Journal Name

RSC Publishing

COMMUNICATION

Cite this: DOI: 10.1039/x0xx00000x

Thiyl Radicals Are Co-Products of Dinitrosyl Iron Complex (DNIC) Formation

Received 00th January 2019,

Daniela R. Truzzi,*[ab] Ohara Augusto [b] and Peter C. Ford *[a]

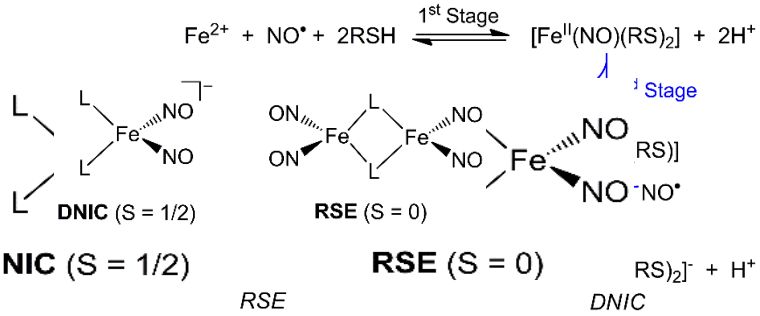
[a] Department of Chemistry and Biochemistry, University of California, Santa Barbara, Santa Barbara, CA, 93106-9510 USA.
[b] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, 05508-000, Brazil.
*Corresponding authors: dtruzzi@iq.usp.br and ford@chem.ucsb.edu

Electronic supplementary information (ESI) available: Materials and method, experimental procedures and 2 figures (5 pages total, PDF).

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] Notably, 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 nitric oxide, 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 complexes 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]

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.

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 1 illustrates the temporal absorbance changes at 350 nm (Abs_{350}) as observed with a stopped-flow spectrophotometer. In both cases, the two stage reaction sequence noted in earlier[16] and ongoing[29] studies is evident. Under the conditions described in Figure 1, 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.

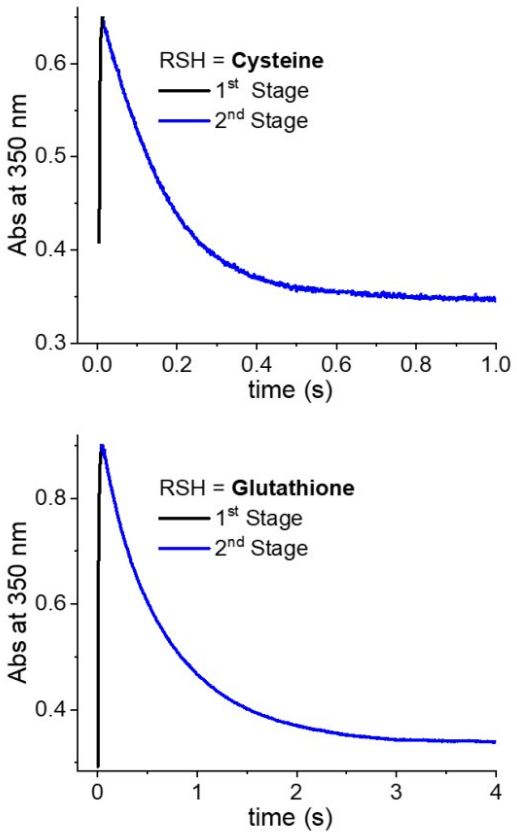


Figure 1 Temporal absorbance changes at 350 nm upon stopped-flow mixing of solutions with final concentrations of [Fe] = 0.090 mM, [NO] = 0.93 mM and *Upper:* [Cys] = 10.0 mM. *Bottom*: [GSH] = 10.0 mM in pH 7.4 HEPES buffer (200 mM).

After determining the time scale of each stage for the assembly of the dinitrosyl iron complexes of CysSH and GSH ~~assemble~~, we used continuous flow EPR spectroscopy (Supporting Infromation Fig. S1) under the same experimental condition to record ~~spectra~~ the spectra of potential intermediates upon symmetrical mixing of the reagents at a continuous flow of 0.5 mL/min. Figure 2 (upper) displays the spectrum acquired for CysSH ~~(~~at 140 ms~~)~~ and ~~which~~ 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] to a triplet Fe^{I} mononitrosyl complex, presumably either $Fe^{I}(NO)(CysS)_2^{-}$ or $Fe^{I}(NO)(CysS)$. A~~fter~~ 140 ms the reaction with CysSH is already well into the second stage as evidenced both by the absorbance changes seen in Figure 1 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. 2 bottom) shows the DNIC to be the only detectable paramagnetic product.[31] 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.

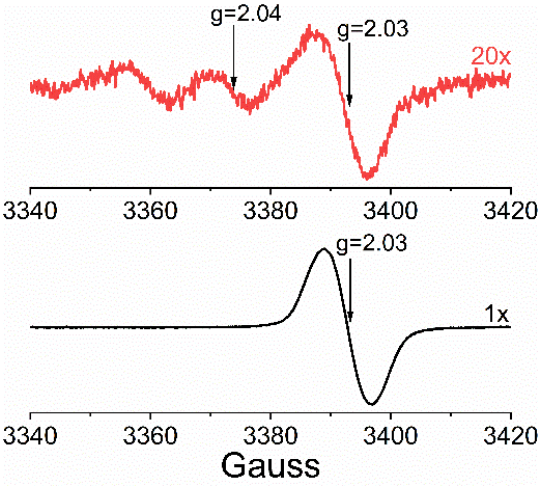
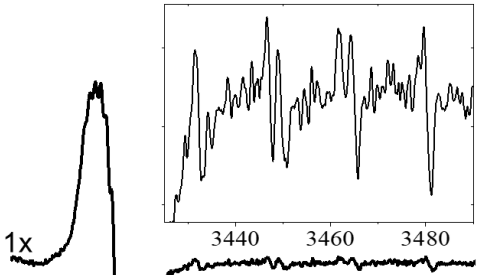


Figure 2 Temporal EPR spectra recorded using a flow cell mixer to prepare reaction solutions with final concentrations [Fe] = 0.090 mM, [NO•] = 0.93 mM and [CysSH] = 10.0 mM in pH 7.4 HEPES buffer (200 mM). *Upper:* EPR spectrum acquired 140 ms after mixing solutions at continuous flow of 0.5 ml/min. *Bottom*: EPR spectrum acquired 5 s after stopping the solution flow. Instrumental conditions: microwave power, 2 mW; time constant, 81.9 ms; scan rate, 0.6 G/s; modulation amplitude, 5 G.

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. Under the reaction conditions such radicals would be expected to be trapped by NO to form RSNO products. 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}.[32,33] Under the reaction conditions it was necessary to use a very large excess of DMPO in order to compete for any thiyl radicals formed with the fast trapping by NO (k = 3 × 10^9 M^{-1} s^{-1}).[34] Figure 3 (upper panel) displays the EPR spectrum obtained when solutions at pH 7.4 containing Fe^{2+} (0.090 mM), NO (0.36 mM), CysSH (10 mM) and DMPO (140 mM) were mixed and promptly transferred to a flat cell. 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 = 15.2 G, a_H = 17.4 G, a_N/a_H = 0.87) is evident.[32,35] The EPR spectrum obtained in an analogous solution with GSH (10 mM) (Figure 3, 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).[35,36] 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). As a control, solutions prepared without Fe(II), that is, containing just [NO] = 0.36 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~~ suggest 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.[37,38] 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•~~RSNO~~ can explain studies showing that DNICs ~~RSNOs~~ and RSNOs ~~DNICs~~ are formed in parallel ~~in~~ when macrophages are exposed to NO



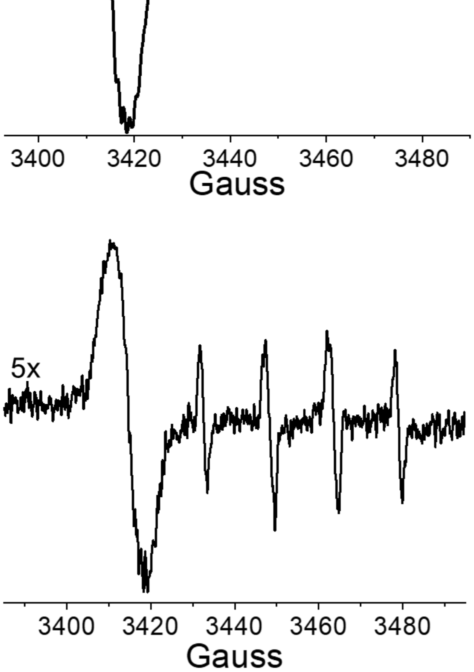


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Figure 3 EPR spectra immediately after mixing room temperature solutions with final concentrations [Fe(II)] = 0.090 mM, [NO] = 0.36 mM, [RSH] = 10 mM and [DMPO] = 140 mM in pH 7.4 HEPES buffer (200 mM). *Top:* RSH = Cys (DMPO/•SCys; a_N = 15.2 G and a_H = 17.4 G). *Insert:* expanded about 6-fold. *Bottom:* RSH = GSH (DMPO/•SG; a_N = 15.3 G and a_H = 16.0 G). Instrumental conditions: microwave power, 20 mW; time constant, 81.9 ms; scan rate, 1.4 G/s; modulation amplitude, 1 G.

Notes and references

1 L. J. Ignarro, Ed., *Nitric oxide: biology and pathobiology*, Academic Press, London, 2 edition., 2009.
2 J. C. Toledo Jr and O. Augusto, *Chem. Res. Toxicol.*, 2012, **25**, 975–989.
3 T. A. Heinrich, R. S. da Silva, K. M. Miranda, C. H. Switzer, D. A. Wink and J. M. Fukuto, *Br. J. Pharmacol.*, 2013, **169**, 1417–1429.
4 N. Hogg, *Annu. Rev. Pharmacol. Toxicol.*, 2002, **42**, 585–600.
5 J. C. Toledo, C. A. Bosworth, S. W. Hennon, H. A. Mahtani, H. A. Bergonia and J. R. Lancaster, *J. Biol. Chem.*, 2008, **283**, 28926–28933.
6 O. Augusto, S. Goldstein, J. K. Hurst, J. Lind, S. V. Lymar, G. Merenyi and R. Radi, *Free Radic. Biol. Med.*, 2019, **135**, 210–215.
7 J. R. Hickok, S. Sahni, H. Shen, A. Arvind, C. Antoniou, L. W. M. Fung and D. D. Thomas, *Free Rad. Biol. Med.*, 2011, **51**, 1558–1566.
8 A. Vanin, V. Serezhenkov, V. Mikoyan and M. Genkin, *Nitric Oxide*, 1998, **2**, 224–234.
9 A. J. Vithayathil, J. L. Ternberg and B. Commoner, *Nature*, 1965, **207**, 1246–1249.
10 A. F. Vanin and R. M. Nalbandian, *Biofizika*, 1965, **10**, 167–168.
11 J. R. Lancaster and J. B. Hibbs, *Proc. Natl. Acad. Sci. U.S.A.*, 1990, **87**, 1223–1227.
12 Dietmar. Seyferth, M. K. Gallagher and Martin. Cowie, *Organometallics*, 1986, **5**, 539–548.
13 A. R. Butler, C. Glidewell and I. L. Johnson, *Polyhedron*, 1987, **6**, 1147–1148.
14 M. C. Kennedy, W. E. Antholine and H. Beinert, *J. Biol. Chem.*, 1997, **272**, 20340–20347.
15 C. E. Tinberg, Z. J. Tonzetich, H. Wang, L. H. Do, Y. Yoda, S. P. Cramer and S. J. Lippard, *J. Am. Chem. Soc.*, 2010, **132**, 18168–18176.
16 J. C. M. Pereira, A. V. Iretskii, R.-M. Han and P. C. Ford, *J. Am. Chem. Soc.*, 2015, **137**, 328–336.
17 A. F. Vanin, A. P. Poltorakov, V. D. Mikoyan, L. N. Kubrina and D. S. Burbaev, *Nitric Oxide*, 2010, **23**, 136–149.
18 B. Muller, A. L. Kleschyov and J. C. Stoclet, *Br. J. Pharmacol.*, 1996, **119**, 1281–1285.
19 D. R. Richardson and H. C. Lok, *Biochim. Biophys. Acta*, 2008, **1780**, 638–651.
20 H. C. Lok, S. Sahni, P. J. Jansson, Z. Kovacevic, C. L. Hawkins and D. R. Richardson, *J. Biol. Chem.*, 2016, **291**, 27042–27061.
21 S. Sahni, J. R. Hickok and D. D. Thomas, *Nitric Oxide*, 2018, **76**, 37–44.
22 Q. Li, C. Li, H. K. Mahtani, J. Du, A. R. Patel and J. R. Lancaster, *J. Biol. Chem.*, 2014, **289**, 19917–19927.
23 F. C. Damasceno, A. L. Condeles, A. K. B. Lopes, R. R. Facci, E. Linares, D. R. Truzzi, O. Augusto and J. C. Toledo, *J. Biol. Chem.*, 2018, **293**, 8530–8542.
24 A. F. Vanin, V. P. Mokh, V. A. Serezhenkov and E. I. Chazov, *Nitric Oxide*, 2007, **16**, 322–330.
25 E. V. Shamova, L. M. Shishlo, I. V. Gorudko, E. N. Aleksandrova, S. N. Cherenkevich and K. B. Shumaev, *Bull. Exp. Biol. Med.*, 2011, **150**, 372–374.
26 A. B. Shekhter, T. G. Rudenko, L. P. Istranov, A. E. Guller, R. R. Borodulin and A. F. Vanin, *Eur. J. Pharm. Sci.*, 2015, **78**, 8–18.
27 S.-C. Wu, C.-Y. Lu, Y.-L. Chen, F.-C. Lo, T.-Y. Wang, Y.-J. Chen, S.-S. Yuan, W.-F. Liaw and Y.-M. Wang, *Inorg. Chem.*, 2016, **55**, 9383–9392.
28 C. A. Bosworth, J. C. Toledo, J. W. Zmijewski, Q. Li and J. R. Lancaster, *Proc. Natl. Acad. Sci. U.S.A.*, 2009, **106**, 4671–4676.
29 D. R. Truzzi, O. Augusto and P. C. Ford, manuscript in preparation.
30 A. F. Vanin, A. A. Papina, V. A. Serezhenkov and W. H. Koppenol, *Nitric Oxide*, 2004, **10**, 60–73.
31 It should be noted that the other dinitrosyl product, the corresponding RSE, which is present in a pH and [RSH] dependent equilibrium with the DNIC is EPR silent.
32 M. J. Davies, L. G. Forni and S. L. Shuter, *Chem. Biol. Interact.*, 1987, **61**, 177–188.
33 D. I. Potapenko, E. G. Bagryanskaya, Y. P. Tsentalovich, V. A. Reznikov, T. L. Clanton and V. V. Khramtsov, *J. Phys. Chem. B*, 2004, **108**, 9315–9324.
34 E. Madej, L. K. Folkes, P. Wardman, G. Czapski and S. Goldstein, *Free Radic. Biol. Med.*, 2008, **44**, 2013–2018.
35 M. G. Bonini and O. Augusto, *J. Biol. Chem.*, 2001, **276**, 9749–9754.
36 B. Kalyanaraman, *Biochem. Soc. Symp.*, 1995, **61**, 55–63.
37 D. T. Hess and J. S. Stamler, *J. Biol. Chem.*, 2012, **287**, 4411–4418.

38 B. C. Smith and M. A. Marletta, *Curr. Opin. Chem. Biol.*, 2012, **16**, 498–506.

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