, the existence of an intermediate $\{MnOO\}^6$ is consistent with reactivity studies previously performed in anhydrous DMSO. A di-µ-oxo dimer [(IPG)Mn^{III}(O)$_2$Mn^{IV}-(IPG)]$^+$ has been obtained upon addition of 0.5 equiv. of superoxide on the manganese complex, and characterised by using UV/Visible spectroscopy and EPR. Its formation has been rationalised by the reaction of a postulated adduct $\{MnOO\}^6$ with the remaining monomeric manganese(II) complex.[19]

3. Multipulse Experiment on a Noncirculating Solution: Is the Behaviour of the Complex towards Superoxide Reproducible?

Experiments were recorded at different time scales ($t \le 8.4\times10^{-5}$ s, $t \le 8.4\times10^{-4}$ s, $t \le 8.4\times10^{-3}$ s, $t \le 8.4\times10^{-2}$ s), indicating that the transient *T* decays with a half-life of about 0.04 s.

To obtain more information on the mechanism of the reaction of **1** with $O_2^{\cdot-}$ and check the reproducibility of the behaviour of **1** towards $O_2^{\bullet-}$, a multi-pulse experiment[30] was performed. 11 repeated irradiations were applied to a noncirculating complex solution. This solution thus received successive doses of superoxide, approximately 0.75 equiv. each.

The trace recorded at 300 nm is provided in Figure 3. After the superoxide pulse, an absorption peak S (for spike) was observed with a decrease and a stabilisation of the absorbance on a plateau P. This behaviour was reproducibly recorded up to 11 times, and the absorbance reached a higher level on successive plateaus. It should also be noted that the size of the peak was decreasing upon repetitive pulses.

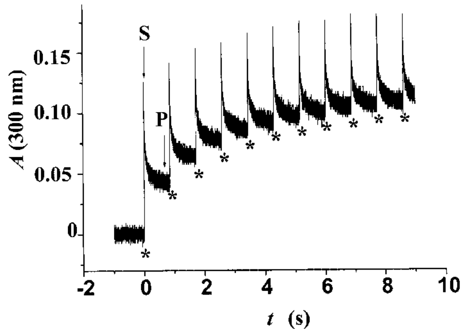


Figure 3. Variation of absorbance with time at 300 nm, during a multipulse experiment. [**1**] = 10^{-4} mol L^{-1}; propan-2-ol 0.1 mol L^{-1}; phosphate buffer 5 mmol L^{-1}; irradiation pulse 1 µs; [$O_2^{\bullet-}$] = 7.3×10^{-5} mol L^{-1}; irradiation frequency 1 Hz. Arrows indicate the first spike S and the plateau P. Stars indicate irradiations.

Difference absorption spectra, obtained at the spike S (2.6 ms ≤ t ≤ 22.6 ms) and at the plateau P (800 ms ≤ t ≤ 820 ms) after the first pulse are shown in Figure 4 and compared to that of the short time transient T. The S and P spectra are both characterised by an intense absorption maximum at 280 nm. It was checked that both S and P spectra are reproducibly obtained after each further pulse. The comparison of spectra labelled S, P and T seems to indicate that at the spike S, the solution is a mixture of the transient T and of the product characterised by the spectrum measured at the plateau P. The absorbance decay after the spike was completed within 0.5 s, and had a half-life (0.02 s) of the same order of magnitude as the half-life of the transient T.

A tentative attempt was made to analyse and simulate the behaviour of the successive plateaus.[31] This behaviour can be described by a catalytic cycle, involving an adduct $\{MnOO\}^6$ as the transient T [ε_T(300 nm)[32] = 1500 L mol^{-1} cm^{-1}] and a species R responsible for the residual absorbance at the plateaus (Scheme 1). Electrochemical oxidation of the manganese(II) complex **1** leads to a yellow solution, whose UV/Visible spectrum is consistent with the main peaks observed at the plateaus.[33] The species R may thus be ascribed to the manganese(III) complex [Mn^{III}(IPG)]$^{2+}$ (a rough estimation from the electrochemical generation leads to an ε_P(300 nm) of about 1800 L mol^{-1} cm^{-1}). A simple ping-pong mechanism cannot reproduce the behaviour of the plateaus upon successive irradiations: as ε_P(300 nm) and ε_T(300 nm) are close, the decay in absorbance to reach the plateau P cannot be assigned solely to the formation of [Mn^{III}(IPG)]$^{2+}$. A pathway back to Mn^{II} has to be considered (see Scheme 1).

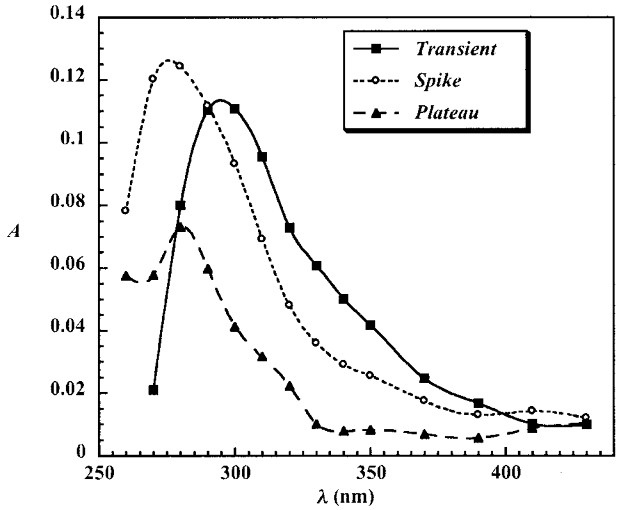
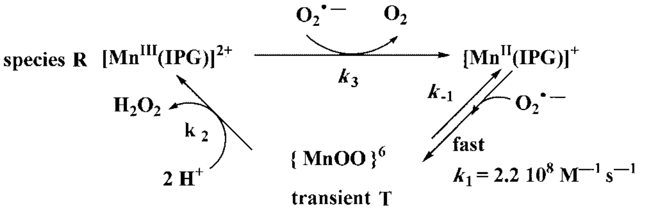


Figure 4. Difference absorption spectra of the solution during the reaction of **1** with superoxide. Absorbance was read at the top of the spike S (2.6 ms ≤ t ≤ 22.6 ms) and at the plateau P (800 ms ≤ t ≤ 820 ms) after the first pulse. The spectrum of transient T is added for the sake of comparison. [**1**] = 10^{-4} mol L^{-1}; propan-2-ol 0.1 mol L^{-1}; phosphate buffer 5 mmol L^{-1}; irradiation pulse 1 µs; [$O_2^{\bullet-}$] = 7.3×10^{-5} mol L^{-1}; irradiation frequency 1 Hz.



Scheme 1. Proposed reaction scheme for the reaction of **1** with superoxide. It consists of a catalytic cycle involving a manganese(II)–superoxide adduct as a transient and a manganese(III) complex as the species R responsible for the spectrum at the plateau.

From the schematic mechanism in Scheme 1, an iterative formula for X_n, representing the fraction of Mn^{II} at plateau n can be derived, see Equation (1).

$$X_{n+1} = X_n - \frac{k_1 X_n \times 0.75}{k_3(1-X_n)+k_1 X_n} \times \frac{k_2}{k_{-1}+k_2} + \frac{k_3(1-X_n)\times 0.75}{k_3(1-X_n)+k_1 X_n} \qquad (1)$$

The second term stands for the fraction of transient T decaying back to [Mn^{II}(IPG)]$^{+}$ (k_{-1}), as T may decay either to [Mn^{II}(IPG)]$^{+}$ (k_{-1}) or to [Mn^{III}(IPG)]$^{2+}$ (R) (k_2). The third term stands for the transformation of [Mn^{III}(IPG)]$^{2+}$ (R) to [Mn^{II}(IPG)]$^{+}$, which is assumed here to be complete. At each pulse, 0.75 equiv. of superoxide is introduced into the system.

The best fit, up to the sixth iteration, (see Figure 5) was obtained for $k_1 = 3k_3$ and $k_{-1} = 2.2k_2$.[34]

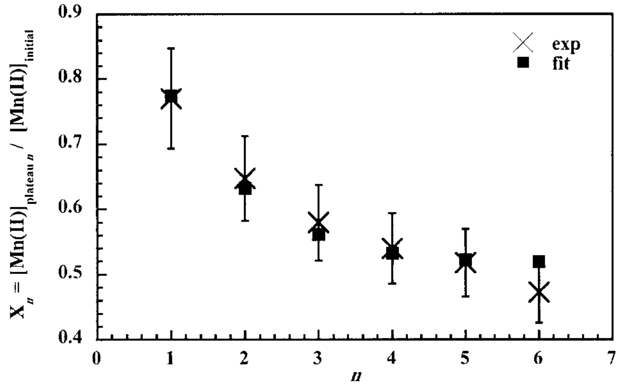


Figure 5. Cross marks: iterative values for the Mn^{II} fraction at plateau *n*, deduced from the absorbance measured at the plateau, assuming only **1** and $[Mn^{III}(IPG)]^{2+}$ are present at the plateau (error arbitrarily fixed at 10%). Square marks: simulation using successive iterations for X_n (see text).

4. Superoxide Decay at 270 nm under Conditions for which $[\mathbf{1}]_0 < [O_2^{\cdot-}]_0$: Overall Catalytic Rate Constant

The catalytic nature of the reaction was confirmed by following the decay of the absorbance of superoxide at 270 nm in the presence of **1**, with $[\mathbf{1}]_0 < [O_2^{\cdot-}]_0$ ($[\mathbf{1}]_0 = 2\times10^{-7}$ mol·L^{-1} and 10^{-5} mol·L^{-1} and $[O_2^{\cdot-}]_0 = 7.3\times10^{-5}$ mol·L^{-1}). It should be noted that only a limited range of concentrations for **1** were informative, as the adduct $\{MnOO\}^6$ itself absorbs at that wavelength.

The decay of superoxide is faster at high concentrations of complex **1** and follows apparent first-order kinetics. The deduced catalytic rate constant $k_{cat} = 3.8\pm0.2\times10^6$ mol^{-1}Ls^{-1} is of the same order of magnitude as that calculated from the indirect xanthine-xanthine oxidase-cytochrome c test ($2.9\pm0.3\times10^6$ mol^{-1}Ls^{-1}).[19]

Conclusions

This pulse radiolysis study allows us to propose a catalytic mechanism involving a transient adduct $\{MnOO\}^6$, and a manganese(III) complex for the reaction of complex **1** with superoxide. These two species were characterised by their differential absorption spectra. The formation rate of the adduct is among the fastest reported in the literature. The multipulse experiment showed unambiguous reproducible behaviour upon successive superoxide pulses (up to 11). Complex **1** efficiently catalyses the dismutation of superoxide with a rate constant equal to 1% of that of the manganese superoxide-dismutase. The present study provides convergent pieces of evidence for catalytic activity. To complete the mechanistic scheme suggested here for the reactivity of the manganese(II) compounds, further research is now underway on the reactivity of the Mn^{III} species. Moreover, further research to improve the catalytic activity of the manganese SOD mimics is now in progress.

Experimental Section

The pulse radiolysis experiments were carried out with a 10 MeV Titan β linear accelerator at the Laboratory of Radiolysis, CEA Saclay. Pulses from 5 ns to 1 µs are available, and the pulse frequency is adjustable. A 10 ns pulse corresponds to a 20 Gray dose. Solutions saturated with oxygen are driven in a quartz circulating microcell (spectral path length 1 cm, volume 400 µL) at a constant flux (1 mL/min). The spectroscopic and temporal analysis is mediated by an optical fibre, linked to a monochromator, then to a photomultiplicator and lastly to a Tektronix numerical oscilloscope allowing the observation of the temporal evolution of absorbance. Absorbance was averaged over 100 pulses as it provides an improvement of the signal-to-noise ratio, relative to the single-pulse experiments generally used. The amount of superoxide generated during pulse radiolysis was established by the absorbance value at 260 nm, assuming that $\varepsilon_{260} = 1940$ L mol^{-1} cm^{-1}.[23]

Measurements were carried out in a 5 mmol L^{-1} phosphate buffer, propan-2-ol 100 mmol L^{-1} at pH 7.8 and 25 °C at different concentrations of Mn^{II} complex. Solutions were oxygen saturated by bubbling O_2 for at least 60 min. A 1 µs-pulse, generating a 7.3×10^{-5} mol L^{-1} superoxide concentration, was imposed upon the solution. The irradiation frequency was 1 Hz, so that the cell content was totally renewed between two pulses. The disappearance of superoxide was followed at 270 nm (($\varepsilon_{270} = 1479$ L mol^{-1} cm^{-1} at pH 7.8 and 23 °C[21]). Spectra are differential, the reference absorbance being recorded before irradiation.

Rates were derived assuming either second-order kinetics corresponding to the self-dismutation of superoxide ($A = A_0/[1 + k_{obs}A_0 t]$)) or pseudo-first-order kinetics in the presence of the complexes ($A = A_0[\exp(-k_{obs}t)] + B$, and $k_{obs} = k_{cat}$ [complex]). Simulations of kinetic traces are performed using Gepasi3 (self-dismutation) or Kaleidagraph (complex catalysed dismutation; see the Supporting Information for a Figure providing $k_{obs} = f([\mathbf{1}])$; see also the footnote on the first page of this article).

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[32] Estimate assuming that 100% of **1** has been converted into the transient over the first 40 ms.

[33] The electrochemical generation of the Mn^{III} species was done in a two-compartment cell, with a three-electrode set up (working electrode: vitreous carbon ($15\ \text{cm}^2$), reference electrode: SCE, KCl sat., counter electrode: Pt grid) collidine/collidinium buffer (50 mM, pH 7.5) (collidine: 2,4,6-pyridine) in MeOH, $[\text{Mn(IPG)}^+] = 2$ mM, potential of the electrolysis: 1V/SCE). The Mn^{III} was unstable and the Mn^{II} species was recovered within a few hours.

[34] The quality of the fit was greatly decreased after the sixth iteration (not shown). This could be due to several experimental factors: cumulative doses, bleaching by UV analysis light, accumulation of hydrogen peroxide and the oversimplified description in the scheme of the reactivity of the Mn^{III} species (R).

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