Distortion of the [FeNO]₂ Core in Flavodiiron Nitric Oxide Reductase Models Inhibits N-N Bond Formation and Promotes Formation of Unusual Dinitrosyl Iron Complexes: Implications for Catalysis and Reactivity

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ABSTRACT:

Flavodiiron nitric oxide reductases carry out the reduction of nitric oxide (NO) to nitrous oxide (N₂O), allowing infectious pathogens to mitigate toxic levels of NO generated in the human immune response. We previously reported the model complex [Fe₂(BPMP)(OPr)(NO)₂](OTf)₂ (1, OPr⁻ = propionate) that contains two coplanar NO ligands and that is capable of quantitative NO reduction to N₂O [White et al., J. Am. Chem. Soc. 2018, 140, 2562-2574]. Here we investigate, for the first time, how a distortion of the active site affects the ability of the diiron core to mediate N_2O formation. For this purpose, we prepared several analogs of 1 that contain two monodentate ligands in place of the bridging carboxylate, $[Fe_2(BPMP)(X)_2(NO)_2]^{3+/1+}$ (2-X; X = triflate, 1methylimidazole, or methanol). Structural data of 2-X show that without the bridging carboxylate, the diiron core expands, leading to elongated (O)N-N(O) distances (from 2.80 Å in 1 to 3.00-3.96 Å in 2-X) and distorted (O)N-Fe-Fe-N(O) dihedral angles (from coplanarity (5.9°) in 1 to 52.9-85.1° in 2-X). Whereas 1 produces quantitative amounts of N₂O upon one-electron reduction, N₂O production is substantially impeded in 2-X, to an initial 5-10% N₂O yield. The main products after reduction are unprecedented hs-Fe^{II}/{Fe(NO)₂}^{9/10} dinitrosyl iron complexes (DNICs). Whereas mononuclear DNICs are stable and do not show N-N coupling (since it is a spin-forbidden process), the hs-Fe^{II}/{Fe(NO)₂}^{9/10} DNICs obtained from 2-X show unexpected reactivity and produce up to quantitative N₂O yields after 2 hours. The implications of these results for the active site structure of FNORs are discussed.

INTRODUCTION

Flavodiiron proteins (FDPs) were first recognized for their ability to reduce O₂ to water in a purely protective, not metabolic, function.¹ Later, Gardner realized that some FDPs are designated NO reductases (flavodiiron NORs, or FNORs).^{2, 3} In general, FDPs can catalyze the reduction of both NO and O₂, but there is usually a catalytic preference – those that preferentially reduce NO are considered FNORs.^{2,4} Here, different protein environments play a crucial role in determining the preference for NO or O₂, but the details of how FDPs accomplish their substrate specificity are currently not well understood.⁵⁻⁷ Further studies have shown that FNORs are found in many pathogens, for example *Escherichia coli, Salmonella enterica, Treponema denticola, Desulfovibrio vulgaris, Trichomonas vaginalis*, and *Giardia intestinalis*,⁸⁻¹⁷ and they equip these microbes with the ability to detoxify the NO released by the human immune system,¹⁸⁻²¹ by reduction to less toxic N₂O:

$$2 \text{ NO} + 2 \text{ e} + 2 \text{ H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$$

In this way, FNORs support microbial pathogenesis, leading to chronic infections that are harder to cure for the human immune system. Hence, FNORs are potential drug targets, and mechanistic studies on these enzymes contribute to the greater goal of finding new strategies to combat drugresistant pathogens.

All structurally characterized FDPs, including FNORs, have very similar active sites. FDPs from organisms such as *Escherichia coli*, *Desulfovibrio gigas*, *Rhodobacter capsulatus*, *Methanobacterium thermoautotrophicum*, and *Treponema denticola* have been studied.^{6,12,22,23} These FDPs exist as head-to-tail homodimers, where every monomer has a non-heme diiron site in the N-terminal domain and a flavin (FMN) binding site in the C-terminal domain. In the headto tail arrangement of the homodimer, the diiron site of one subunit is only about 4–6 Å away from the FMN cofactor of the other subunit. In the diiron core of the *Moorella thermoacetica* and *Thermotoga maritima* enzymes, each iron center is coordinated by two histidine ligands and a monodentate carboxylate, as shown in Figure 1.^{24,25}



Figure 1. Crystal structure of the non-heme diiron active site of the *M. thermoacetica* FNOR (PDB ID: 1YCG)²⁴, including the secondary coordination sphere Y195 and H25^{26,27}

In addition, the two irons are bridged by a hydroxide and a carboxylate ligand, but the significance of these bridging ligands for FNOR catalysis has so far remained unclear.²⁸

While the activation of NO by diiron sites has been studied in both FNORs and in several synthetic model complexes,²⁹ the mechanism by which NO is reduced to N₂O is still contested. Rapid freeze-quench Mössbauer and EPR studies from Kurtz and co-workers on the *T. maritima* FDP have shown that while a diiron mononitrosyl hs-Fe^{II}/hs-{FeNO}⁷ is generated in this enzyme upon sequential addition of NO equivalents, the diiron dinitrosyl complex, [hs-{FeNO}⁷]2,^{30,31} is the catalytically relevant intermediate that accumulates before any N₂O is formed.^{32,33} From the diiron dinitrosyl intermediate, three different pathways for N–N coupling are feasible, all revolving around the question of how the FMN cofactor participates in catalysis (see Scheme 1).

Scheme 1. Proposed mechanisms for NO reduction in FNORs.



Interestingly, Kurtz and co-workers have shown that even in the absence of the FMN cofactor, the *Tm* FDP is still capable of a single turnover of 2NO to N₂O from the diferrous state.³³ However, this occurs at a slower rate compared to the flavinated FDP as monitored by stopped-flow UV-Vis spectroscopy. Here, both reductive equivalents originate from the diferrous core in a direct reduction mechanism (Scheme 1, top), implying that the FMN cofactor acts solely as a means of re-reducing the active site after N-N coupling. This is surprising, given that numerous reported hs-{FeNO}⁷ model complexes are stable towards N-N coupling,³⁴⁻⁴¹ in part due to the high covalency of the Fe-NO bond.⁴² Studies on *Mt* FDP further revealed a ~7-fold and ~34-fold reduction in NOR reactivity when two second coordination sphere (SCS) residues in the active site, Tyr195 and His25 (both in hydrogen-bonding distance to the putative FeNO units), respectively, are substituted for non-hydrogen bonding side chains (Figure 1).²⁴ Correspondingly,

replacement of the analogous Tyr197 residue in *Tm* FDP by Phe leads to an enzyme with no detectable NOR activity.²⁷ Recent DFT studies identify potential roles for the SCS Tyr residue, and show that a H-bonding interaction to one of the FeNO units assists in catalysis by lowering activation barriers for both N-N coupling and N-O cleavage along the catalytic pathway.²⁶ In another study, it was proposed that this hydrogen bond assists in a critical hyponitrite rotation (reorientation) during catalysis.²⁷ However, *the exact geometric and electronic requirements of the diiron core that allow for fast and efficient N-N coupling in FNORs are still elusive*.

In previous work, we have demonstrated that the donicity of the primary coordination sphere in our diiron model complexes dictates the mechanism by which N-N coupling occurs in these systems. While our initially reported [hs-{FeNO}⁷]₂ complex [Fe₂(BPMP)(OPr)(NO)₂]²⁺(1) carries out rapid and quantitative N₂O generation after one-electron reduction (via semireduction; see Scheme 1, bottom),³⁴ the [Fe₂(Pv₂PhO₂MP)(OAc)₂]⁻ derivative with two phenolate ligands in place of two pyridine groups carries out rapid and quantitative direct coupling of NO from the diferrous state, without the need for reduction of the putative [hs-{FeNO}⁷]₂ intermediate.⁴³ Besides the importance of redox-tuning for the mechanism of NO reduction,⁴ we expect that the coplanar (*syn*) geometry of the [FeNO]₂ core ((O)N-Fe-Fe-N(O) dihedral angle: 5.9°) in complex 1 is potentially important for its rapid and quantitative NO reduction to N2O. However, *it is not* clear at this point how sensitive N-N coupling and N₂O formation are to distortions of the [FeNO]₂ core. Interestingly, the related [hs-{FeNO}⁷]₂ diiron complex with trans NO ligation, [L{Fe(NO)}2(µ-OOCR)](BPh4)2 ((O)N-Fe-Fe-N(O) dihedral angle: 168.6°), does not carry out NO reduction upon one-electron reduction, but instead, undergoes disproportionation to form 1 equivalent of a [{Fe(NO)₂}⁹]₂ dimer and 1 equivalent of the diferrous complex.⁴⁴ These products appear stable and show no further reactivity. Furthermore, the non-heme diiron enzymes

ribonucleotide reductase (RNR) and soluble methane monooxygenase (sMMO) are able to form [hs-{FeNO}⁷]₂ adducts in their active sites, but they lack the ability to mediate N₂O generation.^{45,46} This finding has puzzled researchers for a long time. Based on our results, we now propose that this lack of reactivity is related to the geometric structures of their diiron dinitrosyl intermediates.

In this paper, we investigate, for the first time, the sensitivity of N-N coupling and N_2O generation to a distortion of the [FeNO]2 core. For this purpose, we developed a series of $[Fe_2(BPMP)(X)_2(NO)_2]^{3+/1+}$ complexes (2-X; where X = triflate (OTf⁻), 1-methylimidazole (MI), or methanol (MeOH)) with two monodentate coligands X (instead of a bridging carboxylate), which contain non-coplanar (O)N-Fe-Fe-N(O) units and expanded (O)N-N(O) distances compared to 1 (see Figure 2 for a ChemDraw of 1 and 2-X). We then demonstrate that the NO reductase activity of these model complexes is highly sensitive to slight distortions of their [FeNO]₂ cores (see Figure S1), where even moderate increases in the (O)N-Fe-Fe-N(O) dihedral angle and/or (O)N-N(O) distance greatly reduce N₂O yields. These results have important implications for the optimal active site design in FDPs that are designated for NO reduction and point towards a key difference in the active site rigidity of these enzymes, distinguishing them from FDPs that are designated O_2 reductases. Instead of producing N_2O , the model complexes 2-X mostly generate novel dinitrosyl iron complexes (DNICs) as initial products after reduction, which, in an unprecedented reaction, are then able to mediate additional N2O generation (over the course of 2 hours). Importantly, as we show in this paper, it is the unusual, dinuclear structure that allows these DNICs to circumvent the spin-forbidden nature of the N-N coupling reaction of their two NO ligands. In this way, our results demonstrate how DNICs could in fact mediate NO reduction. The latter reaction is potentially relevant for the de-nitrosylation of DNICs formed by the reaction of NO with 2Fe2S sites in NO sensing iron-sulfur proteins (transcription factors), to regenerate the 2Fe2S cluster at low NO concentrations.^{47,48}



Figure 2. ChemDraw comparison between complex 1 and complexes 2-X (see also Figure S1).

RESULTS

Synthesis and Basic Structural and Spectroscopic Characterization.

The synthesis, purification, and nitrosylation of BPMP⁻ complexes with two monodentate ligands can be directly adapted from our previously reported procedure for the synthesis of $[Fe_2(BPMP)(OPr)(NO)_2]^{2+}$ (1), where the bridging propionate (OPr⁻) is either substituted for two equivalents of the monodentate ligand (in case of the X = MI (1-methylimidazole) complex), or where no monodentate ligand is added in the synthesis (for the X = MeOH (methanol) and OTf⁻ (triflate) complexes).³⁴ In the latter two cases, the metallation is still carried out in MeOH followed by precipitation with Et₂O. The precipitate is then filtered off and washed with CH₂Cl₂ to remove Na/KOTf salt byproducts. To generate the OTf⁻ precursor complex, the CH₂Cl₂ filtrate is directly precipitated with hexanes to afford [Fe₂(BPMP)(OTf)₂](OTf). In order to prepare the MeOH complex, the CH₂Cl₂ filtrate must first be evaporated and the solid residue re-dissolved in MeOH and precipitated with Et₂O to generate the [Fe₂(BPMP)(MeOH)₂](OTf)₃ analog. The X = OTf⁻ and MI derivatives are then nitrosylated in CH₂Cl₂ and precipitated with hexanes to afford [Fe₂(BPMP)(OTf)₂(NO)₂](OTf) (**2-OTf**) and [Fe₂(BPMP)(MI)₂(NO)₂](OTf)₃ (**2-MI**), respectively, whereas the X = MeOH complex is nitrosylated in MeOH and precipitated with Et₂O to afford [Fe₂(BPMP)(MeOH)₂(NO)₂](OTf)₃ (**2-MeOH**). Complexes **2-X** show typical UV-Vis spectra for this class of compounds, with broad absorption bands in the 350-450 nm region (Figure S2), which are assigned to NO⁻ to Fe^{III} charge transfer and d-d transitions.^{42,49} The complexes are easily distinguished by IR spectroscopy, where **2-OTf** with weakly coordinating triflate ions shows the highest energy N-O stretch, v(N-O), at 1800 cm⁻¹ (in the solid state). As the donating ability of the coligands X increases, v(N-O) shifts to lower energy, 1776 cm⁻¹ in **2-MeOH** and finally 1756 cm⁻¹ in **2-MI** (1710 cm⁻¹ in **2-MI**(¹⁵NO); see Figure 3).



Figure 3. Overlay of the IR spectra (taken on KBr disks) of the series of complexes **2-X** and **2-MI**(¹⁵**NO**), highlighting their discrete NO stretching frequencies. Figure S3 shows the full spectra.

Note that while NO⁻ can bind to metal centers in either the singlet or triplet state, the electronic structure of non-heme hs- $\{FeNO\}^7$ complexes has been previously determined to be Fe(III)- $^3NO^-$ in character. ^{4,49,50} The IR data therefore follow the general electronic structure description of these

complexes, where the π -donicity of the ³NO⁻ ligand is tuned by the donating abilities of the other coligands of the iron center.⁵¹ In comparison, the N-O stretch of the propionate-bridged complex **1** is observed at 1760 cm⁻¹, which shows that electronically, the hs-{FeNO}⁷ units in **1** and **2-MI** have very similar properties.

The X-Ray crystallographic characterization of these complexes was met with various degrees of success. The precursor complex [Fe₂(BPMP)(MI)₂(OTf)](OTf)₂ was successfully crystallized (Figure S4) and shows two six-coordinate (6C) iron centers containing N₅O ligation in the primary coordination sphere and non-coplanar MI ligands with a (MI)N-Fe-Fe-N(MI) dihedral angle of 58.3°. Unfortunately, the corresponding NO complex **2-MI** was typically obtained as an oily solid and single crystals of this compound could not be obtained, despite several attempts. However, both **2-OTf** and **2-MeOH** were structurally characterized, as shown in Figure 4.



Figure 4. Left: Crystal structure of **2-OTf** (CCDC # 2103072) obtained via the vapor diffusion of Et₂O into a NO-saturated CH₃CN solution of **2-OTf** at room temperature. Right: Crystal structure of **2-MeOH** (CCDC # 2059419) obtained by vapor diffusion of Et₂O into a NO-saturated MeOH solution of **2-MeOH** at room temperature. Cocrystallized solvents, counterions, and hydrogen atoms (except for MeOH) are omitted for clarity

The complex 2-OTf contains two 6C hs- $\{FeNO\}^7$ units with identical (typical) Fe-NO bond distances of 1.77 Å and Fe-N-O angles of 157.3 and 153.2° and, importantly, a non-coplanar [FeNO]₂ core with a (O)N-Fe-Fe-N(O) dihedral angle of 52.9° and an (O)N-N(O) distance of 3.00 Å. The complex 2-MeOH was fortuitously crystallized from our initial attempts to metallate BPMP- in the presence of 2,4,6-trimethylpyridine (TMP): upon vapor diffusion of Et₂O into a NOsaturated MeOH solution of this reaction mixture, crystals of 2-MeOH were obtained.⁵² In this complex, each hs-{FeNO}⁷ unit is 6C with one solvent molecule bound, which was quite surprising. In this complex, the Fe-NO bond distances are 1.76 Å, the Fe-N-O angles are 153.7°, and the (O)N-Fe-Fe-N(O) dihedral angle is the largest of our series at 85.1°. The resulting (O)N-N(O) distance is 3.96 Å. Interestingly, this complex features two H-bonding interactions between each MeOH group and the adjacent NO ligand, with MeOH---N(O) H-bonding distances of 2.73 Å. These unusual hydrogen bonds are likely the reason for the further distortion of the [FeNO]2 core in 2-MeOH compared to 2-OTf. Accordingly, one must be mindful that the structure of 2-MeOH is subject to larger changes in solution, if, for example, the MeOH molecules engage in hydrogen-bonding to solvent molecules instead of the NO ligands. The exact structure of 2-MeOH is therefore somewhat uncertain in solution, which needs to be considered when analyzing the reactivity data of this complex. Please note that 2-OTf and 2-MeOH show clearly different N-O stretching frequencies, and in this way, these complexes can easily be distinguished when prepared in bulk. The structural parameters of all [hs-{FeNO}⁷]₂ complexes are compared in Table 1.

	1 ^a		2-OTf		2-MI		2-MeOH		3	
	DFT	Expt	DFT	Expt	DFT	Expt	DFT	Expt	DFT	Expt
Fe-Fe (Å)	3.54	3.47	3.74	3.63	3.90		3.86	3.72		
Fe-NO (Å)	1.74, 1.76	1.77, 1.80	1.75	1.77, 1.77	1.74		1.73	1.76	1.72	1.76
N-O (Å)	1.18, 1.18	1.16, 1.17	1.17	1.16, 1.15	1.18		1.17	1.18	1.14	1.12
Fe-N-O (°)	147.7, 143.0	155.5, 144.7	150.7, 147.2	153.2, 157.6	147.5		149	153.7	178.4	180.0
(O)N-N(O) (Å)	2.64	2.80	3.04	3.00	3.78		3.36	3.96		
Dihedral (O)N-Fe-Fe-N(O) (°)	4.1	5.9	51.5	52.9	76.8		64.7	85.1		
Dihedral O-N-N-O (°)	4.5	4.9	21.3	23.9	18.2		72.2	90.4		
v(N-O) (cm ⁻¹) ^b	1733, (1665)	1760	1789, (1765)	1800	1728, (1713)	1756	1765, (1747)	1776	1793	1848

Table 1. Overview of key experimental and DFT-optimized (BP86/TZVP) structural and vibrational parameters for complexes **2-X** and **3**.

^a Structural parameters for **1** are from previous publications.^{34,37 b} Experimental N-O stretching frequencies are reported from solid state IR measurements in KBr disks.

Since the structures of the [hs-{FeNO}⁷]² cores are of importance in correlating NO binding geometries and (O)N-N(O) distances to their N-N coupling reactivity, DFT optimizations and frequency calculations were employed to supplement the X-ray crystallographic data discussed above. Using the crystal structures of **1**, **2-OTf** and **2-MeOH** as starting points, Gaussian 09⁵³ optimizations were performed on these complexes using both BP86/6-311G(d) and BP86/TZVP to evaluate how well these methods predict the relevant core structural features of these complexes. The fully optimized structures generally reproduce the key Fe-Fe, Fe-NO and N-O distances and Fe-N-O angles well (see Tables 1 and S1). For **2-OTf**, the experimental (O)N-Fe-Fe-N(O) and (O)N-N(O) angles of 53° and 24°, respectively, are reproduced with very high accuracy (within 3°). Similar precision is also observed for **1**, as shown in Table 1. Larger deviations are observed for **2-MeOH**, reflecting the critical role of the MeOH---N(O) hydrogen bonds for the core structure, which are harder to reproduce by DFT. Here, the experimental (O)N-Fe-Fe-N(O) and (O)N-N(O) angles of 85° and 90°, respectively, are underestimated in the DFT

calculations by about 10-20°, while the experimental MeOH----N(O) distance of 2.73 Å is overestimated by DFT at 3.31 Å. In the case of **2-MeOH**, we speculate that the DFT-optimized structure might actually better reflect the solution structure of the complex (with de-emphasized H-bonds). In any case, these results reasonably reproduce the core structural trends between **1**,³⁷ **2-OTf** and **2-MeOH**, as shown in Table 1.

Finally, in order to obtain a structural model for **2-MI**, we also optimized the geometry of this complex, using the fully optimized structure of **2-OTf** as a starting point. The DFT results indicate that this complex has a more distorted structure compared to **1** and **2-OTf**, with predicted (O)N-Fe-Fe-N(O) and (O)N-N(O) angles of 77-79° and 18-21°, respectively. We expect the DFT structure of **2-MI** to be highly accurate (with a typical error $< \pm 10^{\circ}$), as in the case of **2-OTf** and **1** (in the absence of hydrogen bonding between ligand X and the coordinated NO, which causes the larger deviations in **2-MeOH**). As a further indicator of the overall accuracy of the DFT results, we note that the DFT-calculated N-O stretching frequencies for our **2-X** series exactly reproduce the experimental trend, with v(N-O) decreasing in the order: **2-OTf** > **2-MeOH** > **2-MI** ≈ **1** (see Table 1).

Chemical and Electrochemical Reduction of Complexes 2-X.

Cyclic Voltammograms of Complexes **2-X** *and IR-Spectroelectrochemistry.* The cyclic voltammograms (CVs) of complexes **2-X** all exhibit two main reductive events. For **2-OTf** and **2-MeOH**, these are irreversible reductions that occur at -0.69 and -0.95 V, and -0.80 and -1.12 V (vs Fc^+/Fc), respectively. In contrast, the reduction of **2-MI** leads to a more stable product, with quasi-reversible reductive events at -0.90 and -1.12 V vs Fc^+/Fc (Figure S6). Based on the reduction potentials determined by CV, further chemical and electrochemical experiments were then

performed to analyze the reduction products. As a first step towards this goal, we carried out IRspectroelectrochemistry (IR-SEC) experiments on a ~5 mM solution of **2-MI** in CH₂Cl₂, containing ~0.1 M [TBA][OTf] as the supporting electrolyte. Under these conditions, the broad N-O stretching band of **2-MI** at 1756 cm⁻¹ in the solid state resolves into two features at 1747 and 1766 cm⁻¹ (see Figure S7), which belong to the corresponding symmetric and antisymmetric combinations. Upon holding the cell at a potential of -1.4 V vs. Ag wire, the N-O stretching bands of **2-MI** decayed with the formation of numerous new bands, first at 1789 and 1716 cm⁻¹ and then at 1682 and 1620 cm⁻¹, accompanied by the formation of N₂O (evident from the rising peak at 2222 cm⁻¹) as the reduction proceeded (Figure S7). These data indicate that numerous reduced species are sequentially formed upon the electrochemical reduction of the initial [hs-{FeNO}⁷]₂ complex, and that **2-MI** is still capable of generating N₂O upon reduction, but in significantly lower amounts compared to **1**. These data demonstrate that the distortion of the [FeNO]² core in **2-MI** (compared to **1**) has a detrimental effect on the N-N coupling reaction, and therefore significantly inhibits N₂O generation. Instead, new NO complexes are formed.

Chemical Reduction Experiments and Spectroscopic Characterization. The nature of these new product complexes was clarified by monitoring the bulk one- and two-electron chemical reduction of **2-MI** with CoCp₂ in CH₂Cl₂ at -70 °C by solution IR spectroscopy. In these experiments, the addition of one equivalent of CoCp₂ to **2-MI** cleanly generates two new NO bands at 1788 and 1715 cm⁻¹ that shift to 1689 and 1632 cm⁻¹ upon addition of the second equivalent of CoCp₂ (see Figure 5).



Figure 5. Solution IR overlay of complex **2-MI** (~ 5 mM) and its reduced hs-Fe^{II}/{Fe(NO)₂}^{9/10} products upon chemical reduction with either one or two equiv of CoCp₂ at -70 °C in CH₂Cl₂.

These two-band signals are a fingerprint of dinitrosyl iron complexes $(DNICs)^{54-58}$ and suggest that a $\{Fe(NO)_2\}^9$ and a $\{Fe(NO)_2\}^{10}$ species sequentially forms upon addition of one and two equivalents of reductant, respectively, as well as some N₂O. It is known from the literature that many mononuclear hs- $\{FeNO\}^7$ complexes disproportionate upon one-electron reduction, to generate 1 equivalent of a $\{Fe(NO)_2\}^{10}$ DNIC and 1 equivalent of the corresponding hs-Fe(II) complex,^{35,55,59,60} following the eqn:

$$2 \text{ hs-} \{\text{FeNO}\}^7 + 2 \text{ e}^- \rightarrow 2 \text{ hs-} \{\text{FeNO}\}^8 \rightarrow \{\text{Fe(NO)}_2\}^{10} + \text{hs-Fe}^{\text{II}}$$
(1)

Here, one NO⁻ ligand is transferred to one hs-{FeNO}⁸ complex via a dimeric intermediate to form the DNIC.⁴ Complex **1**, with a coplanar [FeNO]² unit, does not show any DNIC formation, but instead, produces quantitative amounts of N₂O upon one-electron reduction. Therefore, it is the distortion of the [FeNO]² core in **2-X** to a non-coplanar structure that suppresses N₂O generation, and instead, promotes the DNIC formation by opening this new reaction channel. We propose that upon one-electron reduction, one NO⁻ ligand is transferred from the hs-{FeNO}⁸ unit to the adjacent hs-{FeNO}⁷ center within the dimer, to generate a hs-Fe^{II}/{Fe(NO)₂}⁹ complex in a unimolecular reaction (see also Scheme 2):

$$[hs-{FeNO}^{7}]_{2} + e^{-} \rightarrow [hs-{FeNO}^{8}/hs-{FeNO}^{7}] \rightarrow hs-Fe^{II}/{Fe(NO)_{2}}^{9}$$
(2a)

Addition of a second equiv of reductant then reduces the DNIC:

hs-Fe^{II}/{Fe(NO)₂}⁹ + e⁻
$$\rightarrow$$
 hs-Fe^{II}/{Fe(NO)₂}¹⁰ (2b)

Importantly, further low-temperature IR studies show that the three complexes 2-Xproduce the exact same DNICs upon one- and two-electron reduction at -70 °C in CH₂Cl₂, as evident from their exactly identical N-O stretching bands (see Figure S8), implying that the monodentate ligands X are no longer coordinated to the DNIC-containing iron center. This is not entirely unexpected since the starting [hs-{FeNO}⁷]₂ complexes all contain 6C iron centers, and would require the displacement of at least one ligand X to coordinate the second NO ligand. While there are a few cases of 6C DNICs reported in the literature, the majority of DNICs are either fourcoordinate (4C)^{44,61,62} or five-coordinate (5C),^{61,63} supporting the idea that the monodentate ligands X are in fact lost from the DNIC-containing iron centers, allowing identical DNICs to form independent of the nature of the starting complex 2-X. A useful diagnostic feature that is helpful in distinguishing between DNICs of different coordination numbers is the splitting between the symmetric and antisymmetric N-O stretching bands, Δv (N-O), of the DNIC.⁵⁴ Here, 6C DNICs have a $\Delta v(N-O)$ of 100-110 cm⁻¹, while 5C {Fe(NO)₂}⁹ DNICs have $\Delta v(N-O)$ values of 70-80 cm⁻¹ ¹, and 4C {Fe(NO)₂}¹⁰ DNICs have Δv (N-O) values of 45-70 cm⁻¹. Experimentally, the hs- $Fe^{II}/{Fe(NO)_2}^9$ complex has a $\Delta v(N-O)$ of 73 cm⁻¹, corresponding to a 5C DNIC. In the hs- $Fe^{II}/{Fe(NO)_2}^{10}$ case, we observe a $\Delta v(N-O)$ of 57 cm⁻¹, which is consistent with a corresponding 4C complex. In summary, we propose that the DNICs formed from the (electro)chemical reduction

Scheme 2. Proposed mechanism for the sequential one- and two-electron reduction of 2-X, including key spectroscopic parameters for all experimentally captured intermediates.



EPR spectroscopy lends additional support to these conclusions. The [hs-{FeNO}⁷]₂ starting material contains two antiferromagnetically coupled S = 3/2 centers, giving a $S_t = 0$ ground state electronic structure. The proposed hs-Fe^{II}/{Fe(NO)₂}¹⁰ intermediate contains a hs-Fe^{II} center (S = 2) and a {Fe(NO)₂}¹⁰ DNIC with S = 0, both of which are similarly EPR silent. However, the hs-Fe^{II}/{Fe(NO)₂}⁹ intermediate contains a S = 1/2 {Fe(NO)₂}⁹ DNIC fragment, and should therefore be EPR active. Indeed, upon one-electron reduction of **2-MI** at -70 °C in CH₂Cl₂, a large signal appears in the EPR spectrum at g = 2.037, as shown in Figure 6 (see Figure S9 for a fit of the data).



Figure 6. Overlay of the EPR spectra of the precursor $[Fe_2(BPMP)(MI)_2](OTf)_3$, **2-MI**, and of its one-/two-electron reduced hs-Fe^{II}/{FeNO}^{9/10} products. All species other than the hs-Fe^{II}/{Fe(NO)_2}⁹ complex are EPR silent. Conditions: $[Fe_2(BPMP)(MI)_2](OTf)_3 = 2 \text{ mM}$ dissolved in CH₂Cl₂ at RT; **[2-MI]** = 2 mM dissolved in CH₂Cl₂ at RT; reaction of **2-MI** + 1 or 2 eq CoCp₂ was carried out at -70 °C in CH₂Cl₂ and samples were immediately frozen in liquid nitrogen. Spectra were taken at 4-7 K, with 2 mW power, and 9.326 Gz frequency. Figure S9 contains the fit of the S = 1/2 data.

Importantly, the g = 2.037 signal implies that the {Fe(NO)₂}⁹ fragment is essentially <u>uncoupled</u> from the hs-Fe^{II} center (S = 2), because the spin-coupled system would be expected to either have an S = 5/2 or 3/2 ground state. This result is significant, as it implies that the phenolate bridge between the hs-Fe^{II} center and the {Fe(NO)₂}⁹ DNIC is broken, likely shifting to a monodentate coordination of the hs-Fe^{II} center. This would then generate a 5C {Fe(NO)₂}⁹ DNIC, in direct agreement with the vibrational properties of this species (see above, $\Delta v(N-O) = 73 \text{ cm}^{-1}$). Here, the {Fe(NO)₂}⁹ center would then be coordinated by the tertiary amine (N_{t-amine}) and two pyridine (N_{Py}) groups from the BPMP⁻ ligand, and two NO as the 4th and 5th ligands. Based on the identical N-O stretching frequencies (from solution IR spectroscopy) for the one- and two-electron reduced products of all **2-X** complexes, we further conclude that the monodentate ligand X must have

shifted to the hs-Fe^{II} center, completing its coordination sphere. The g-value of 2.037 is within the typical range of monoiron DNICs, which have isotropic g-values ranging from about 2.01-2.03⁵⁴ (in contrast to dimeric {Fe(NO)₂}⁹/{Fe(NO)₂}¹⁰ DNICs, which tend to have axial signals with g_{\parallel} < 2).⁶⁴ Within this bracket of g-values, 4C {Fe(NO)₂}⁹ DNICs tend to have $g \sim 2.03$, whereas the reported 5C and 6C {Fe(NO)₂}⁹ DNICs have $g \sim 2.01$ -2.02, placing the g-value of our proposed hs-Fe^{II}/{Fe(NO)₂}⁹ intermediate slightly outside of the typical range for 5C DNICs (see further analysis in the Experimental Modeling section below).

Notably, a related diiron dinitrosyl complex containing a bridging pyrazolate ligand from the Meyer group was found to have minimal coupling between the two Fe centers (Fe1--Fe2 = 4.34 Å; $J = -0.1 \text{ cm}^{-1}$, as determined by SQUID magnetometry) in the hs-{FeNO}⁷/hs-{FeNO}⁷ state,⁴⁴ despite the presence of the pyrazolate bridge. This further supports our conclusion that in the absence of any bridging ligand, the {Fe(NO)₂}⁹ fragment is essentially uncoupled (J < |0.04|cm⁻¹; see Figure S27) from the hs-Fe^{II} center, as observed in the EPR data for the hs-Fe^{II}/{Fe(NO)₂}⁹ product of **2-MI**.

As discussed below, our DFT modeling further supports these conclusions about the structures of the hs-Fe^{II}/{Fe(NO)₂}⁹ and hs-Fe^{II}/{Fe(NO)₂}¹⁰ intermediates. Interestingly, the hs-Fe^{II}/{Fe(NO)₂}⁹ and hs-Fe^{II}/{Fe(NO)₂}¹⁰ DNICs formed from the reduction of **2-X** are quite unstable in CH₂Cl₂, and decay within minutes at room temperature. This is unusual, as corresponding mononuclear species are usually stable in solution for hours as long as they are kept under anaerobic conditions (see below). Interestingly, the DNICs generated from the **2-X** series are more stable in CH₃CN. Their solution reactivity and slightly different mechanistic subtleties are discussed in the SI.

Quantification of N₂O Yields and Analysis

Gas-headspace N₂O yields provide an additional means of evaluating the reactivity of the complexes **2-X** under different conditions, including reductive equivalents, solvents, and time. A new gas-headspace N₂O yield calibration curve was generated via the semireduction of a clean (by elemental analysis) batch of **1** as the N₂O standard. A fit of the data, taken in triplicate at each data point, where $y = \mu mol$ of N₂O and x = integrated area under the N₂O IR band, gives a linear fit with y = 1.858x - 0.019 (Figure S10). Based on the different solution stabilities of the DNIC products from **2-X**, N₂O yields were measured in CH₂Cl₂ and CH₃CN (see page S29-30 in the SI for a discussion of the CH₃CN data), using either 1 or 2 eq of CoCp₂. Data were collected after 5 minutes to quantify N₂O production directly from the diiron dinitrosyl complex, and after 2 hours to quantify N₂O produced from DNIC decomposition. These data are combined in Table 2 and visualized in Figure 8.

		% N ₂ O Yields ^a (± STD)						
		1 eq CoCp ₂				2 eq CoCp ₂		
Solvent	Time	2-OTf	2-MI	2-MeOH	1	2-OTf ^b	2-MI	2-MeOH
CH ₂ Cl ₂	5 min	61.8 ± 2.3	20.0 ± 0.7	64.8 ± 3.1	$\begin{array}{c} 90.0 \\ \pm \ 0.6^{b} \end{array}$	21.8 ± 0.4	16.8 ± 2.5	21.7 ± 0.5
	2 hrs	106.3 ± 3.8	$38.1\ \pm 1.7$	103.7 ± 2.6		93.8 ± 12.8	28.3 ± 4.8	89.5 ± 2.3
CH ₃ CN	5 min	31.5 ± 6.0	$4.7\ \pm 0.7$	57.9 ± 12.1		$7.4\ \pm 0.6$	$4.7\ \pm 0.7$	$8.7\pm0.2^{\rm c}$
	2 hrs	77.0 ± 7.5	$38.6\ \pm 3.2$	$82.3\pm2.3^{\rm c}$		$14.4\ \pm 2.9$	6.1 ± 1.3	$71.1 \pm 14.3^{\texttt{c}}$

Table 2. Compiled N₂O yields for **2-X** taken under different experimental conditions. N₂O yield data for the propionate-bridged complex **1** are also included for comparison.

^a Unless otherwise noted, these are averages of triplicate experiments. ^b Data for 1 were collected after 1 minute. ^c Average of duplicate experiments.



Figure 8. Graphical representation of the gas-headspace N₂O yields for **2-X** with 1 or 2 eq of CoCp₂ added, after 5 minutes and 2 hours in CH₂Cl₂ (left) and CH₃CN (right). Error bars represent \pm standard deviation.

Several comparisons can be made based on these data sets that directly complement the spectroscopic findings discussed above. First, the N₂O generation occurs in two phases, where the N₂O yields after 5 minutes are substantially lower than those obtained for 1, discussed in our previous publications,^{34,37} with 64.8 % as the highest yield from **2-MeOH** with 1 eq of CoCp₂ in CH₂Cl₂, whereas **2-OTf** and **2-MI** generate 61.8 and 20.0 % of N₂O, respectively, under these conditions. In line with the enhanced stabilities of the DNIC products in CH₃CN compared to CH₂Cl₂, the N₂O yields after 5 minutes decrease to 57.9% for **2-MeOH**, 31.5% for **2-OTf** and 4.7% for **2-MI**, respectively. Still, these data do not represent the N₂O solely produced from the initially formed, one-electron reduced hs-{FeNO}⁷/hs-{FeNO}⁸ dimer, as decomposition of the hs-Fe^{II}/{Fe(NO)₂}¹⁰ DNIC is still quite fast under these conditions at room temperature. Since the hs-Fe^{II}/{Fe(NO)₂}¹⁰ DNIC is significantly more stable, a better way to access the amount of N₂O produced solely from the hs-{FeNO}⁷/hs-{FeNO}⁸ intermediate in the first phase of the reaction (see Scheme 2) is therefore to look at the two-electron reduction data, especially in CH₃CN where

the DNIC products show the highest stability. Indeed, the N₂O yields after 5 minutes are (a) smaller in CH₃CN and (b) smaller after two-electron reduction (see Table 2), which are all conditions that stabilize the DNIC products. Upon two-electron reduction at room temperature for 5 minutes in CH₂Cl₂, **2-X** produce 21.8, 16.8, and 21.7 % N₂O for X = OTf⁻, MI, and MeOH, respectively, and only 7.4, 4.7, and 8.7 % N₂O for X = OTf⁻, MI, and MeOH, respectively, in CH₃CN under the same conditions. Since the hs-Fe^{II}/{Fe(NO)₂}¹⁰ DNIC is the most stable in CH₃CN, the latter data therefore provide the best estimate of the N₂O produced only from the direct N-N coupling in the hs-{FeNO}⁷/hs-{FeNO}⁸ intermediate (see Scheme 2), without any contributions from DNIC decomposition. Under these conditions, **2-X** produce about 5-10% N₂O from direct semireduction, which sharply contrasts with the quantitative yield of N₂O obtained with **1** under identical conditions. This result therefore underscores the conclusion that the distortions of the [FeNO]₂ core in complexes **2-X**, especially the enlarged (O)N-Fe-Fe-N(O) dihedral angle and the increased (O)N-N(O) distance, have a detrimental effect on the ability of the complexes to mediate fast and efficient N-N coupling. This aspect is further analyzed in the Discussion section.

Despite forming the exact same DNIC products, the N₂O yields over 2 hours differ across the series of complexes **2-X**. First and foremost, this suggests that the DNIC is not a dead end, but can be activated for N₂O formation, a surprising finding based on the general stability of hs- ${Fe(NO)_2}^{9/10}$ species towards N-N coupling, due to the spin-forbidden nature of this reaction.⁵⁵ Here, the N₂O yield for the hs-Fe^{II}/{Fe(NO)₂}⁹ DNIC after 2 hours is larger compared to that of the hs-Fe^{II}/{Fe(NO)₂}¹⁰ complex. For example, complexes **2-X** generate 106.3, 38.1, and 103.7% N₂O with 1 eq CoCp₂ in CH₂Cl₂ at room temperature after 2 hrs (for X = OTf⁻, MI, and MeOH, respectively), compared to 93.8, 28.3, and 89.5 % N₂O, respectively, with 2 eq CoCp₂ under otherwise identical conditions. This finding agrees with the electronic structure descriptions of these two redox states, whereby the hs-Fe^{II}/{Fe(NO)₂}⁹ complex contains weaker Fe-NO bonds with larger NO dissociation constants, and decomposes more rapidly than the hs-Fe^{II}/{Fe(NO)₂}¹⁰ DNIC.^{55,65} Since the DNIC products are generally more stable in CH₃CN, as discussed above (based on solution IR spectroscopy), N₂O yields decrease in this solvent in the 2 hour reactions as well. For example, the two-electron reduction of **2-X** in CH₃CN at room temperature results in 14.4, 6.1, and 71.1 % N₂O yields for X = OTf⁻, MI, and MeOH, respectively, after 2 hrs (see Table 2).

Taken together, these data show an interesting trend, where the N₂O yields show a strong dependence on the nature of X, even though our data demonstrate that X is not actually bound to the DNIC iron in these intermediates (evident from the fact that the DNICs show identical N-O stretching bands independent of X). The general trend is that N₂O yields decrease along the series: **2-MeOH > 2-OTf > 2-MI**, for the t = 2 hr data, in CH₃CN or CH₂Cl₂, and with 1 or 2 equivalents of CoCp₂. The trend therefore correlates with the ability of X to serve as a ligand, and since X does not bind to the DNIC, this indicates that the trend is dominated by the affinity of X for the hs-Fe(II) center. The hs-Fe(II) center must therefore somehow be involved in DNIC decomposition and affect the ability of the hs-Fe^{II}/{Fe(NO)₂}^{9/10} DNIC intermediates to produce N₂O. These findings are further analyzed in the Discussion section.

Theoretical Modeling of the Proposed DNIC Products

Suitable crystals of both proposed 5C hs-Fe^{II}/{Fe(NO)₂}⁹ and 4C hs-Fe^{II}/{Fe(NO)₂}¹⁰ intermediates could not be obtained due to their decomposition accompanied by N₂O formation, so we turned to DFT modeling to predict potential structures and vibrational frequencies for these intermediates. The starting geometry for the 5C hs-Fe^{II}/{Fe(NO)₂}⁹ species was constructed from

the DFT-optimized structure of **2-MI**, by creating the 5C DNIC (S = 1/2) on one iron center (with the N_{t-amine} and N_{Py} groups as additional ligands), and twisting this group away from the central phenolate. The other hs-Fe(II) center (S = 2) is 6C with two MI coordinated as additional ligands. This structure was then fully optimized with uncoupled spins (as observed experimentally), and subsequently used as the starting geometry for the optimization of the hs-Fe^{II}/{Fe(NO)₂}¹⁰ analog. The structures obtained in this way are shown in Figure 9.



Figure 9. Left: DFT-optimized structure of the proposed 5C hs-Fe^{II}/{Fe(NO)₂}⁹ intermediate with X = MI, illustrating a quite large (DNIC)Fe-O(PhO) distance, in support of a 5C geometry at the DNIC. Right: The analogous hs-Fe^{II}/{Fe(NO)₂}¹⁰ optimized structure contains an elongated Fe-N_{t-amine} bond, and takes on a pseudo-tetrahedral geometry, in accordance with the Δv (N-O) for this species. Select bond distances and angles are listed in Table 3. Calculated with B3LYP/TZVP.

In the ${Fe(NO)_2}^9$ oxidation state, the DNIC center is trigonal bipyramidal with one NO bound approximately trans to the tertiary amine, while the other NO rests in the trigonal plane, together with the two pyridine ligands. Here, the Fe-N_{t-amine} distance is 2.39 Å, whereas the two Fe-N_{Py} bond lengths are similar at 2.17 and 2.13 Å (see Table 3).

	hs-Fe ^{II} /-	{Fe(NO) ₂ } ⁹	hs-Fe ^{II} /{Fe(NO) ₂ } ¹⁰			
	hs-Fe ^{II}	{Fe(NO) ₂ } ⁹	hs-Fe ^{II}	${Fe(NO)_2}^{10}$		
Fe-O (PhO) (Å)	2.02	4.11	1.99	4.19		
Fe-Fe (Å)	4	5.86	5.89			
Fe-N _{t-amine} (Å)	2.31	2.39	2.33	2.61		
Fe-N _{Py} (Å)	2.35, 2.27	2.17, 2.15	2.34, 2.28	2.23, 2.13		
Fe-MI (Å)	2.28, 2.22		2.26, 2.21			
Fe-N(O) (Å)		1.80, 1.80		1.85, 1.79		
(O)N-Fe-N(O) (°)		102.8		110.2		
v(N-O) (cm ⁻¹)		1835, 1739		1653, 1616		

Table 3. Overview of key structural parameters for the DFT-optimized (B3LYP/TZVP) structures of the hs-Fe^{II}/{Fe(NO)₂}⁹ and hs-Fe^{II}/{Fe(NO)₂}¹⁰ intermediates of **2-MI**.

Upon reduction to the hs-Fe^{II}/{Fe(NO)₂}¹⁰ oxidation state, the DNIC further distorts towards a tetrahedral geometry, consistent with the experimental $\Delta v(NO)$ of the hs-Fe^{II}/{Fe(NO)₂}¹⁰ species, with the two NO and two pyridine ligands in the first coordination sphere. The structure is twisted due to the steric restrictions imposed by the presence of the central tertiary amine group (the Fe-N_{t-amine} distance expands to 2.61Å, which moves this group out of the primary coordination sphere). One of the NO ligands is bound approximately trans to the tertiary amine, while the other NO occupies a position that is about trans to one of the pyridines. The Fe-N_{py} bond lengths are 2.23 and 2.13 Å. Frequency calculations on these two DNICs predict symmetric and antisymmetric N-O stretching frequencies of 1835 and 1739 cm⁻¹ for the hs-Fe^{II}/{Fe(NO)₂}⁹ and 1653 and 1616 cm⁻¹ for the hs-Fe^{II}/{Fe(NO)₂}¹⁰ complex, respectively. The key geometric and vibrational parameters of the two optimized structures are summarized in Table 3.

Experimental Modeling of the Proposed DNIC Products

In tandem with DFT studies, we also sought to chemically model the DNIC products. As described above, the dinuclear complexes hs-Fe^{II}/{Fe(NO)₂}^{9/10} effectively contain completely uncoupled DNIC complexes that are solely ligated by one of the bis(methylpyridyl)amine (BMPA) arms of the BPMP⁻ ligand. With this in mind, we targeted a purely mononuclear DNIC model that contains a similar coordination sphere, but lacks the tethered hs-Fe(II) center. For this purpose, we used the simple BMPA ligand itself for the preparation of mononuclear DNIC analogs of the hs-Fe^{II}/{Fe(NO)₂}^{9/10} intermediates. We first synthesized the hs-{FeNO}⁷ complex, [Fe(BMPA)(OTf)₂(NO)] (**3**), and then reduced this complex by 0.5 or 1 eq of CoCp₂ in CH₂Cl₂ to generate the corresponding mononuclear {Fe(NO)₂}^{9/10} DNICs via disproportionation of the initially formed hs-{FeNO}⁸ intermediate (eqns 3a and 3b),^{4,55,59,60} which is analogous to the process that we used to obtain the hs-Fe^{II}/{Fe(NO)₂}^{9/10} species:

$$2 \text{ hs-}\{\text{FeNO}\}^7 + e^- \rightarrow \text{hs-}\{\text{FeNO}\}^8 + \text{hs-}\{\text{FeNO}\}^7 \rightarrow \text{hs-}\text{Fe}^{\text{II}} + \{\text{Fe}(\text{NO})_2\}^9$$
(3a)

hs-Fe^{II} + {Fe(NO)₂}⁹ + e⁻
$$\rightarrow$$
 hs-Fe^{II} + {Fe(NO)₂}¹⁰ (3b)

Following this route, H[BMPA] was first metallated with iron(II) triflate in CH₃CN, followed by immediate nitrosylation of the precursor, affording complex **3** as a microcrystalline solid. This complex exhibits an intense N-O stretching band at 1848 cm⁻¹ by IR (KBr), while in the ¹⁵NO isotopolog, v(N-O) shifts by 34 cm⁻¹ to 1814 cm⁻¹ (Figure 10).



Figure 10. IR(KBr) spectra of 3 and 3(¹⁵NO), illustrating a 34 cm⁻¹ shift in the N-O stretching frequency upon labelling with ¹⁵NO.

Due to limited solubility of **3** in CH₂Cl₂, the solution IR spectrum of **3** was measured in CH₃CN, affording the N-O stretch at 1817 cm⁻¹ (Figure S24), potentially indicating replacement of one or both triflate ligand(s) with CH₃CN. Such shifts in N-O stretching frequencies between solid and solution IR spectra are not uncommon for these types of complexes, and related shifts in v(N-O) have been previously reported from our laboratory.^{35,66} Owing to its quite electron poor ligand sphere, **3** has one of the highest N-O stretching frequencies reported so far for a hs-{FeNO}⁷ complex.⁶⁷ In line with this, the X-ray crystal structure of **3** reveals that the [Fe(BMPA)(NO)] core is square planar, containing a linear Fe-NO unit (Fe-N-O angle = 180° ; due to symmetry constraints), with the two OTf⁻ counter ions occupying the two axial positions (see Figure 11).



Figure 11. The X-ray crystal structure of 3 (CCDC # 2059421) shows a square planar [Fe(BMPA)(NO)] coordination sphere with two axial OTf⁻ ligands, and a completely linear Fe-NO bond (due to the C₂v symmetry of the molecule), in line with an extremely electron poor hs- $\{FeNO\}^7$ complex. Hydrogen atoms are omitted for clarity.

Interestingly, the NRVS spectrum of $3(^{57}Fe)$ shows the Fe-NO stretching frequency of this complex at 484 cm⁻¹ (Figure S11), about 26 cm⁻¹ lower than would be predicted based on our previously established v(N-O)/v(Fe-NO) correlation.⁵¹ This point requires further study.

The cyclic voltammogram of **3** shows two irreversible reductive events at -0.57 and -1.05 $V vs Fc^+/Fc$, with a new oxidative peak at 0.42 V on the reverse scan, followed by a larger oxidative feature at +1.00 V (see Figure 12).



Figure 12. Cyclic voltammogram of 3 (\sim 5 mM) in CH₃CN with 0.1 M tetrabutylammonium triflate, [TBA](OTf), as the supporting electrolyte. The measurement was conducted using a glassy carbon working electrode, a platinum counter electrode, and a silver wire pseudoreference electrode. Potentials are referenced to an Fc/Fc+ standard

The reductive event at -0.57 V is attributed to the reduction of the hs-{FeNO}⁷ complex to hs-{FeNO}⁸, followed by fast disproportionation to yield 1/2 eq of the {Fe(NO)₂}⁹ DNIC plus 1/2 eq of the hs-Fe(II) complex (1:1 ratio, see eqn 3a). The -1.05 V reductive event is then attributed to the following reduction of {Fe(NO)₂}⁹ to {Fe(NO)₂}¹⁰ (eqn 3b). On the reverse (oxidative) scan the hs-Fe(II) byproduct is irreversibly oxidized to the hs-Fe(III) state at 0.42 V. Finally, the irreversible oxidation of the hs-{FeNO}⁷ complex occurs at 1.00 V. These assignments are supported by several other cyclic voltammograms taken with different scan windows (Figure S12), as well as current integration of the redox events (Figure S13). The assignments of the reductive events at -0.57 V and -1.05 V were additionally confirmed by IR-SEC experiments (see Figure S14), illustrating that the {Fe(NO)₂}⁹ DNIC forms when the potential is held -0.50 V and that the {Fe(NO)₂}¹⁰ DNIC forms when the potential is held -1.00 V. In this way, the analysis of the CV data for complex **3** also provides a rationale for the CVs of complexes **2-X** (see Figure S6), which we propose follow the analogous mechanism whereby the first one-electron reduction results in the formation of the hs- $Fe^{II}/{Fe(NO)_2}^9$ intermediate, while the second one-electron reduction at more negative potentials then generates the hs- $Fe^{II}/{Fe(NO)_2}^{10}$ complex (following eqns 2a and 2b).

The chemical reduction of **3** with 0.5 eq CoCp₂ at room temperature in CH₂Cl₂ leads to the formation of a stable {Fe(NO)₂}⁹ species with N-O stretching frequencies of 1789 and 1713 cm⁻¹ (Figure 13),⁶⁸ shifted *only 1-2 cm⁻¹ higher* in frequency than those observed for the hs-Fe^{II}/{Fe(NO)₂}⁹ intermediate from complexes **2-X** (see Table 4).



Figure 13. Solution IR measurements for the reduction of **3** (10 mM) at RT in CH₂Cl₂ with 0.5 eq CoCp₂, which generates a {Fe(NO)₂}⁹ DNIC with N-O stretching frequencies of 1789 and 1713 cm⁻¹.

		${Fe(NO)_2}^9$	${Fe(NO)_2}^{10}$			
	2-OTf, 2- MeOH	2-MI	3	2-OTf, 2- MeOH	2-MI	3
CH_2Cl_2	1788, 1715	1788, 1715	1789, 1713	1689, 1632	1689, 1632	1694, 1636
CH ₃ CN	1785, 1709	1785, 1709	1789, 1713	[1716, 1659] [1687, 1636]	1689, 1636	[1714, 1659] [1693, 1637]

Table 4. Summary of N-O stretching frequencies (in cm⁻¹) for the DNIC product of 2-X and 3.

This is a very important result, as it confirms that the coordination environment of the DNIC is identical in hs-Fe^{II}/{Fe(NO)₂}⁹ and the {Fe(NO)₂}⁹ DNIC formed from **3**. The latter species was also characterized by EPR, as shown in Figure S15. Interestingly, while the EPR signal of this DNIC is similarly isotropic as that of the dimeric hs-Fe^{II}/{Fe(NO)₂}⁹ species, a fit of the EPR spectrum affords g-values of 1.992 for this compound (see Figure S15), compared to g = 2.0365for hs-Fe^{II}/{Fe(NO)₂}⁹. In order to evaluate this difference in effective g-values, EasySpin simulations were performed to determine whether very small exchange coupling between the hs-Fe(II) center and the $\{Fe(NO)_2\}^9$ unit in the dimer could account for this difference. These simulations, shown in Figure S27 (see also Table S3), illustrate that very weak ferromagnetic coupling $(J < 0.04 \text{ cm}^{-1})$ between the hs-Fe^{II} (S = 2) and the {Fe(NO)₂}⁹ (S = $\frac{1}{2}$) center indeed increases the effective g-values of the dimer, while maintaining an isotropic EPR signal. Once the exchange coupling constant increases above J > |0.04| cm⁻¹, the EPR signal starts to split, as shown in Figure 27, which is not observed experimentally. Our EPR results therefore support our structural assignment for the hs-Fe^{II}/ $\{Fe(NO)_2\}^9$ complex, as well as our DFT model of this species shown in Figure 9. The analogous chemical reduction of 3 at room temperature in CH₂Cl₂ with 1 eq of CoCp₂ leads to the rapid formation of a {Fe(NO)₂}¹⁰ DNIC, again with N-O stretching bands similarly shifted only 4 cm⁻¹ higher in frequency, to 1694 and 1636 cm⁻¹, compared to those of the hs-Fe^{II}/{Fe(NO)₂}¹⁰ complex obtained from **2-X** (Figure S16). This result again indicates that the

simple mononuclear BMPA complex exactly models the coordination environment of the DNIC in the hs-Fe^{II}/{Fe(NO)₂}¹⁰ dimer.⁶⁹ Importantly, <u>the mononuclear DNIC products that are</u> <u>generated upon reduction of 3 with both 0.5 and 1 eq of CoCp₂ at room temperature are stable in</u> <u>CH₂Cl₂ with no discernable N₂O formation</u> (as determined from gas headspace IR analysis, see Figure 14), in sharp contrast to the dinuclear analogs obtained from the reduction of **2-X** at -70 °C in CH₂Cl₂. This result indicates that the presence of the hs-Fe(II) center in the same complex as the DNIC, not just present in solution (which is the case for the DNICs obtained by reduction of **3**), is responsible for the slow, but sustained formation of N₂O from the hs-Fe^{II}/{Fe(NO)₂}^{9/10} complexes. The latter is a new reaction that has not been previously reported. These results clearly point to the significance of the tethered hs-Fe(II) center in the dimeric hs-Fe^{II}/{Fe(NO)₂}^{9/10} complexes for the decomposition of the DNICs, accompanied by N₂O release, as discussed above.



Figure 14. Gas-headspace IR measurements for **3** (10 μ mol) with 0.5 or 1 eq CoCp₂ in CH₂Cl₂, taken after 5 minutes and 2 hours. The solvent background (red) and N₂O yield from **2-MeOH** under related conditions (blue) are provided as a reference.

DISCUSSION

Mechanistic Implications for FNORs

Our mechanistic interpretation of the results obtained in this manuscript, and taking into consideration previous (published) work, is summarized in Scheme 3.

Scheme 3. Schematic overview of the proposed reaction intermediates and pathways leading to N₂O formation upon one- and two-electron reduction of the [hs-{Fe(NO)}⁷]₂ complexes 2-X.



The one-electron reduction of the stable $[hs-{FeNO}^7]_2$ complexes $[Fe_2(BPMP)(OPr)(NO)_2]^{2+}(1)$ and $[Fe_2(BPMP)(X)_2(NO)_2]^{3+/1+}(2-X;$ where $X = triflate (OTf^-), 1-$ methylimidazole (MI), or methanol (MeOH)) leads to the generation of a hs- $\{FeNO\}^7/hs \{FeNO\}^8$ intermediate. As we have shown previously, hs- $\{FeNO\}^8$ complexes are unstable and reactive,³⁵ and correspondingly, the hs- $\{FeNO\}^7/hs-\{FeNO\}^8$ intermediate is very reactive and can therefore not be observed directly, even in low-temperature studies.³⁴ In the case of our original compound 1 (with two coplanar FeNO units), this intermediate then proceeds to generate

quantitative amounts of N₂O via a semireduced pathway within 150 ms,³⁴ which corresponds to the pathway with rate constant k_1 in Scheme 3. However, in the analogous complexes 2-X (with two monodentate ligands X, instead of the bridging carboxylate in 1, and non-coplanar FeNO units), two divergent reaction pathways are observed, resulting in both the formation of a hs- $Fe^{II}/{Fe(NO)_2}^9$ DNIC (the pathway with rate constant k₂ in Scheme 3) via intramolecular disproportionation, as well as the formation of some initial N_2O (pathway with k_1). The formation of a dinuclear hs- $Fe^{II}/{Fe(NO)_2}^9$ DNIC in these reactions is supported by the shift in the effective g-value of this species to g = 2.037, caused by very weak ferromagnetic exchange coupling between the hs-Fe^{II} and the {Fe(NO)₂}⁹ unit (see Figure S27 and Table S3). The only other DNIC products that could form from the reduction of 2-X under our experimental conditions would be dimeric DNICs (so both iron centers in the dimer correspond to Fe(NO)₂ units), and then a dinuclear hs-Fe^{II} complex in a 1:1 mixture. However, dimeric DNICs have been shown in the past to not mediate N-N coupling and N₂O formation, even in the presence of a dimeric hs-Fe(II) complex. In fact, from such reaction mixtures, dimeric DNICs have previously been isolated and characterized by X-ray crystallography.⁴⁴

Our studies demonstrate that the kinetic competition between the two reaction pathways, formation of N₂O or a hs-Fe^{II}/{Fe(NO)₂}⁹ DNIC, is extremely sensitive to the structure of the [FeNO]₂ core (see Figure S1). In the coplanar system **1**, k₁ dominates and quantitative N₂O formation via semireduction occurs, whereas in **2-X** a kinetic competition arises that is sensitive to X. In order to estimate how much N₂O is produced solely from pathway k₁ and not the following decomposition of the DNICs (see below), the t = 5 min N₂O generation data (see Table 2 and Figure 8) need to be analyzed under conditions that provide maximum stability for the initially formed DNIC product. The most stable DNIC is the hs-Fe^{II}/{Fe(NO)₂}¹⁰ complex in CH₃CN; these

data therefore provide quantitative insight into the amount of N₂O formed from the hs- $\{FeNO\}^{7}/hs-\{FeNO\}^{8}$ intermediate of 2-X via the k₁ pathway, and in this way, allow us to further estimate the ratio of complexes that follow pathway k₁ vs. k₂. Inspection of Table 2 shows that the complexes 2-X produce 7.4, 4.7 and 8.7% N_2O for X = OTf⁻, MI, and MeOH, respectively, which means that >90% of the initially formed {FeNO}⁷/hs-{FeNO}⁸ intermediates follow pathway k₂. This result roughly correlates with the structural distortions observed in these complexes. Here, 2-MI produces significantly less N₂O than 2-OTf, due to its increased Fe-Fe distance (3.90 Å) and (O)N-Fe-Fe-N(O) dihedral angle (77°; estimated from BP86/TZVP calculations) that lead to an increase in the (O)N-N(O) distance (3.78 Å), compared to **2-OTf**, which has Fe-Fe and (O)N-N(O) distances of 3.63 and 3.00 Å, respectively, and an (O)N-Fe-Fe-N(O) dihedral angle of 53° in (from X-ray crystallography). On the other hand, 2-MeOH is an outlier, producing the highest yields of N2O despite having geometric parameters that are somewhat in-between 2-MI and 2-OTf (the Fe-Fe distance is 3.72 Å and the (O)N-Fe-Fe-N(O) dihedral angle is 85°), and, in particular, having the longest (O)N-N(O) distance (3.96 Å). We propose that the geometry of the [FeNO]₂ core in the crystal structure of **2-MeOH** is critically influenced by hydrogen bonding between the MeOH and NO ligands (MeOH---NO = 2.73 Å in the crystal structure). These hydrogen bonds could break in solution, leading to a change in the geometry of the [FeNO]₂ core of this complex, which would then influence the N₂O yields. Alternatively, the protic MeOH ligands might assist in the N-N coupling reaction, promoting N₂O formation in this complex.⁷⁰

Despite this outlier, our data clearly demonstrate that N-N coupling and N₂O formation by diiron cores is strongly retarded even upon a moderate distortion of the [FeNO]₂ core, as in the case of **2-OTf**, resulting in new reaction channels. These results underline the necessity of the two NO units to bind in close proximity in FNORs, within a very limited range of (O)N-Fe-Fe-N(O)

dihedral angles and (O)N-N(O) distances. Here, an increase of the (O)N-Fe-Fe-N(O) dihedral angle to ~53°, as in the case of **2-OTf**, is in fact enough to almost completely suppress efficient N₂O generation, compared to complex **1**, with a coplanar [FeNO]² core. This sensitivity of the N-N coupling reaction is certainly unexpected, and shows that in FNORs, the core conformation must be precisely controlled to allow for efficient N-N coupling and quantitative N₂O formation to occur and suppress undesired side reactions. This also points to the importance of the bridging carboxylate ligand in FNORs to lock in the catalytically productive conformation of the diiron core. In contrast, the structural requirement for O₂ activation is less stringent, and this could be a distinction between primarily O₂ versus primarily NO activating FDPs and also other, O₂-activating non-heme diiron enzymes. For example, whereas the *Mt* FDP contains an active site pocket with two 5C Fe^{II} centers containing adjacent (coplanar) vacant sites for NO binding,²⁴ ribonucleotide reductase (RNR) contains two weakly bound water molecules that sit nearly orthogonal to each other, which explains the lack of NO reductase activity in RNRs.⁷¹

Mechanism of N₂O Formation from the DNIC Products

The comparison of the DNIC stabilities between 2-X and 3 is mechanistically interesting and can be based on the N₂O production data after a 2 hr time period. While the bulk material in both the dinculear and mononuclear systems contains an equivalent of DNIC and hs-Fe(II), <u>the key</u> <u>distinction is that in the dinuclear systems 2-X the hs-Fe(II) center is tethered and held in proximity</u> <u>to the DNIC center</u>, whereas these components are independent complexes in the case of 3. Note that N-N bond formation from the two NO units within a DNIC center is spin-forbidden and therefore, energetically unfeasible.⁵⁵ Accordingly, DNICs with nitrogen-based coligands are usually stable in solution with respect to N-N coupling, though both hs-{Fe(NO)₂}^{9/10} DNICs are

known to decompose via NO loss in solution (under anaerobic conditions). Here, the hs- $\{Fe(NO)_2\}^9$ typically decompose more rapidly than the hs- $\{Fe(NO)_2\}^{10}$ DNICs.^{72,73} For **3**, both hs- $\{Fe(NO)_2\}^{9/10}$ DNICs are stable in solution compared to their dinuclear analogs in 2-X. Despite the presence of 1 equivalent of a free hs-Fe(II) complex in solution, the DNIC products of 3 do not produce any N₂O. The free hs-Fe(II) complex in solution has therefore very little influence on the solution stability of the mononuclear DNICs. In contrast, the dinuclear hs-Fe^{II}/{Fe(NO)₂}^{9/10} complexes are actually quite reactive towards N2O formation, especially in the non-coordinating solvent CH₂Cl₂ (up to 100% N₂O yield in 2 hours; see Table 2), which is highly unusual. We propose that this significant difference in reactivity, under otherwise identical conditions, is due to the tethered hs-Fe(II) center in the hs-Fe^{II}/ $\{Fe(NO)_2\}^{9/10}$ complexes. As shown in Scheme 3, we therefore propose that after the initial formation of the $Fe^{II}/{Fe(NO)_2}^{9/10}$ DNICs, N₂O production in the dinuclear systems proceeds by NO or NO⁻ transfer from the DNIC to the tethered hs-Fe(II) center. This process is facilitated by loss of one ligand X from the hs-Fe(II) center, and then attack of the 5C hs-Fe(II) on the DNIC. This leads to the generation of reactive hs-{FeNO}⁷/hs-{FeNO}⁸ and $[hs-{FeNO}]^{8}]_{2}$ intermediates for the $Fe^{II}/{Fe(NO)_{2}}^{9}$ and $Fe^{II}/{Fe(NO)_{2}}^{10}$ DNICs, respectively. These intermediates can either react back to the corresponding DNICs, which is therefore best described by two equilibria with equilibrium constants K1 and K2 in Scheme 3, or, in a less likely process (due to the distortion of the [FeNO]₂ cores in the hs-{FeNO}⁷/hs-{FeNO}⁸ and $[hs-{FeNO}]^{8}]_{2}$ intermediates) they can proceed to generate N₂O via rate constants k₃ and k₄. Based on our computational studies, we have previously found that semireduction (pathway k₃) and superreduction (pathway k4) have essentially identical barriers for the BPMP- diiron complexes,⁷⁴ and therefore, we expect k₃ and k₄ to be rather similar. In other words, the observed differences in reactivity between the $Fe^{II}/{Fe(NO)_2}^9$ and $Fe^{II}/{Fe(NO)_2}^{10}$ DNICs with the different coligands X and in the different solvents must then relate to differences in the equilibria K₁ and K₂, which are therefore the primary factors that affect the N₂O yields over 2 hours in these systems (see Scheme 3).

These mechanistic conclusions are supported by several experimental observations. First, {Fe(NO)₂}⁹ DNICs are generally more susceptible (and less stable) towards NO/NO⁻ loss than $\{Fe(NO)_2\}^{10}$ DNICs, which means that K₁>>K₂. With k₃≈k₄≈k₁ we would therefore expect that the hs-Fe^{II}{Fe(NO)₂}⁹ complexes produce significantly more N₂O per time than the hs- Fe^{II} {Fe(NO)₂}¹⁰ species, which is indeed observed under all conditions for each coligand X (see Table 2). Second, since NO/NO⁻ transfer is related to the loss of ligand X from the hs-Fe(II) center, a less coordinating ligand X should therefore promote N₂O generation. This is in fact observed, with N₂O yields decreasing in the order 2-MeOH > 2-OTf > 2-MI for the t = 2 hr data (CH₃CN or CH₂Cl₂, and 1 or 2 eq of CoCp₂). Finally, in a coordinating solvent, the 5C hs-Fe(II) intermediate could be intercepted by solvent coordination, therefore shifting the equilibria K1 and K₂ towards the DNIC side, and in this way, slowing down N₂O generation. In line with this, the amounts of N₂O produced after 2 hrs are dramatically decreased in CH₃CN compared to CH₂Cl₂. All available data therefore support the mechanistic scenario in Scheme 3, which shows that DNICs can in fact be activated for N₂O generation in the presence of a second iron center, which allows the system to enter the established semi- and superreduced pathways for N-N coupling and ultimately, N₂O formation. However, the overall rate of this process is very much dependent on other (external) factors, like available coligands and/or solvents. This is a new reaction for DNICs that has not been described so far in the literature, and that has potential biological significance for NO sensing by transcription factors that contain 2Fe2S cluster.^{75,76} Here, NO binding leads to a breaking up of the 2Fe2S cluster accompanied by DNIC formation; however, how the 2Fe2S

cluster is regenerated at low NO concentrations is not known. In this regard, N₂O elimination could be a pathway that allows for the regeneration of the 2Fe2S cluster at low NO concentrations, as demonstrated here.

CONCLUSIONS:

In this paper, we demonstrate that efficient N₂O generation is highly sensitive to the structural properties of a diiron complex, where small deviations from coplanarity of the two coordinated NO ligands have a detrimental effect on the N₂O yields. This provides key insight into the optimal active site layout of FNORs, with surprisingly little room for variation, and suggests that this could be a key feature that further distinguishes primarily NO versus primarily O₂ activating FDPs (where the latter reaction tolerates much larger variations in the diiron core geometry). This result puts a special spotlight on a conserved, bridging carboxylate ligand in the active sites of FNORs, which is crucial in (a) constraining the Fe-Fe distance and (b) keeping the two NO binding sites in These properties are reflected in the model complex а perfect cis position. $[Fe_2(BPMP)(OPr)(NO)_2]^{2+}$ (1), which mediates NO reduction with high efficiency (on the millisecond time scale) and produces quantitative N₂O yields. Upon replacement of the bridging carboxylate ligand in this compound with monodentate ligands, N₂O yields drop dramatically, particularly for 2-OTf and 2-MI. Here, we report a new reaction mechanism by which DNICs can indirectly catalyze N-N coupling and N₂O formation. Intrinsically, a mononuclear DNIC cannot support this reaction, due to its spin forbidden nature. However, as we demonstrate in this paper, a second iron center in close proximity to the DNIC and with labile ligands allows this process to go forward smoothly, via NO/NO⁻ transfer from the $\{Fe(NO)_2\}^{9/10}$ DNIC to the appended hs-Fe^{II} center, followed by a sequential semi- or superreduction reaction from the hs{FeNO}⁷/hs{FeNO}⁸ or hs-{FeNO}⁸/hs-{FeNO}⁸ intermediate, respectively.⁴ Hence, it is the unusual, dinuclear structure that allows these DNICs to circumvent the spin-forbidden nature of the N-N coupling reaction of their two NO ligands to generate N₂O. Furthermore, the N-N coupling is significantly inhibited in complexes **2-X** due to the conformational flexibility of the diiron core within these complexes and accessibility of non-coplanar NO-binding geometries.

DNICs are important NO-containing species in biology that are generated by reaction of NO with iron-sulfur proteins, for example in the active sites of transcription factors that are NO sensors. The novel reactivity of DNICs reported here is of potential biological relevance in these systems. It is known that the 2Fe2S cluster in the active sites of NO-sensing transcription factors can bind NO reversibly to form DNICs, but the mechanism by with the 2Fe2S cluster are regenerated at low NO concentrations is not known. In this regard, the novel reaction of DNICs discovered here could constitute a pathway for the regeneration of the 2Fe2S cluster from DNICs by NO transfer and following N₂O elimination.^{48,75} Going forward, it will be interesting to see whether similar chemistry does indeed exist in biology.

ASSOCIATED CONTENT:

Supporting Information:

The Supporting Information is available free of charge at _____ and contains information on the following: Experimental Section, UV-Vis, IR, NRVS, and CV data, additional XRD and DFT data (PDF). Supplementary crystallographic data can be found at CCDC deposition #2059419-2059421, 2103072 and can be obtained free of charge.

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68. In this experiment, the N-O stretching bands of the $\{Fe(NO)^2\}^9$ DNIC increase over time, which we rationalize in the following way: The hs- $\{FeNO\}^7$ complex **3** is minimally soluble in CH²Cl². The addition of CoCp² pulls some of the hs- $\{FeNO\}^7$ complex into solution; however, the hs- $\{FeNO\}^7$ complex never completely dissolved initially. The increase in the N-O stretching bands is attributed to the slow dissolution of the hs- $\{FeNO\}^7$ complex over the course of the IR experiment, and subsequent reduction to $\{Fe(NO)^2\}^9$, which leads to a slow increase in intensity of the N-O stretching bands of this product.

69. The $\{Fe(NO)^2\}^{10}$ DNIC is slowly oxidized to the $\{Fe(NO)^2\}^9$ complex over the course of 2 hrs, as indicated by the appearance of N-O stretching bands at 1789 and 1713 cm⁻¹. Since the reduction of the starting hs- $\{FeNO\}^7$ complex only generates two fully reduced species: the $\{Fe(NO)^2\}^{10}$ and hs-Fe(II) complexes, it is unclear how this re-oxidation occurs.

70. To test this hypothesis, the structure of **2-X** with $X = CH_3CN$ was also optimized using BP86/TZVP, since this structure does not contain any hydrogen bonds (see Figure S23). Interestingly, the core structure of the CH₃CN-bound complex remains largely intact and comparable to **2-MeOH**, with only minimal deviations in key structural parameters (see Table S2). The higher N₂O yields from **2-MeOH** may therefore indeed arise from the presence of the protic (and H-bonding) MeOH ligands, which could play a role in a proton-assisted mechanism for N-N coupling and N₂O formation. In this regard, note that recent studies have highlighted the role of the second coordination sphere (SCS) Y197 in the active site of Tm. FDP as a hydrogen bond donor, leading to a decreases in the activation barriers for N₂O formation (see refs 26 and 27)

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[Fe₂(BPMP)(OTf)₂(NO)₂](OTf) (O)N-Fe-Fe-N(O) Angle: 53° DNIC