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Water-soluble nitroxyl porphyrin complexes Fe^{II}TPPSHNO and Fe^{II}TPPSNO⁻ obtained from isolated Fe^{II}TPPSNO⁻

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KEYWORDS: Nitroxyl complexes, porphyrin, water-soluble.

ABSTRACT: The first biomimetic water soluble Fe^{II}-porphyrin nitroxyl complexes were obtained and characterized by UV-Vis in protonated and deprotonated forms by reduction of previously isolated and characterized Fe^{II}TPPSNO[•]. The pK_a involved in the Fe^{II}-HNO \rightleftharpoons Fe^{II}-NO⁻ + H⁺ equilibrium was estimated to be around 9.7. The Fe^{II}TPPSHNO complex spontaneously reoxidizes to the nitrosyl form following a first order kinetic decay with a measured kinetic constant of $k = 0.017 \text{ s}^{-1}$. Experiments show that the HNO adduct undergoes unimolecular homolytic cleavage of the H-NO bond. DFT calculations suggest a *phlorin radical* intermediate for this reaction. The deprotonated NO⁻ complex resulted more stable, with a half-life of about 10 minutes.

Introduction

Nitric oxide (NO[•]) and nitroxyl (HNO/NO⁻) have been consistently studied during recent years due to their biological as well as chemical relevance. Previously only regarded as an environmental pollutant, nitric oxide's role as a signaling agent in living organisms has already been established, acting in vasodilation and nerve signal transduction at low concentrations.^{1,2} More recent investigations have also suggested that endogenous generation of the reduced nitroxyl species is possible at physiological conditions, from many biological metabolites,³⁻⁶ which implies that HNO/NO⁻ could have independent signaling activity of its own, and might also be responsible for some of the physiological effects initially attributed to NO[•], like protection against ischemia related injuries.7-9 Heme proteins are one of the principal targets for these molecules in biological systems, as they tend to bind to metal centers through the nitrogen atom.¹⁰

In addition, such heme-protein adducts of metal-site coordinated NO•/HNO/NO- species are key intermediates in the mechanism of action of different enzymes of the nitrogen biogeochemical cycle, such as nitrite or nitric oxide reductases.¹¹⁻¹³ These considerations make it vital to develop biomimetic models of such active sites in order to study their reactivity and structure. Thus, nitrosyl/nitroxyl porphyrin iron complexes have become particularly interesting to study from a bioinorganic point of view.

The increasing number of reports on {FeNO}⁸ (according to the Enermark-Feltham notation)¹⁴ complexes have contributed to the spectroscopic characterization of this initially elusive species but still much more investigation is needed to elucidate mechanistic issues.¹⁵ The first porphyrinate iron-nitroxyl complexes were reported by Kadish et al., using TPP (meso-tetraphenylporphyrin) and OEP (B-ACS Paragon Plus Environment

octaethyl porphyrin), by one-electron UV electrochemical reductions of the nitrosyl {FeNO}⁷ analogues, in dichloromethane.^{16,17} Later on, both complexes could be obtained chemically and electrochemically by Ryan's group, and further stabilized in THF solution, which allowed their characterization by FT-IR spectroscopy.^{18,19} Evidence for the protonated [(OEP)Fe(HNO)] complex was also obtained, resulting stable for hours in organic media in the presence of phenols as proton sources.²⁰ The hexacoordinated derivative [(OEP)Fe(HNO)(5-MeIm)] was prepared via hydride attack on the corresponding ferric nitrosyl by Richter-Addo et al. and characterized by ¹H NMR, although in 11% yield and at -20°C.²¹ A particularly stable {FeNO}⁸ complex reported by our group could be isolated and characterized by UV-Vis, IR and ¹⁵N NMR, using the perhalogenated porphyrin TFPPBr₈ (β-octabromo-mesotetra(pentafluorophenyl porphyrin)) in organic media.²² Nevertheless, attempts to protonate the species to give the expected {FeHNO}⁸ complex were unsuccessful, reacting to give the {FeNO}⁷ complex (Fe^{II}-NO[•]). Lehnert et al. observed analogous results with other electron-poor porphyrins, and more interestingly, reported the first ferrousheme HNO complex using a very sterically hindered bis picket fence porphyrin which resulted of great stability, decomposing completely only after ~ 20 h. They concluded that the bulky substituents on the porphyrin ring disfavored a bimolecular reoxidation pathway, proposed for unhindered porphyrins in organic media.23

Regarding aqueous chemistry, the only examples reported consist of the nitroxyl-myoglobin adduct, Mb-HNO, and other globin adducts studied by Farmer and coworkers. 24, ²⁵ The Mb-HNO adduct could even be characterized by Raman, NMR and X-ray absorption.²⁶ These complexes did not decompose for weeks, suggesting that the protein's

distal pocket residues could stabilize the HNO moiety by hydrogen bonding. No biomimetic water-soluble iron heme complex has been stabilized or characterized in aqueous solution.

On the other hand, a very stable water-soluble (although non-heme) {FeNO}⁸ complex could be obtained upon twoelectron reduction of $[Fe(CN)_5(NO)]^2$, and characterized spectroscopically, in both its protonated and nonprotonated form.²⁷ The p K_a for $[Fe(CN)_5(HNO)]^3$ · was estimated via ¹H NMR to be about 7.7, giving the first experimental value for bound HNO. The special stability reported for this complex led us to consider that other factors, like hydrogen bonds, could stabilize the Fe-HNO moiety.

In this work we isolated for the first time the {FeNO}⁷ adduct of the water-soluble porphyrin TPPS (meso-tetra(4-sulfonatophenyl)porphine), which has been widely used in previous works on NO chemistry (Figure 1).^{28,29} Moreover, we chemically obtained the corresponding {FeNO}⁸ elusive adducts, Fe^{II}TPPSHNO and Fe^{II}TPPSNO⁻, making these complexes the first ferrous-heme water-soluble nitroxyl models. The products have been characterized by UV-Vis, and cyclic voltammetry experiments. The p K_a for Fe^{II}TPPSHNO deprotonation was estimated using spectrophotometric and electrochemical methods. The spontaneous reoxidation reaction of the protonated form to the {FeNO}⁷ adduct Fe^{II}TPPSNO⁻ was kinetically studied via experimental measurements and DFT calculations.



Figure 1. Fe^{II}TPPSNO• anionic moiety. Sodium counterions were omitted for clarity.

Experimental Section

Equipment and measurements

All UV-Vis measurements were carried out using a Hewlett-Packard HP8453 diode array spectrometer with 1 cm or 0.2 cm pathlength cuvettes. All NMR measurements were carried out in a BRUKER AVANCE NEO 500 MHz spectrometer. All IR measurements were made using a Thermo Nicolet Avatar FT-IR spectrophotometer. Elemental analysis was carried out in a Carlo Erba EA 1108 system for C, N and H by INQUIMAE. The sample was fully

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combusted. CO_2 , H_2O and N_2 generated were separated by gas chromatography and quantified after thermal conductivity detection. Cyclic voltammetry experiments were carried out using a Princeton Applied Research (PAR) 273A instrument and a PAR303A mercury drop electrode (area 0.024 cm²), a Ag/AgCl reference electrode (3.0 M KCl), and a platinum wire counter electrode.

Materials

Meso-tetra(4-sulfonatophenyl)porphine in the form of the tetrasodium, dodecahydrate salt ($Na_4TPPS \cdot 12H_2O$) was purchased from Frontier Scientific and used as received.

Buffers were freshly prepared with recently degassed water before every experiment. Sodium phosphate monobasic and dibasic salts were purchased from Carlo Erba. Citric acid, sodium carbonate and sodium tetraborate salts were purchased from Biopack. Sodium hydroxide was purchased from Dorwil.

Sodium dithionite (82%) was purchased from Sigma Aldrich and recrystallized following literature procedures³⁰ in order to increase its purity and separate unwanted by-products. This reagent was stored always in a freezer under argon. Solutions of this compound were always made basic (pH \geq 10) in order to minimize decomposition reactions.

Gaseous NO was produced anaerobically from a mixture of NaNO₂, NaBr, FeSO₄ (Biopack) and 5 ml purged mQ water according to literature techniques.³¹

2,2,6,6-Tetramethylpiperidine 1-oxyl, (TEMPO) was purchased from Sigma Aldrich and used as received for qualitative experiments.

Tetrafluoro boric acid 48% wt. water solution was purchased from Sigma Aldrich, and was deoxygenated by argon bubbling before use.

DFT calculations

All calculations were carried out with the program package Gaussian 09. Meso-tetraphenyl porphyrin (TPP) was used as a model. The structures of all molecules were fully optimized at the DFT level, using the PBE exchangecorrelation functional. LANDL2DZ basis set and pseudopotential were used for the Fe atom. For the remaining atoms (H, N, O, C) the 6-31G** basis set was used. Energy scans were performed, and the QST3 method was used as implemented to optimize the geometry of the transition state.

Anoxic atmosphere considerations

All experiments involving Fe^{II}TPPSNO• were carried out under strict anaerobic conditions. A Schlenk argon-vacuum line and appropriate Schlenk glassware were used throughout the development of this work. When necessary, air-sensitive compounds were manipulated inside a nitrogen glove box. All solids were purged with various argonvacuum cycles, and liquid reagents and solvents were deoxygenated using techniques varying from argon bubbling (acid solutions) to freeze-pump-thaw (organic sol-

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vents, except methanol) or direct vacuum methods (water and methanol). Quartz cuvettes of 1 cm and 2 mm pathlengths topped with new septa were used each time to avoid oxygen filtration. Gastight Hamilton syringes ranging from 10 μ L to 5 mL were used for the correct manipulation of the air-sensitive solutions and gases.

Preparation and isolation of $Fe^{II}TPPSNO^{\bullet}Na_4(1)$

Na₄TPPS•12H₂O was metallated according to literature techniques,³² but replacing the tenfold excess of FeSO₄ salt with less than 3 eq. of FeCl₂. This gave chloride salts as byproducts, which resulted much easier to wash than their sulphate analogues. Approximately 200 mg (0.16 mmol) of free porphyrin were mixed with 1.5 equivalents of FeCl₂ and 2 eq. NaOH. The reaction was carried out in a water reflux, under argon flow, in order to avoid early oxidation of the ferrous reactant. The process was monitored by UV-Vis spectroscopy until full conversion was achieved. Extra base or FeCl₂ were added when necessary. The resulting solution was passed through a DOWEX cation exchange column to remove excess Fe3+ ions and afterwards alkalinized to pH = 5. The solvent was pumped out and the resulting solid was repeatedly recrystallized by adding distilled acetone to a saturated aqueous solution of the compound until no more traces of chloride were found in the supernatant (checked by AgCl formation after addition of AgNO₃). The product, ferric meso-tetra(4sulfonatophenyl)porphine (FeIIITPPS) was dried under vacuum and stored at room temperature. Yield: 124 mg, 62 %.

29 The five coordinated nitrosyl ferrous complex (Fe^{II}TPP-30 SNO•) was obtained by reaction of the ferric porphyrinate with an HNO donor, as reported in literature for porphyrin 31 nitrosyls in organic media.³³ Approximately 40 mg of 32 Fe^{III}TPPS were dissolved in 0.5 ml water along with 1.5 33 equivalents of 4-fluoro-N-hydroxybenzenesulfonamide 34 (fluoro-Piloty's Acid, F-PA, Figure S1) in the presence of 35 two equivalents of NaOH (See Results, Scheme 1). F-PA 36 was synthesized according to literature methods³⁴ and 37 further purified using a silica gel chromatography column 38 as reported independently.³⁵ The identity and purity of the 39 resulting compound was monitored by ¹H in d⁶-DMSO 40 (Sigma Aldrich). The nitrosylation reaction was carried out 41 in water at room temperature, under argon atmosphere 42 and constant stirring. A different Piloty's acid derivative, 43 nitro-PA (synthesized analogously) also proved useful for this reaction, and other variants may be tried, as long as 44 they are at least partially water soluble. Conversion was 45 followed by monitoring the Soret bands corresponding to 46 the reactant and the product in the UV-vis region (395 and 47 414 nm respectively). Additional base and Piloty's acid 48 equivalents were added when needed. The product was 49 precipitated by addition of freshly degassed acetone. The 50 remaining solid was recrystallized twice analogously 51 (H₂O/acetone) and washed with acetone until Piloty's acid 52 byproducts were no longer detectable by spectrophotome-53 try in the UV region. The resulting red solid, Fe^{II}TPP-54 SNO•·Na₄, **1**, was stored under argon in a Schlenk tube. 55 This product, although very air-sensitive when in solution,

proved to be quite stable as a solid, and was characterized by IR in Fluorolube and elemental analysis. N-O stretching was observed at 1668 cm ⁻¹ (Figure S2). This value is in good agreement with literature data of iron nitrosyl porphyrin compounds.⁶ Elemental analysis: **Anal. Calc. for Fe^{II}TPPSNO-Na4 • 5(H₂O) • 3(CH₃COCH₃): %C** 46.8 **%N** 5.1 **%H** 3.8 **Exp: %C** 46.4 **%N** 5.1 **%H** 3.7 A 3/1 acetone/porphyrin molar proportion was verified by ¹H NMR spectroscopy in d⁴-MeOH (Figure S3, Table S1). It is soluble in water and, to a lesser extent, in methanol; it is insoluble in other organic solvents.

Kinetic measurements

Experiments to determine the kinetic parameters of the Fe^{II}TPPSHNO (2) reoxidation reaction were carried out in the UV-Vis range using a 2 mm pathlength quartz cuvette. Absorbance traces at 414 and 418 nm (1 and 2 Soret band peak wavelength respectively) were monitored. Kinetic measurements were made after 1.5-2 eq. of dithionite were added to a solution of 1. Cycle time was set to 1 s and the experiments ran for about 5 minutes. These experiments were carried out in a variety of conditions, of which the most studied was in 2 mM phosphate buffer at pH = 6. They were also carried out in phosphate at pH = 7 and citrate buffers at pH = 5 of the same concentration and in plain distilled and degassed water, using in this case a non-buffered, pH neutral dithionite solution prepared immediately before use.

The kinetic data were analyzed making a deconvolution of the total absorbance registered at 418 nm. Considering that initially the only species present is **1**, whose absorption spectra and molar absorptivity were already reported,³⁶ and having calculated the molar absorptivity coefficient of **2** (See Results section), the molar fraction of the nitroxyl compound and its absorbance was calculated as a function of time. Integral and differential kinetic method plots and fitting were made in order to determine whether the decay followed an order 0, 1 or 2 behavior. Afterwards, appropriate non-linear fittings were made with Origin Pro 8 to estimate the associated rate constant. Multiwavelength analyses were also performed using MatLab and proved to give according results.

Results and Discussion

Preparation and isolation of Fe^{II}TPPSNO··Na₄

The product was successfully prepared and isolated as a solid as shown in the reactions shown in Scheme 1, following reaction path A using an HNO donor, or reaction path B, involving reduction followed by NO gas bubbling. In order to isolate relatively large amounts of compound (20-30 mg), path A was chosen over path B, since the HNO donor byproduct is easily washed off the product by acetone, unlike dithionite byproducts. However, path B, either for *in situ* or isolation purposes resulted useful for UV experiments and particular bulk experiments. Other reducing agents such as Cr^{II}EDTA and BH₄⁻ also proved useful for reduction of Fe^{III}TPPS.

Scheme 1. Preparation and isolation of Fe^{II}TPP-SNO··Na₄. Sodium counterions were omitted for clarity. $R=4-F-C_6H_4$

A)

$$Fe^{III}TPPS^{3-}(aq) + R-SO_2NHOH (aq) \xrightarrow{+OH^-}$$

 $\xrightarrow{-H_2O}$ $Fe^{II}TPPSNO^*(aq) + R-SO_2H (aq)$
B)
1) $Fe^{III}TPPS^{3-}(aq) + 1/2 S_2O_4^{2-}(aq) + H_2O \longrightarrow$
 $Fe^{II}TPPS (aq) + HSO_3^- (aq) + H^+(aq)$
2) $Fe^{II}TPPS (aq) + NO(g) \longrightarrow Fe^{II}TPPSNO^*(aq)$
 $Fe^{II}TPPSNO^*(aq) \xrightarrow{\bigcirc}$ $Fe^{II}TPPSNO^*(s)$

Once precipitated by acetone addition, the product was vacuum-dried and stored in a Schlenk tube under argon. This allowed us to manipulate the nitrosyl compound in a relatively simple manner by directly weighing the solid inside the glovebox before the experiments. Furthermore, it allowed us to characterize the compound through IR and elemental analysis techniques for the first time, and carry out cyclic voltammetry and UV-vis experiments using solid **1** as the starting material instead of having to generate the species in situ through chemically unclean paths.

UV scale experiments - Obtention and characterization of Fe^{II}TPPSHNO⁴⁻ (**2**) and Fe^{II}TPPSNO⁵⁻ (**3**) in aqueous media

A deoxygenized solution (approx. 5-6 µM) of Fe^{III}TPPS in 2 mM phosphate buffer solution at pH = 6 was treated with 1.5-2 equivalents of sodium dithionite to give Fe^{II}TPPS. Excess NO (g) was then added to the mixture to produce 1, and finally 1-2 more equivalents of dithionite were added to give the desired product, **2** (Scheme 2). The observed spectrum peak of the Soret band shifted from 414 to 418 nm and the corresponding molar absorption coefficient increased from 1.55 10⁵ to 2.17 10⁵ M⁻¹ cm⁻¹ (Figure 2). Although this product rapidly reoxidized to **1**, in presence of excess dithionite and NO (g) a steady-state regime was reached, allowing us to determine its molar absorptivity coefficient. Seki et al.³⁶ transiently obtained the same UV spectrum through flash photolysis techniques using benzophenone ketyl radicals and reported the adduct's instability, with a half-life time of 2 seconds. Note that here, we established that under these conditions, the adduct exists in the protonated form, Fe^{II}TPPSHNO (2), and not as Fe^{II}T-PPSNO⁻(**3**): see further experiments below.

Scheme 2. Indirect formation and subsequent reoxidation of Fe^{II}TPPSHNO in phosphate buffer at pH = 6.



Figure 2. UV-Vis Spectral changes observed upon reduction of Fe^{II}TPPSNO• at pH = 6.

The same was observed when 1-2 equivalents of dithionite were added directly to a freshly prepared 5-6 μ M solution of isolated Fe^{II}TPPSNO[•], in a more controlled, cleaner manner. In both cases, the resulting **2** complex spontaneously reoxidizes to the {FeNO}⁷ nitrosyl compound, **1** (Figure 3).



Figure 3. Observed reoxidation of Fe^{II}TPPSHNO to Fe^{II}TPP-SNO• at pH = 6. The time elapsed between the maximum and minimum peak is ca. 200 s.

The same experiments were carried out at pH = 12 phosphate buffer, and in this case a different spectrum emerged for the {FeNO}⁸ adduct, which we tentatively assigned to the deprotonated complex, Fe^{II}TPPSNO⁻ (3). In this case, a subtle decrease in absorbance was observed for

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the Soret band, at 413 nm, as well as a slight widening of the band. The most important change was observed in the Q band zone, resulting in a shift from \approx 541 nm to \approx 533 nm, and a noticeable absorbance increase and shape change of the most intense band (Figure 4). Both spectral changes (especially in the Q band zone) are surprisingly consistent with previously reported spectra of other ironnitroxyl heme complexes in organic media.²³ **3** also eventually oxidizes to **1**, with a half-life of about 10 minutes, although further analysis and reactivity studies will be pursued in a different work.



Figure 4. UV-Vis Spectral changes observed upon reduction of Fe^{II}TPPSNO• at pH = 12.

In order to confirm the identity of **2** and **3**, NaOH was added to the cuvette once **2** was formed at pH 6, rapidly rising the pH to 12. The spectrum assigned to **3** was instantly observed as a result (Figure S5, Scheme 3A). Correspondingly, the formation of **2** was observed after lowering the pH from 11 to 6 by adding HBF₄ to a cuvette containing initially **3**. (Figure S5, Scheme 3B) Therefore, the UV-vis spectra could be correctly assigned to both Fe^{II}T-PPSHNO and Fe^{II}TPPSNO⁻ species. Spectroscopic data for the Soret and the main Q band for compounds **1-3** can be found in Table 1.

Scheme 3. Fe^{II}TPPSHNO and Fe^{II}TPPSNO– formation and deprotonation/protonation reactions.

| A) | $Fe^{II}TPPSNO' \xrightarrow{+ \operatorname{Na}_2 S_2 O_4, \ pH=6} Fe^{II}TPPSHNO$ |
|----|--|
| | $Fe^{II}TPPSHNO \xrightarrow{+ \text{ NaOH to } pH=12} Fe^{II}TPPSNO^{-}$ |
| B) | $\mathbf{Fe^{II}TPPSNO'} \xrightarrow{+\operatorname{Na_2S_2O_4, pH=12}} \mathbf{Fe^{II}TPPSNO^-}$ |
| | $Fe^{II}TPPSNO^{-} \xrightarrow{+ HBF_4 \text{ to } pH=6} Fe^{II}TPPSHNO$ |
| | Fe ^{II} TPPSHNO |

Table 1. UV-Vis spectroscopic data for Fe^{II}TPPS-NO compounds.

| | | λ_{max}/nm ($\epsilon/M^{-1}cm^{-1}$) |
|--------------------------------------|-----|--|
| Fe ^{II} TPPSNO [.] | (1) | 414 (155000) ^a , 543 (11300) ^a |
| Fe ^{II} TPPSHNO pH = 6 | (2) | 418 (217000), 541(12600) |
| Fe ⁿ TPPSNO- pH = 12 | (3) | 413 (146000), 533 (12000) |

Table 1. UV-vis spectroscopic data for Fe^{II}TPPS nitrosyl and nitroxyl compounds in aqueous phosphate buffer solutions. a: Reference 35.

pK_a estimation

In order to estimate the pK_a value associated to the Fe^{II}TPPSHNO/Fe^{II}TPPSNO⁻ acid-base pair, experiments were carried out in different buffers ranging from pH 5 to 11. On one hand, chemical reductions of **1** with dithionite were followed by UV-Vis spectrophotometry in order to stablish at which point the obtained spectra for the {FeNO}⁸ adduct changed. UV-Vis spectral changes were monitored at pH range 5-11 (See Figures S6-S12 in Supporting Information). Spectral evidence for the protonated adduct could be observed at pH = 9, but not at pH = 10. On the other hand, evidence for the deprotonated species was observed at pH = 10, but not below. This suggests that the pK_a value for **2** would be between 9 and 10, as reported for a ruthenium nitroxyl complex. ³⁷

On the other hand, cyclic voltammetry experiments were made to study the pH dependence of the reduction potential involved. A Fe^{II}TPPSNO• to Fe^{II}TPPSHNO reduction process would depend on H⁺ concentration, whereas an Fe^{II}TPPSNO•/ Fe^{II}TPPSNO⁻ reduction would not. At pH = 6, an irreversible strong reduction peak assignable to the Fe^{II}TPPSNO•, H⁺/Fe^{II}TPPSHNO couple was observed at -0.665 V vs. Ag/AgCl, in concordance with previous reports.³⁸ At pH = 11.3, the irreversible reductive current wave had effectively shifted to more negative potentials, reaching the peak at -0.885 V for the Fe^{II}TPP-SNO•/Fe^{II}TPPSNO⁻ couple vs. Ag/AgCl. The complete set of reductive waves at different pHs values are shown in Figure 5.

The peak current potential shifts as a function of pH can also be used to estimate the pK_a of **2**. Nernst's equation for the Fe^{II}TPPSNO•, H⁺/Fe^{II}TPPSHNO redox process involving one electron and one proton at 298K, predicts a shift of -59 mV per pH unit until the pK_a is reached. This behavior is clearly observed for pHs 6-9 (Figure 6), with an experimental slope of -52 mV (see Figure S13). The curve shown in Figure 6 represents the expected behavior for the reduction potential assuming an acidity constant, K_a, of 2x10⁻¹⁰, estimating a pK_a of about 9.7 for Fe^{II}TPPSHNO, consistent with observations done in the UV-vis experiments previously described. It is important to note that irreversible current peaks were used for this estimation. Previous reports show the use of irreversible peak data to obtain related useful thermochemical data.³⁹ Therefore, we believe the method can be used as a semiquantitative estimation alongside the UV-measurements.



Figure 5. Reductive electrochemical wave assigned to the ${FeTPPSNO}^{7}/{FeTPPSNO}^{8}$ couple for pHs 6 – 11. Current intensities are normalized.



Figure 6. Reduction potential for {FeTPPSNO}⁷/{FeTPPSNO}⁸ as a function of pH. Experimental measurements are shown along with expected fitting for $pK_a = 9.7$. Linear fitting for pH 6-9 and extra information about CV experiments are shown in Supplementary Information.

The p*K*_a value of 9.7 for Fe^{II}TPPSHNO resulted two units lower than the revised value of 11.5 reported for free HNO, ⁴⁰ in agreement with the expected stabilization of the NO⁻ moiety upon coordination. The obtained estimate, however, strongly differs from the results previously obtained from electrochemical experiments by Meyer and coworkers, where they report a pH dependent reduction for Fe^{II}T-PPSNO• at pH < 2.6, implying a p*K*_a close to this value for the HNO complex.³⁸ Such value probably seemed reasonable for the authors at that time, considering that the accepted p K_a value for free HNO was 4.7, obtained by pulse radiolysis experiments.⁴¹ However, this was later corrected to 7.2, and it has been settled at 11.5 since 2001.^{42, 40} It is also important to note that the studies in Meyer's work were carried out in conditions very different from ours, forming the nitrosyl complex in situ in presence of excess nitrite instead of using the isolated solid in a clean manner. Moreover, the species' behavior at such acidic pHs may affect the nitrosyl's stability and even imply a second protonation process at the HNO ligand and at the sulfonate groups of the porphyrin substituents.

On the other hand, the pK_a value of 9.7 for coordinated HNO is in excellent agreement with the one reported for $[Ru(Me_3[9]aneN_3)(bpy)(NO)]^{+1}$, which displayed a pK_a of 9.8 ³⁷, and falls within the 8-10 range for the pK_a value for Fe^{II}OEP(HNO) estimated by FTIR spectroscopy.²⁰

Reoxidation reaction studies: kinetic measurements

Many hypotheses were considered in order to elucidate why spontaneous reoxidation took place for the protonated **2** adduct. Although complete absence of oxygen traces cannot be guaranteed, the reproducibility in the experiments led us to think that something other than adventitious oxygen was driving this process. Besides, the expectedly more air-sensitive deprotonated adduct, **3**, seemed to last longer in the cuvette.

Another hypothesis contemplated the possibility of excess NO (g) present in the cuvette displacing HNO as a ligand. However, this was disregarded when the same results were observed using isolated $\mathbf{1}$ as the starting material, NO gas not being present in excess anymore. In other experiments, when excess NO (g) was purposely added to $\mathbf{2}$ formed *in situ*, $\mathbf{1}$ was formed instantly, indicating a probable ligand exchange reaction.

A third hypothesis included the bimolecular decomposition:

2 Fe^{II}TPPSHNO (aq)
$$\rightarrow$$
 2 Fe^{II}TPPSNO[•](aq) + H₂(g) (1)

In order to test this last case, the system was properly optimized in order to make robust kinetic measurements, since processes of this kind have been previously proposed in literature in organic media, but not tested directly.²³

Kinetic measurements of the reoxidation reaction were carried out under different conditions, being the most robust those experiments carried out in 2 mM phosphate buffer at pH = 6, using a 2 mm pathlength quartz cuvette at room temperature. Integral and differential method plots showed that the decomposition reaction followed a first order decay and not a second order decay, as expected for the reaction shown in Eq. 1. (See Figures S14-S17). Further fitting gave an exponential decay as shown in Figure 7.

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Figure 7. Fe^{II}TPPSHNO absorbance time traces at 418 nm for one selected experiment, which fit a monoexponential decay. See SI (Figures S18-S25) for more representative fittings under different conditions.

The observed first order kinetic constant under these conditions is $k= 0.017 \pm 0.003 \text{ s}^{-1}$, which accounts for a half-life time of approximately 41 s, considerably longer that the previously reported lifetime of 2 s.³⁶ It is important to point out that in those conditions, **2** was generated in the presence of glucose and benzophenone radicals, which possibly interfered in the stability of the HNO moiety resulting in a shorter lifespan.

Temperature studies of the reaction under these conditions in the 8 - 48°C range were also performed, and an activation energy of 13 ± 8 kcal mol⁻¹ could be estimated. The corresponding Arrhenius plot is shown in Figure S26.

These results ruled the bimolecular reaction hypothesis out and left us with two possibilities: either the ratelimiting step of the HNO complex reoxidation was an intrinsic first order decomposition of **2**, or a *pseudo* first order decay, involving water or buffer species available in the system. However, no significant differences were observed when buffer was excluded and pure water was used as a solvent, or different buffer compositions were used (Table 2).

The rate constant was determined under different experimental conditions in order to find possible reoxidation paths (Table 2). The pHs = 5, 6 and 7 gave out virtually the same reoxidation rate. Surprisingly, the only significantly different rate, greater by an order of magnitude, was observed when using Cr^{II}EDTA as a reducing agent (Table 2, row 4). This led us to think that either the metallic traces in Cr^{II}EDTA or the chromium complex itself catalyzed the oxidation reaction, or that, inversely, when dithionite was used as a reducing agent, the reaction proceeded more slowly. This could be a consequence of dithionite byproducts, such as sulfite, coordinating to **2** in the sixth position. However, dithionite experiments in presence of metallic traces (Cr³⁺, Fe³⁺) did not show significant differences; the same was observed when excess sulfite was added to the cuvette before reduction.

The reaction was also carried out in presence of excess imidazole at pH 7 to determine if coordination of an axial sixth ligand would relatively stabilize **2**. The obtained kinetic constant was fairly smaller than in its absence (Table 2, row 7), suggesting a somewhat although not substantial stabilizing effect of the axial ligand.

Finally, when the experiment was carried out in phosphate buffer dissolved in D_2O , the rate constant was significantly smaller than in H_2O (Table 2, row 3), k_H/k_D being 2.7, which seems to correspond to a primary kinetic isotope effect. Therefore, the H-NO bond breaking is involved in the limiting step of the reoxidation reaction. However, it could also implicate that water attacks **2** to give **1** and byproducts through a *pseudo* first order mechanism.

The observed results suggest that the reoxidation of **2** to **1** occurs by loss of an H atom through a rate-determining homolytic rupture of the N-H bond. The significantly different value obtained for the reduction with $Cr^{II}EDTA$ could be explained by a possible hydrogen atom abstraction activity carried out by this compound or its byproducts.

 Table 2. Measured kinetic constants for Fe^{III}TPPSHNO

 reoxidation under different contidions

| Row n° | Experimental conditions | k (s ⁻¹) | Ν |
|--------|---|---------------------------|----|
| 1 | Phosphate buffer pH 6, 2 mM | 0.017 ± 0.002 | 13 |
| | Reducing agent: Na ₂ S ₂ O ₄ | 0.017 ± 0.003 | |
| | Phosphate buffer pH 6 2 mM | | 3 |
| 2 | Reducing agent: Na ₂ S ₂ O ₄ | 0.014 ± 0.003 | |
| | Sulphite excess | | |
| | Phosphate buffer pH 6, 2 mM | | |
| 3 | in D ₂ O | in D_2O 0.0064 ± 0.0003 | |
| | Reducing agent: Na ₂ S ₂ O ₄ | | |
| 4 | Phosphate buffer pH 6, 2 mM | 0.17 + 0.06 | 10 |
| | Reducing agent: Cr ^{II} (EDTA) | 0.17 ± 0.00 | |
| 5 | Citrate buffer pH 5, 2 mM | 0.010 ± 0.000 | 3 |
| | Reducing agent: Na ₂ S ₂ O ₄ | 0.019 ± 0.009 | |
| 6 | Phosphate buffer pH 7, 2 mM | 0.017 + 0.002 | 4 |
| | Reducing agent: Na ₂ S ₂ O ₄ | 0.017 ± 0.002 | |
| 7 | Phosphate buffer pH 7, 2 mM | | |
| | Reducing agent: Na ₂ S ₂ O ₄ | 0.010 ± 0.002 | 3 |
| | Imidazole excess | | |
| 8 | Deionized water | 0.025 + 0.007 | 7 |
| | Reducing agent: Na ₂ S ₂ O ₄ | 0.025 ± 0.007 | |

Table 2. Kinetic constants for the spontaneous reoxidation of Fe^{II}TPPSHNO to Fe^{II}TPPSNO• measured under different experimental conditions, along with their standard deviation. N is the number of experiments considered for each experimental condition.

 \rightarrow Fe^{II}TPPNO[•](aq) + H₂(g) + OH[•](aq) (2)

In order to indirectly test this hypothesis, UV reduction experiments were carried out in the presence of the freeradical TEMPO, which has the ability to abstract H[•] radicals and form TEMPOH. Previous studies show that the H-N bond energy in HNO (49.9 kcal/mol) is substantially smaller than the average H-N bond (90 kcal/mol), making it relatively easy to break.43 The reaction of free HNO with TEMPOL has already been studied, resulting in a hydrogen atom abstraction to give NO¹ and TEMPOL-H.44 With this in mind, 2 eq. of TEMPO were added to a cuvette containing freshly formed **2** at pH = 6 (by dithionite reduction of **1**). Complete and instantaneous formation of **1** was observed instead of the progressive oxidation witnessed in analogous experiments in the absence of TEMPO. This qualitative experiment shows that the H[•] in the Fe-HNO moiety can readily abstracted, evidencing the relative weakness of the H-N bond in the complex, supporting the suggested homolytic rupture as the rate-limiting step.

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In order to evaluate the feasibility of the proposed mechanism, the reoxidation reaction was further explored through DFT calculations.

DFT calculations – formation of a *phlorin radical* intermediate

DFT calculations were performed using the FeTPP model in order to gain more insight into the reoxidation mechanism of 2. The structure for Fe^{II}TPPHNO was optimized, and energy scans along the H-N bond of the HNO moiety were performed with increasing bond distance. The final structure showed that the hydrogen atom from the HNO moiety had migrated to the most proximate meso carbon of the porphyrin ring. This unexpected result led us to optimize the structures of the product and the transition state to see if it was a plausible rate-limiting step for a unimolecular reaction. Surprisingly, the final *phlorin radical* intermediate resulted only 2.4 kcal mol ⁻¹ higher in energy than the initial Fe^{II}TPPHNO structure, while the calculated activation energy for the interconversion of these tautomers resulted in 19.4 kcal mol⁻¹, in good agreement with the experimentally measured value of 13 ± 8 kcal mol⁻¹ (Figure 8). Intramolecular proton-coupled electron transfers of this kind have been recently reported for other porphyrins, where similar bent phlorin intermediates were theoretically proposed and detected.^{46–49} This *phlorin* radical can be best interpreted as a 1 electron oxidized phlorin, rather than a 1 electron reduced porphyrin, since it bears two substituents on the sp³ hybridized carbon. Previous literature reports show that free phlorin macrocycles can undergo oxidative processes of up to 3 electrons at modest potentials, which makes the postulation of the proposed intermediate reasonable.50,51

The energy associated with reaction (2) was also calculated, in order to dismiss a *pseudo* first order mechanism involving water. As expected for the formation of a reactive OH• radical, the reaction resulted thermodynamically uphill by 93.1 kcal mol ⁻¹.

 $Fe^{II}TPPHNO (aq) + H_2O \rightarrow$



Figure 8. Optimized structures and calculated energy differences for Fe^{II}TPPHNO, the final *phlorin radical* intermediate and transition state.

The performed calculations suggest a decomposition mechanism in which the rate-determining step is first order in the Fe^{II}TPPSHNO complex, in agreement with the experimental measurements, and consists in the formation of an unstable phlorin radical intermediate (Scheme 4). This species, which was not detected via UV-vis measurements, would rapidly react re-donating the H[•] entity to give unspecified products and the Fe^{II}NO' complex, reestablishing the aromaticity of the porphyrin ring. Even if we cannot specify the fate of the H atom, we are aware that hydrogen gas would be the most reasonable product. Preliminary hydrogen measurement experiments were intended, although they resulted inconclusive and should be pursued more carefully in a different work. However, previous work by Abucayon et al. shows H₂ formation following the decomposition of Fe(OEP)(HNO)(MeIm).45

Scheme 4. Proposed reoxidation pathway for Fe^{II}T-PPSHNO.



The proposed mechanism, suggested for the first time for the decomposition of HNO complexes, could be supported by evaluating if the stability of Fe-HNO complexes is enhanced by the use of porphyrinoid platforms that already bear substituted sp³ carbons, such as phlorins, porphodimethenes and calixpirroles, which are reported to prevent the oxidation to the corresponding porphyrins.^{52,53} If this turns out to be the case, the obtained complexes might provide a stable platform for performing interesting reactivity studies of coordinated HNO/NO⁻, even if the *heme* reminiscence is somewhat lost.

In addition, the newly proposed mechanism allows for the re-interpretation of previous results. For instance, although the presented results do not support the previously suggested bimolecular decomposition pathway in

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organic media (1), the greater stability of the sterically hindered *bis picket fence* porphyrin could also be due to the fact that the bulky substituents prevent the structure from bending and transferring the H^{*} atom to the *meso* carbon in an effective manner. Nevertheless, we are aware that aqueous and organic media chemistry are different and therefore decomposition mechanisms might not be the same.

Additional investigations should be carried out to further study these systems, and more importantly, evaluate the implications of similar reaction pathways and in biologically relevant conditions for enzymatic mechanisms.

Conclusions and perspectives

A water-soluble iron nitrosyl porphyrin complex, Fe^{II}T-PPSNO• was obtained through two different routes and successfully isolated for the first time as a and characterized by FT-IR, cyclic voltammetry, elemental analysis and UV-visible techniques. This compound turned out to be stable for many months if stored under argon.

Reduction of previously isolated Fe^{II}TPPSNO[•] at different pHs provided UV-Vis spectroscopic evidence for the formation of the first water-soluble ferrous heme nitroxyl complexes Fe^{II}TPPSHNO and Fe^{II}TPPSNO⁻. This information, along with data from electrochemical measurements allowed us to estimate the pK_a value for the Fe^{II}T-PPSHNO/Fe^{II}TPPSNO⁻ equilibrium to be around 9.7.

Both complexes eventually reoxidize to the initial nitrosyl species, with an estimated half-time life of 10 min for Fe^{II}TPPSNO⁻ and less than a minute for Fe^{II}TPPSHNO.

Plausible causes for Fe^{II}TPPSHNO instability were evaluated, and most of them were ruled out. The reoxidation reaction is an order 1 decay in pH = 6 phosphate buffer, with an estimated $k = 0.017 \text{ s}^{-1}$ ($t_{1/2} = 41 \text{ s}$, considerably greater than the one previously reported)¹⁴. Our results strongly indicate that the Fe^{II}TPPSHNO moiety undergoes a rate-limiting homolytic bond break, resulting in the observed Fe^{II}TPPSNO[•] and H[•] which due to its high reactivity further reacts with solvent and/or other species (such as buffer and/or the byproducts of the reducing agents). This hypothesis is supported by experiments carried out in the presence of H[•] withdrawing compounds (TEMPO) and DFT calculations that predict homolytic H-NO bond cleavage and intramolecular addition to the porphyrin *meso* carbon to give a *phlorin radical* intermediate. Despite this apparently rapid reoxidation, the protonated nitroxyl species turned out to be substantially more stable than its organicmedia analogues previously reported. This observation agrees with the proposed hypothesis that hydrogen bonds with water help to stabilize the Fe-HNO moiety.

Further experiments will be carried out to continue investigating these new important biomimetic complexes, in order to evaluate their reactivity with biologically relevant substrates. The decomposition pathway of Fe^{II}TPPSNO⁻ must also be explored and understood, hoping to increase its stability and carry out more rigorous characterization and reactivity studies.

In addition, other water-soluble platforms will be studied. In particular, experiments with cationic porphyrins like meso-tetra(N-methylpyridyl)porphyrin ($TMPyP^{4+}$) could provide valuable insight on the influence of the charge of the ligand on the stability of nitroxyl compounds.

ASSOCIATED CONTENT

Supporting Information. Complementary IR, NMR and UV spectra, cyclic voltammetry considerations, additional kinetic fittings and useful structures are shown in Supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

TPPS = Meso-tetra(4-sulfonatophenyl)porphine

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TABLE OF CONTENTS

Fe-HNO

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Fe-NO•

Time

radical intermediate

Fe-HNO

Abs I

FIRST

2

Phlorin

ORDER KINETICS

FellTPPSHNO

Fe^{II}TPPSNO⁻

500

λ (nm)

pKa ≈ 9,7

600



1,5 -

Absorbance

0,0

- 55
- 56 57
- 58
- 59