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Thiyl Radicals Are Co-products of Dinitrosyl Iron Complexes (DNICs) Formation

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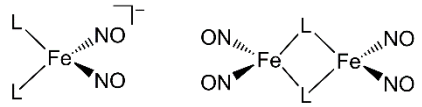
Thiyl radicals are detected by EPR as co-products of dinitrosyl iron complex (DNIC) formation. In demonstrating that DNIC formation generates RS• in a NO rich environment, these results provide a novel route for S-nitroso thiol formation.

Nitric oxide (NO) plays important physiological/pathological roles in mammalian biology including vasodilation, inflammation and immune response.[1] Bioregulatory NO concentrations fall into the nanomolar range while pathological concentrations are micromolar.[1–3] NO metabolites include S-nitroso thiols (RSNOs), nitrite, peroxynitrite and dinitrosyl-iron complexes.[4–6] The dinitrosyl iron complexes are proposed to be the most abundant NO-derived adducts in cells exposed to either physiological or pathological concentrations of NO.[7] Mononuclear dinitrosyl iron complexes (DNICs) with the spin state S_{total} = ½ are electron paramagnetic resonance (EPR) active (Fig. 1) showing a characteristic EPR signal at g = 2.03[8] that was observed decades ago in cells, including activated mammalian macrophages.[9–11] Other dinitrosyl iron complexes are the EPR-inactive binuclear species $Fe_2(NO)_4(\mu\text{-}L)_2$ also known as Roussin's red salt esters (RSEs).[12–15] When L is cysteine (CysSH) or glutathione (GSH), there is an equilibrium between the DNIC and RSE forms in aqueous media that is both pH and thiol concentration dependent.[16,17]

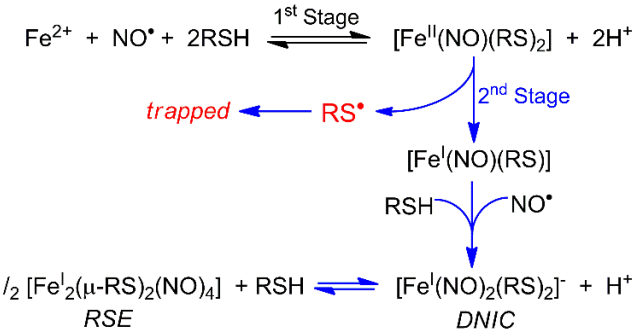
Proposed physiological roles of such complexes include serving as less reactive reservoirs of NO[18–20] and as sequesters of free iron, thereby reducing Fe-mediated oxidations,[21,22] although free iron may also serve a protective role against peroxynitrite damage.[23] Dinitrosyl iron complexes have been shown to induce vasodilation,[24] inhibit platelet aggregation[25] and accelerate wound healing,[26] as well as drawing attention as having therapeutic potential.[27] Notably, increases on DNIC cellular levels were demonstrated to be concomitant to increases in RSNO levels, leading to the proposal that DNICs are able to promote S-nitrosation of biothiols.[22,28]

Despite the importance of mono- and bi-nuclear dinitrosyl iron complexes to the chemical biology of NO, little is known about the dynamics of the generation of these species under physiologically relevant conditions. A previous report from the UCSB laboratory probed the stopped-flow kinetics of the reaction between iron(II), NO and CysSH in pH 7.4 aqueous media and proposed the mechanism for dinitrosyl iron complex formation illustrated in Scheme 1.[16] In brief, the overall reaction occurs via two stages, the first being quite fast and leading to the putative intermediate $Fe^{II}(NO)(RS)_2$. During the slower second stage, this intermediate undergoes unimolecular autoreduction to form an RSE/DNIC mixture, but also generating a thiyl radical as co-product. Although this mechanism rationalizes well the kinetics behavior with CysSH, specific intermediates of the proposed mechanism have not yet been identified. Notably, the kinetics of the analogous reaction with GSH indicates a similar sequence.[29]



DNIC (S = 1/2) **RSE** (S = 0)

Fig. 1 General formulae for dinitrosyl iron complexes (DNICs) and the binuclear Roussin's red salt esters (RSE).



Scheme 1 Model proposed (ref 16) for the formation of mono- and bi-nuclear DNICs directly from Fe(II), RSH and NO in aqueous media. Black rows represent the 1st stage while blue rows represent reactions taking place during the 2nd stage.

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Herein we report EPR studies demonstrating the intermediacy of thiyl radicals during the reaction between iron(II), NO and the low molecular weight thiols CysSH and GSH in aqueous pH 7.4 media leading to formation of the respective dinitrosyl iron complexes. We also describe the detection of another EPR active species that we attribute to a Fe^{I} mononitrosyl intermediate, either $Fe^{I}(NO)(RS)$ or $Fe^{I}(NO)(RS)_2^{-}$.

The reactions described here were initiated by rapid mixing of a deaerated aqueous solution containing NO with another deaerated solution containing ferrous sulfate and a low molecular weight thiol (CysSH or GSH). Both solutions were maintained at pH 7.4 with HEPES buffer. Figure 2 illustrates the temporal absorbance changes at 350 nm (Abs_{350}) obtained with a stopped-flow spectrophotometer. The displayed kinetics demonstrate the two stage reaction sequence noted in earlier studies for CysSH[16] and in ongoing studies for GSH.[29] Under the conditions described in Figure 2, the rapid rise Abs_{350} reaches a maximum absorbance in a few milliseconds, and this is followed by an exponential decay over a time scale of seconds, the two stages each being somewhat faster for CysSH than for GSH.

After determining the time scale of each stage for the assembly of the dinitrosyl iron complexes of CysSH and GSH, we used continuous flow EPR spectroscopy (Supporting Information Fig. S1) under the same experimental condition to record the spectra of potential intermediates upon symmetrical mixing of the reagents at a continuous flow of 0.5 mL/min. Figure 3 (upper) displays the spectrum acquired for CysSH at

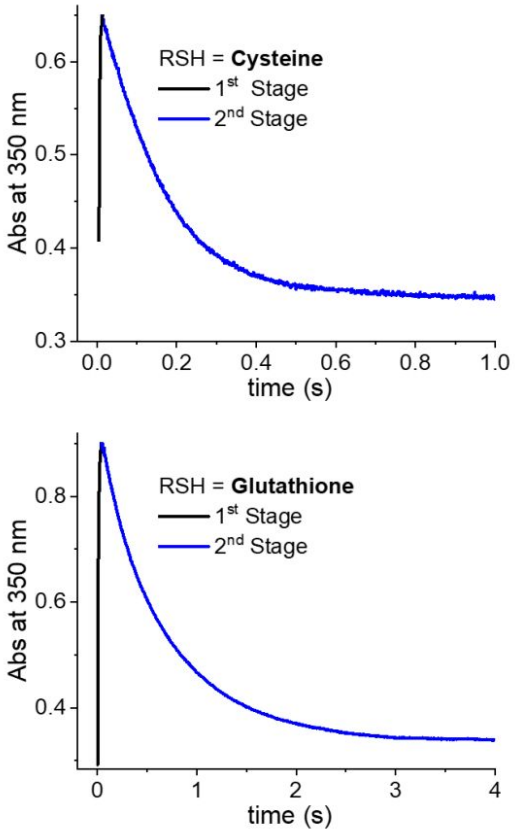

Fig. 2 Temporal absorbance changes at 350 nm upon stopped-flow mixing of solutions with final concentrations of [Fe] = 0.09 mM, [NO] = 0.93 mM and Upper: [Cys] = 10 mM. Bottom: [GSH] = 10 mM in pH 7.4 HEPES buffer (200 mM).

140 ms and clearly shows the appearance of two paramagnetic species, the DNIC with its signature resonance at g = 2.03 and a second species at g = 2.04. This latter signal has been previously attributed[16,30,31] to a triplet Fe^{I} mononitrosyl complex, presumably either $Fe^{I}(NO)(CysS)_2^{-}$ or $Fe^{I}(NO)(CysS)$. At 140 ms the reaction with CysSH is already well into the second stage as evidenced both by the absorbance changes seen in Figure 2 and the appearance of the EPR signal for the DNIC $Fe^{I}(NO)_2(CysS)_2^{-}$. However, although not evident from the absorbance decay at 350 nm, the transient EPR spectrum indicates that the mononitrosyl iron intermediates (Scheme 1) are not instantly captured but can be detected. However, the EPR spectrum recorded 5 s after stopping the flow (Fig. 3 bottom) shows the DNIC to be the only detectable paramagnetic product.[32] Notably, a similar result was observed with GSH (see Supporting Information Fig. S2), although the EPR signals seen at 140 ms were weaker, as one might expect given the slower reaction with this thiol. It is important to note that Fe^{I} mononitrosyl complex (g = 2.04) decays rapidly under our experimental conditions because NO is under excess but this can change in NO limiting conditions.[16,30]

The mechanism described in Scheme 1 also proposes the generation of thiyl radicals as co-products with the dinitrosyl iron complexes formed in stage 2. Therefore, in order to establish the viability of thiyl radical intermediates, we performed EPR spin trapping experiments with the spin trap DMPO (5,5-dimethyl-1-pyrroline N-oxide). This spin trap has been reported to react with RS• radicals to form DMPO/•SR adducts with a second order rate constant of 2.6×10^8 M^{-1} s^{-1}.[33,34] However, thiyl radicals also react fast with NO to form S-nitroso thiols ($k = 3 \times 10^9$ M^{-1} s^{-1}).[35] In order to ensure conditions where DMPO would be competitive with NO in trapping RS•, buffered solutions at pH 7.4 containing [Fe] = 0.09 mM, [NO] = 0.36 mM, [Cys or GSH] = 10 mM and [DMPO] = 140 mM were mixed and promptly transferred to a flat cell.[35] Figure 4 (upper panel) displays the EPR spectrum obtained for CysSH (10 mM). In addition to the characteristic signal of the DNIC at g = 2.03, a six-line signal characteristic of the DMPO/•SCys adduct (a_N =

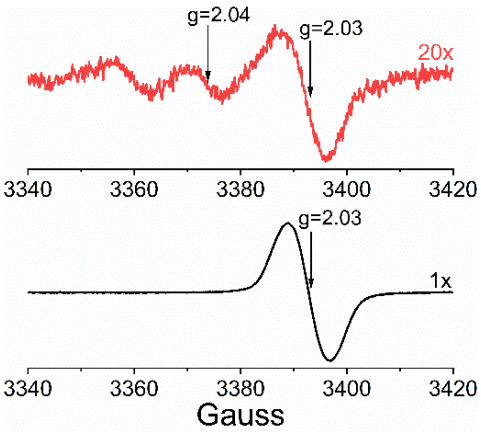

Fig. 3 Temporal EPR spectra recorded using a flow cell mixer to prepare reaction solutions with final concentrations [Fe] = 0.09 mM, [NO] = 0.93 mM and [CysSH] = 10 mM in pH 7.4 HEPES buffer (200 mM). Upper: EPR spectrum acquired 140 ms after mixing solutions at continuous flow of 0.5 ml/min. Bottom: EPR spectrum acquired 5s after stopping the solutions flow. Instrumental conditions: microwave power, 2 mW; time constant, 81.9 ms; scan rate, 0.6 G/s; modulation amplitude, 5 G.

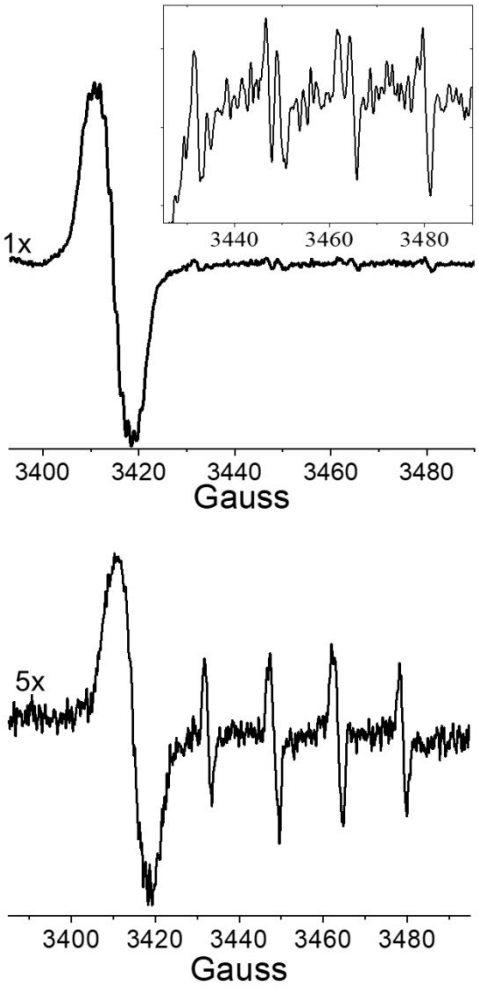

Fig. 4 EPR spectra immediately after mixing room temperature solutions with final concentrations [Fe(II)] = 0.09 mM, [NO] = 0.36 mM, [RSH] = 10 mM and [DMPO] = 140 mM in pH 7.4 HEPES buffer (200 mM). Top: RSH = CysSH (DMPO/•SCys; aN = 15.2 G and aH = 17.4 G). Insert: expanded about 6-fold. Bottom: RSH = GSH (DMPO/•SG; aN = 15.3 G and aH = 16.0 G). Instrumental conditions: microwave power, 20 mW; time constant, 81.9 ms; scan rate, 1.4 G/s; modulation amplitude, 1 G.

15.2 G, a_H = 17.4 G, a_N/a_H = 0.87) is evident.[33,36] The EPR spectrum obtained in an analogous solution with GSH (10 mM) (Fig. 4, botton panel) rendered the DNIC signal at 2.03 plus a four-line signal with a 1:1:1:1 intensity pattern characteristic of the DMPO/•SG adduct (a_N = 15.3 G, a_H = 16.0 G, a_N/a_H = 0.96).[36,37] Although the DMPO adduct seen with CysSH appears weaker than that seen with GSH, it should be noted that the signal for the DMPO/•SCys adduct is split into six-lines, given the impression of lower intensity. Therefore, the amount of thiyl radicals detected are likely similar in both systems (the spectra were not integrated to obtain actual concentrations due the partial overlap with DNIC spectra). It is also evident that the levels of thiyl radicals detected are not stoichiometric with the levels of dinitrosyl iron complex formed (Scheme 1) owing to the reactivity of RS• not only with DMPO but also with NO. In addition, the DMPO/•SR adducts are unstable.[34,38]

All control experiments, solutions prepared without Fe(II), that is, containing just [NO] = 0.36 mM, [GSH or CysSH] = 10 mM and [DMPO] = 140 mM, and solutions prepared without NO ([Fe(II)] = 0.09 mM, [GSH or CysSH] = 10 mM and [DMPO] = 140 mM at pH 7.4), were EPR silent. Moreover, no DMPO-thiyl radical adduct was detected as result of dinitrosyl iron complex breakdown when DMPO was added to solutions in which DNICs formation was completed.

In summary, we have shown that assembly of DNICs from NO, Fe(II) and low molecular weight biothiols occurs in aqueous media, pH 7.4 via the formation of the mono-nitrosyl iron complex intermediate(s) and thiyl radicals as co-products. These results provide a novel pathway for S-nitroso thiol formation *in vivo*. S-nitrosation is a post-translational modification that has gained considerable attention due to its possible involvement in NO-signaling.[39,40] Biological formation of RSNO has been proposed to occur by the reaction of thiols with N_2O_3, peroxynitrite, other S-nitroso thiols (transnitrosation reactions), nitrosylated heme proteins and the direct reaction between thiyl radicals and NO. Since most of these reactions are either slow or have low specificity for a signaling process, S-nitrosation has been proposed to involve transfer of the NO ligands of DNICs to biothiols.[28] To our knowledge, the current study is the first one to demonstrate that the mechanism of DNIC formation can lead directly to formation of RSNO's through the trapping of the RS• radicals by NO. The concurrent formation of DNICs and RS• can explain studies showing that DNICs and RSNOs are formed in parallel in macrophages exposed to NO under anoxia.[28,22] Relevantly, thiyl radical formation by the autoreduction of $[Fe^{II}(NO)(RS)_2]$ could favor certain biothiols, rendering some specificity to RSNO formation. Future efforts will focus on the investigation of autoreduction of this intermediate formed by the reactions with different biothiols.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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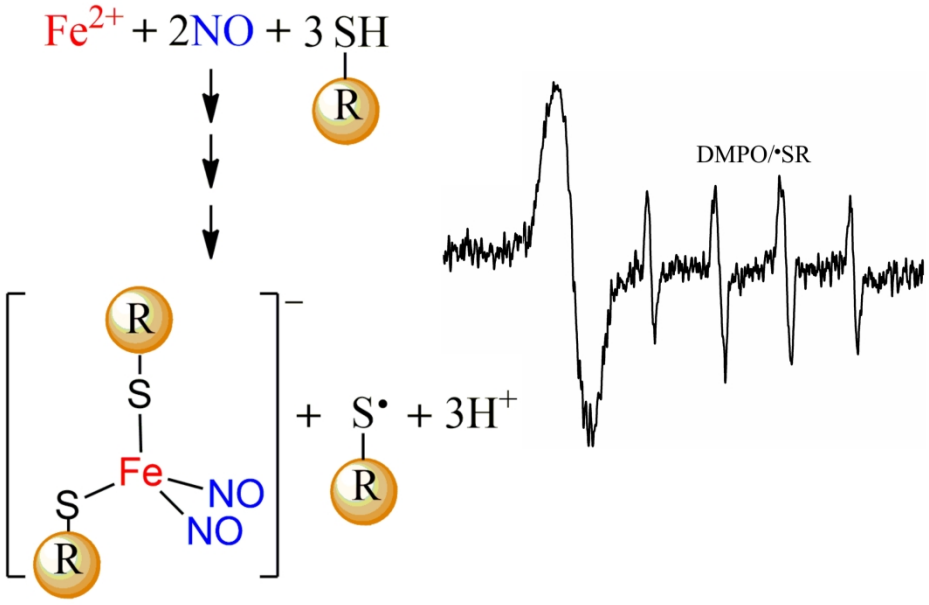

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