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Superoxide Dismutase Mimics

NO Dismutase Activity of Seven-Coordinate Manganese(II) Pentaazamacrocyclic Complexes**

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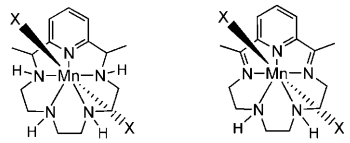
Seven-coordinate Mn^{II} pentaazamacrocyclic complexes represent the most potent synthetic mimics of native superoxide dismutase (SOD), which catalyze dismutation of superoxide ($O_2^{\bullet-}$) into O_2 and H_2O_2 with efficiency that can exceed that of the mitochondrial MnSOD.[1] A number of studies demonstrated the ability of these SOD mimics to protect cells and tissues from oxidative damage caused by superoxide (and/or the product of its reaction with nitric oxide, peroxynitrite), for example in inflammation and oxidation reperfusion injury.[2a–c] The members of this class of SOD mimics have entered Phase II clinical trials in the USA.[2d] It is emphasized that the major advantage of Mn^{II} pentaazamacrocycles over other SOD mimics is their high selectivity for $O_2^{\bullet-}$ and lack of reactivity with NO,[1,2d–g] a key molecule in biological processes.[3] However, direct studies of the reaction between NO and this class of complexes, which would support such claims, have not been reported.

Various metal complexes, including manganese complexes,[4a–d] react readily with NO, either to yield metal nitrosyls, or to produce N_2O and metal nitrite complexes by NO disproportionation.[4a,b] Coordinated NO in metal nitrosyls can exist in one of its three formal redox states NO^+, NO, and NO^-.[4a,c,d] For a number of NO^+ and NO^- complexes reactivity towards selected nucleophiles and electrophiles, respectively, has been documented.[4d] We demonstrated recently that natural MnSOD enzyme reacts with NO according to distinct catalytic NO disproportionation (dismutation) mechanism which yields both reactive species NO^+ and NO^-.[5–7]

Collectively these results prompted us to (re)examine the reaction of these complexes with NO. The chosen approach has been 1) to establish the reactivity of complexes with NO, 2) to establish the mechanistic details of the reaction, and 3) to demonstrate the potential validity of the complex reaction with NO in a biological setting.

In the present study we used $[Mn^{II}(pyane)Cl_2]$ (**1**),[8] as a general representative of this class of SOD mimics,[9] and its



1 X = Cl^-, H_2O **2**

SOD-inactive imine analogue $[Mn^{II}(pydiene)Cl_2]$ (**2**)[1] to probe whether the difference in reactivity towards $O_2^{\bullet-}$ affects their reaction with NO. Herein we present evidence that Mn^{II} pentaazamacrocyclic complexes react with NO and stimulate NO dismutation.[7] The mechanism that would account for our observations is proposed. An interference of Mn^{II} pentaazamacrocyclic complexes with NO in biological ex vivo models is demonstrated.

We studied first the reaction of **1** and **2** with a large excess of NO, measuring its consumption by the complexes in anaerobic aqueous solutions.[10] Since manganese nitrosyl complexes could be light-sensitive[11a] all experiments were performed in the dark. We found that addition of an argon-purged solution of **1** or **2** into an anaerobic aqueous solution of NO caused a rapid disappearance of NO (Figure 1 and Supporting Information Figure SI 1). The effect was significantly more pronounced in the presence of reduced glutathione (GSH; Figure 1 and Supporting Information Figure SI 1). In the presence of GSH the amounts of NO consumed were 10-times greater than the amounts of **1** and **2** present, a result which indicates a catalytic reaction. In a control incubation with GSH (but without the complexes) NO decay was non-detectable (not shown), excluding the reaction of GSH with NO[12] as a source of NO decay. To

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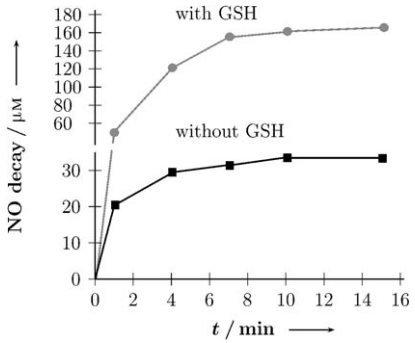

Figure 1. Anaerobic NO (250 μM) decay stimulated by **1** (10 μM) at pH 7.4 and 23 °C in the absence and in the presence of GSH (250 μM) monitored with a NO-sensitive electrode.

probe whether **1** and **2** stimulate NO dismutation[5,6] we determined the amounts of S-nitroso glutathione (GSNO) and hydroxylamine in the reaction mixture, which are the reaction products of GSH with NO^+ and HNO/NO^- species, respectively[13,14] (for experimental conditions see Supporting Information). We found 80 μM of S-nitroso glutathione (GSNO) and 65 μM of hydroxylamine in the presence of **1** (10 μM), and 55 μM of GSNO and 45 μM of hydroxylamine in the presence of **2** (10 μM), which corresponds to the amounts of NO consumed in the reaction with **1** and **2**, respectively (Figure 1 and Supporting Information Figure SI1).

The lower reactivity of NO with **1** and **2** in the absence of GSH (Figure 1 and Supporting Information Figure SI1) deserves a further comment. We found that **1** lost its SOD activity following anaerobic NO treatment in the absence of GSH, suggesting a structural modification of the complex, which renders it inactive as dismutation catalyst for both $O_2^{\bullet-}$ and NO. To elucidate the structural changes of **1**, we bubbled NO through its anaerobic solution ([**1**] = 10 mM) in THF and analyzed the products with the mass spectrometer. The positive ion of ESI mass spectrum of NO-treated **1** (Supporting Information Figure SI2) showed a large parent-ion peak at *m*/*z* 139.6 corresponding to the combination $M^{3+} = \{\mathbf{1}-3\,H+3\,NO\}^{3+}$ and a small peak at *m*/*z* 365.2 corresponding to the modified ligand without manganese, $M^+ = \{(pyane)-3\,H+3\,NO\}^+$. Thus, the product is interpreted as being the triple (presumably N-)nitrosated derivative of **1**. For comparison, the positive ion ESI-MS spectrum of **1** gave a parent-ion peak at *m*/*z* 110.6 corresponding to $M^{3+} = \{\mathbf{1}\}^{3+}$. Anaerobic treatment of **2** (0.5 mg in 1 mL of THF) with NO yielded insoluble products which were not analyzed further.

The results clearly show that GSH, which efficiently scavenges the reactive NO species generated upon anaerobic exposure of **1** and **2** to a high excess of NO, protects the complexes from structural modifications which would cause their inactivation. As a result the amount of NO which can react with the complexes increases. It is important to note that SOD-active **1** is somewhat more efficient in dismutating NO than SOD-inactive **2**, which is related to the generally higher stability of **1** in solution.[8]

In water O_2 reacts readily with NO to yield NO_2^-.[15] Therefore we assessed whether **1** and **2** can compete for NO in the presence of O_2. Figure 2a shows that both complexes

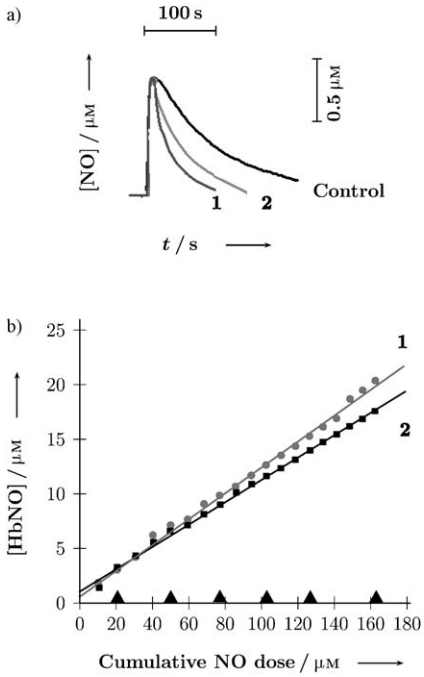

Figure 2. Aerobic reaction of **1** and **2** with NO. a) NO (1 μM) decay under aerobic conditions caused by its reaction with O_2 (control) and after injection of **1** or **2** (15 μM each). The reactions were monitored with the NO-sensitive electrode (pH 7.4, 23 °C). b) Reductive nitrosylation of metHb (50 μM) to HbNO. The aerobic solutions of **1** and **2** (15 μM each, pH 7.4, 23 °C) and control (without the complexes; ▲) were supplemented with metHb and subjected to sequential additions (10 μL each) of NO solution to yield [NO] = 10 μM.

increase the rate of NO decay under aerobic conditions. The plot of the observed rate constants as a function of complex concentration was linear, with the slope corresponding to the second-order rate constants for the aerobic reaction of NO with **1** ($891\,M^{-1}s^{-1}$) and **2** ($466\,M^{-1}s^{-1}$; 23 °C, pseudo first-order conditions with the complex in excess), and the intercept corresponding to the rate of NO decay caused only by O_2 (see Supporting Information Figure SI3).

Since the nitrogen oxides formed upon the reaction of NO with O_2[15] are a source of NO^+ species[13] we examined the conversion of NO into HNO/NO^- species upon aerobic reaction with **1** and **2**. Both the reductive nitrosylation of metHb (methemoglobin) into HbNO (the nitrosyl adduct of hemoglobin)[14] (Figure 2b and Supporting Information Figure SI4a), and thiol-dependent formation of hydroxylamine[14] (Supporting Information Figure SI4b) strongly support the formation of HNO/NO^- species upon aerobic reaction of **1** and **2** with NO.

On the basis of information available for the reactions of NO with other metal complexes, which involve substitution of labile solvent molecules by NO,[4c,e] we propose that **1** and **2** react with NO according to Equations (1) and (2) (L = pentaazamacrocyclic ligand, S = solvent molecule) to form labile manganese–NO adducts, which are carriers of NO^+ and NO^- species. The results of our IR and EPR studies fully support this assumption.

Since **1** does not exhibit IR bands[16] in the range characteristic for NO stretching frequencies (1900–

$$[(L)Mn^{II}(S)_2] \xrightarrow[-S]{+NO} [(L)Mn^{II}(NO)(S)] \xrightarrow{e^-} [(L)Mn^{III}(NO^-)(S)] \rightleftharpoons^{-S} [(L)Mn^{III}(NO^-)] \quad (1)$$

$$[(L)Mn^{III}(S)] \xrightarrow{+NO} [(L)Mn^{II}(NO^+)(S)] \quad (2)$$

1630 cm^{-1}),[4a–d] attenuated total reflection (ATR) FTIR spectroscopy could be applied to monitor the reaction of **1** with NO in THF. When the THF solution of **1** (5 mM) was exposed aerobically to NO (10 mM) three distinct types of NO stretching mode were observed. These bands at 1840 cm^{-1}, 1732 cm^{-1} and a doublet at 1653 and 1647 cm^{-1} (Figure 3)[17]

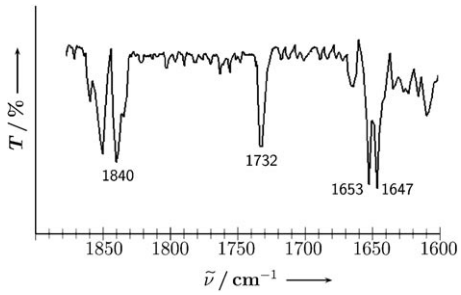

Figure 3. ATR FTIR spectrum (1600–2000 cm^{-1}) taken aerobically at room temperature after addition of THF solution of NO to a THF solution of **1** (initial concentrations in the reaction mixture: [NO] = 10 mM, [**1**] = 5 mM).

correspond to the manganese–nitrosyl species from Equations (1) and (2). The bands are assigned to Mn^{II}-NO^+ (1840 cm^{-1}), Mn^{II}-NO (1732 cm^{-1}), and six- and seven-coordinate forms (1653 and 1647 cm^{-1})[18] of Mn^{III}-NO^- adducts.[4a–d,11]

To verify the changes in oxidation state of the manganese center upon complex reaction with NO [Eqs. (1)–(2)], the EPR spectra of **1** and **2** (not shown) upon their reaction with NO were recorded. Figure 4 shows that peak heights in the

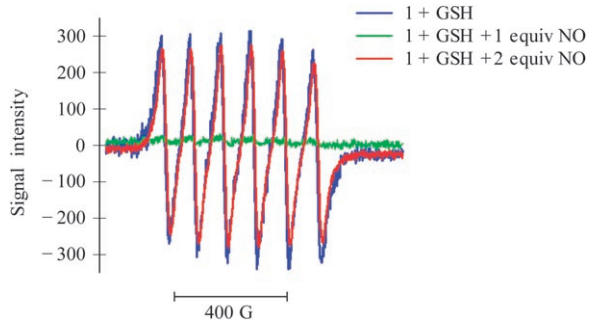

Figure 4. Changes in the EPR spectra recorded during the reaction of NO with **1** (100 μM) in the presence of GSH (1 mM; T = 25 °C, 50 mM potassium phosphate (KPi) buffer at pH 7.4). Synthetic NO donor (DEA-NONOate, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide) was used as a source of NO (1 equiv = 50 μM). Instrument settings: microwave frequency, 9.51 GHz; power 10 mW; modulation amplitude 2 G; gain 2×10^4.

EPR spectrum of **1** (in the presence of GSH) are reduced after the addition of the first equivalent (based on **1**) of synthetic NO donor, suggesting the oxidation of Mn^{II} into the EPR-silent Mn^{III} form of **1**.[2a] It is worth noting that the high excess of GSH present in the reaction mixture does not interfere with this process. When the second equivalent of NO donor was added, Mn^{III} was nearly quantitatively converted into the EPR active Mn^{II} form of **1** (Figure 4). Analogous results were obtained when the experiment was started with the Mn^{III} form of the complex, prepared by electrochemical oxidation of **1** (Supporting Information Figure SI5).

It is emphasized that this class of manganese complexes does not react with NO, this is because of their relatively high redox potential (+0.78 V vs. the standard hydrogen electrode (SHE)).[2f,g] This high potential does not allow their outer-sphere oxidation by NO (the redox potential for the NO/NO^- and $NO,H^+/HNO$ couples are −0.8 and ca. −0.5 vs. SHE respectively).[19] However, these complexes are generally prone to react with different monodentate ligands and coordination of NO is quite feasible.[4e] Once NO coordinates, its redox potential shifts towards significantly more positive values, enabling an inner-sphere electron transfer resulting in the Mn^{III}–NO^- nitrosyl species [Eq. (1)].[4e] By way of comparison, $[Fe^{II}(H_2O)_6]^{2+}$, with a redox potential of +0.77 V versus SHE, reacts with NO producing $[Fe^{III}(H_2O)_5(NO^-)]^{2+}$.[20a] In addition, it has recently been demonstrated that the catalytic cycle of the Mn^{II} pentaazamacrocyclic SOD mimetics proceeds also through an inner-sphere mechanism.[20b]

Under pharmacological conditions, which imply the application of Mn^{II} pentaazamacrocyclic SOD mimics in concentrations[2] exceeding the highest (micromolar) pathological concentrations of NO,[3] the interaction of Mn^{II} pentaazamacrocycles with NO will result in the formation of the NO^- complex. NO^- species can be consumed in reactions with diverse substrates producing various bioeffects.[14,21] NO^- has been found to react readily with cellular low-mass thiols and protein thiols.[14,19,21]

Therefore we considered it important to establish whether the reaction of Mn^{II} pentaazamacrocyclic SOD complexes with NO operates under physiologically relevant conditions. Thus we examined whether **1** and **2** react with NO produced in cell cultures of activated macrophages,[22] and whether they attenuate NO-inhibited platelet aggregation.[23] Figure 5a shows that in the presence of either **1** or **2** the NO concentration in activated macrophages is significantly lower than that in the control. This result suggests that both complexes react with NO generated in the activated cells. In contrast to the findings described above, which showed that **1** is more reactive than **2** towards NO, Figure 5a shows that SOD-inactive **2** was more efficient than **1** in removing NO produced in activated macrophages. Activated macrophages also produce a large amount of $O_2^{\bullet-}$, which combines rapidly with NO to yield peroxynitrite.[22] Therefore the results suggest that Mn^{II} pentaazamacrocyclic complexes could react with NO in biological milieu even in the presence of $O_2^{\bullet-}$. Under such conditions a SOD-active complex, which reacts with both $O_2^{\bullet-}$ and NO, will exhibit lower reactivity towards NO than an SOD-inactive complex which reacts just

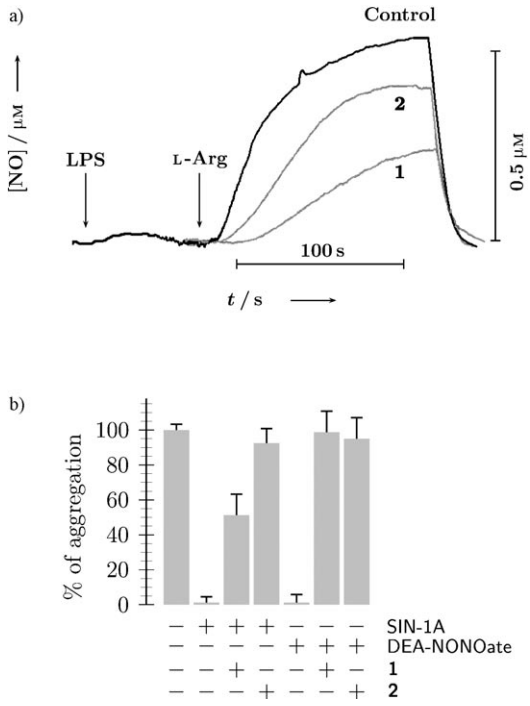

Figure 5. The effect of **1** and **2** on a) NO production in activated macrophages and b) NO-mediated inhibition of platelet aggregation. a) Murine macrophages (3×10^6 cells) were pre-incubated in the respiratory buffer containing **1** or **2** (100 μM each) for 2 h at 37 °C followed by the aspiration of the buffer and resuspension of the cells in the respiration buffer with addition of lipopolysaccharide (LPS; 1 μg mL^{-1}) and L-arginine (50 μM). NO production was measured with a NO-sensitive electrode for 10 min and subsequent addition of hemoglobin solution (20 μM) to scavenge residual NO. b) Platelet-rich plasma (PRP; 500 μL, 2.5×10^8 cell L^{-1}) was pre-incubated at 37 °C with DEA-NONOate (donor of NO) or SIN-1 (donor of NO and $O_2^{\bullet-}$; 10 μM each) prior to activation with collagen. PRP was pre-incubated with 10 μM of **1** or **2**, prior to addition of NO donors and collagen.

with NO. Figure 5b shows that both **1** and **2** attenuated NO-inhibited platelet aggregation in response to collagen. In the presence of SIN-1A, which is a donor of NO and $O_2^{\bullet-}$, SOD-inactive **2** was again more efficient in preventing NO-mediated effects than the SOD-active **1**.

In summary our results demonstrate that both **1** and **2** stimulate NO disproportionation by the catalytic (dismutation) mechanism. This mechanism is based on the formation of labile metal–nitrosyl adducts in which NO bound to the metal center exhibits the character and reactivity of NO^- and NO^+ species, which is associated with the Mn^{II}/Mn^{III} redox cycle [Eqs. (1)–(2)]. This, to our knowledge, novel reactivity behavior of metal complexes with NO seems to be similar to that of the natural MnSOD.[5,6] The concept that the selectivity of Mn^{II} pentaazamacrocyclic SOD mimics for $O_2^{\bullet-}$ and their lack of reactivity towards NO[2] is questioned by our chemical and ex vivo study. These results suggest that cytoprotective effects of Mn^{II} pentaazamacrocyclic SOD mimics against oxidative stress[2] may be better explained by their capacity to remove both $O_2^{\bullet-}$ and NO, and thus efficiently reduce the formation of cytotoxic peroxynitrite. We argue that blocking of hypotension associated with interleukin therapy by Mn^{II} pentaazamacrocyclic SOD mimic[24] may be explained by the capacity of the complex to remove excess NO. We expect that biomedical implication of this study will motivate further design and screening of truly selective SOD mimetics.

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