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Fast Nitroxyl Trapping by Ferric Porphyrins

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Nitroxyl anion (NO^-), the elusive one- electron reduction product of nitric oxide (NO), is currently being considered as a product of the enzyme nitric oxide synthase (NOS).[1] It is also biosynthesized from *N*-hydroxy-L-arginine under oxidative stress[2] or nonenzymatically from the reaction of *S*-nitrosothiols with thiols.[3] Nevertheless, it has been recently demonstrated that the direct one-electron reduction of NO is thermodynamically unfavored under physiological conditions,[4] where the conjugated weak acid nitroxyl (HNO) is the predominant species.[5] The anion or its conjugated acid are capable of reacting with heme or non-heme iron[6] and with thiol residues, respectively, pointing to hemeproteins as relevant biochemical targets.[7] However, no studies have been reported on the reactions of heme model systems with NO^-/HNO. In this work we report for the first time an investigation of the reactions of NO^-/HNO with ferriheme model systems. These reactions not only shed light on the putative mechanisms operating under physiological conditions but could also provide a powerful tool for the discrimination of NO and NO^-/HNO biological effects by selective trapping.

We have studied the reactions of different ferric porphyrins in aqueous and organic media with the widely used NO^- donors sodium trioxodinitrate (Angeli's salt, AS) and toluensulfohydroxamic acid (4-methyl Piloty's acid, TSHA).[8] The former decomposes thermally in acidic media yielding nitrite and HNO;[9] the latter is known to undergo decomposition in alkaline media to yield toluensulfinate and NO^-.[10] In all cases, the corresponding nitrosyl ferrous porphyrins (Fe^{II}(porphyrin)(NO)) were obtained quantitatively (see below).

Up to date, several methods have been described for the synthesis of nitrosyl ferrous porphyrins. Reductive nitrosylation is a widely used method for the obtention of nitrosyl metal complexes,[11] and it has been thoroughly used with several synthetic ferric metalloporphyrins.[12] Iron(II) nitrosyl *meso*-(tetraphenyl) porphyrinate (Fe^{II}TPP(NO)) has already been obtained from $[Fe^{III}TPP]^+$ by different reductive nitrosylation approaches: excess NO and trace amounts of a protic agent such as MeOH or H_2O, Zn amalgam reduction followed by excess NO, or hydroxylamine disproportionation.[13] Reductive nitrosylation with excess NO in organic media affords a mixture of Fe^{II}TPP(NO) and $Fe^{II}(TPP)(NO)_2$.[14] The presence of trace NO_2 in the gas stream is hard to avoid, and $Fe^{II}TPP(NO)(NO_2)$ is sometimes found as a byproduct.[15] In aqueous solution, ferrous nitrosyl porphyrins are obtained by bubbling NO after reduction of the ferric center[16] or by adding a nucleophile.[13a,17] It has been suggested that the peripheral net charge in the porphyrin substituents influence the electrophilicity of the Fe^{III}(NO) intermediate and, hence, the reductive nitrosylation reaction.[18]

In the present work, an appropriate selection of NO^-/HNO donor, media, and pH was required to achieve optimal reaction yield for each ferric porphyrin. $[Fe^{III}TPP]^+$ was assayed with both AS and TSHA in methylene chloride/methanol solutions. Iron(III) *meso*-tetrakis (4-sulfonatophenyl) porphyrinate ($[Fe^{III}TPPS]^{3-}$) could only be assayed with AS in aqueous acidic media, as the porphyrin forms a dimer in alkaline media that interferes with kinetic and mechanistical comparisons. Iron(III) protoporphyrin IX was chosen to study the reaction with TSHA in aqueous alkaline media.

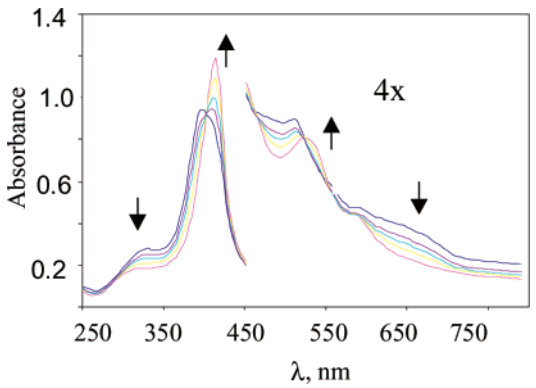

Figure 1. UV/vis spectral changes for $[Fe^{III}TPPS]^{3-}$ 6.5×10^{-6} M, buffer phosphate 2×10^{-3} M, pH 6.4, EDTA 10^{-4} M, upon addition of AS 1.3×10^{-4} M.

The reactions in aqueous media were performed as follows: a solution of $Na_3[Fe^{III}(TPPS)]\cdot 12H_2O$ 6.5×10^{-6} M in phosphate buffer 2×10^{-3} M (pH 6.4), containing EDTA 10^{-4} M was carefully degassed. A solution of AS, at final concentration of 1.3×10^{-4} M in phosphate buffer 2×10^{-3} M (pH 11), was added to the ferric porphyrin at 298 K under Ar atmosphere. The reaction monitored by UV/vis spectroscopy shows a shift of the Soret band from 392 to 412 nm, as reported for the reductive nitrosylation reaction.[19] After 30 s the reaction is considered complete since no more spectral changes are observed (Figure 1). The unstable $[Fe^{III}TPPS(NO)]^{3-}$ derivative and free $[Fe^{II}TPPS]^{4-}$, showing absorption bands at 422 and 425 nm respectively, were not detected in our experiments. In the Q-band region, a blue-shifted, low absorbance, single peak (from 528 to 542 nm) was also observed. A similar procedure was followed for iron(III) protoporphyrin IX. In this case, the reaction was also followed by Raman spectroscopy, showing spectral changes almost identical to those observed for nitrosyl hemeproteins.[20] (see Supporting Information).

For synthetic purposes, Fe^{II}TPP(NO) was obtained by dropwise addition of a solution of AS (1.7 mg, 0.014 mmol) in 3.5 mL of deareated methanol to a stirred solution of Fe^{III}TPPCl (10 mg, 0.014 mmol) in minimum volume of CH_2Cl_2 under Ar. The red-brown precipitate formed within 10 min at room temperature was separated by centrifugation, dissolved in CH_2Cl_2, and washed with water. The organic layer was concentrated in vacuo and recristalyzed from CH_2Cl_2/CH_3OH yielding 7.9 mg of Fe^{II}TPP(NO) (80% yield), characterized by UV/vis, FTIR and microanalysis. The spectroscopic

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data was coincident with that corresponding to $Fe^{II}TPP(NO)$ obtained by reductive nitrosylation with NO or hydroxylamine solutions (λ_{max}= 413 nm, ν_{NO}= 1680 cm^{-1}).[13] TSHA afforded the same product only after 24 h under the same experimental conditions. The reaction time was shortened to 30 min when an organic base (DBU = (1,8-diazabicyclo[5.4.0]undec-7-ene), 5 μL) was added.

The direct binding of NO^-/HNO followed by reduction of the metal ion by internal electron transfer has been described for *N*-methyl-D-glucamine dithiocarbamate iron,[6] metmyoglobin,[7a] and other ferric hemeproteins.[7b,c] The formal description of heme (or non-heme) ferrous nitrosyl complexes depends on both the nature of the coligands and the strength of the Fe−NO bond.[21] In the case of iron porphyrins, density functional theory calculations provided a strong evidence of an iron(II) nitrosyl complex.[22]

Concerning the possible side reactions of NO^-/HNO in aqueous media, anaerobic conditions prevented the formation of peroxynitrite and guaranteed the presence of NO^-/HNO as the unique active species. Examination of the gas products by mass spectrometry indicated that NO evolved, probably as a consequence of $Fe^{II}TPPS(NO)$ decomposition mediated by a nucleophile derived from the reactant or the buffer solution; N_2O from nitroxyl dimerization was also observed. When the reaction was carried out in the presence of oxygen, product formation was diminished, probably due to the reaction of the nitrosyl porphyrin with oxygen.

The rate-limiting step for the overall reaction of ferric porphyrins with AS was considered to be the donor decomposition that was not affected by the presence of the ferric porphyrin. The metalloporphyrin did not catalyze the decomposition of AS, and coordination of AS to the iron center was not favorable, or if it was, it did not contribute to the formation of HNO. Initial rate determinations in aqueous anaerobic media were performed at 298 K, taking into account the parallel reactions described below (eqs 1, 2, and 3).

$$N_2O_3H^- \overset{k_1}{\rightleftharpoons} HNO + NO_2^- \quad (1)$$

$$2HNO \xrightarrow{k_2} H_2N_2O_2 \rightarrow N_2O + H_2O \quad (2)$$

$$HNO + [Fe^{III}(TPPS)(H_2O)_2]^{3-} \xrightarrow{k_{on}} Fe^{II}(TPPS)(H_2O)NO + H^+ + H_2O \quad (3)$$

The product formation rate was calculated from UV/vis measurements of the reaction between AS and $[Fe^{III}TPPS(H_2O)_2]^{3-}$, for varying AS concentrations. Assuming steady-state for [NO^-/HNO] and using the known values of k_1[9] and k_2,[5b,23] k_{on} for eq 3 was estimated to be $1.00 \pm 0.04 \times 10^7$ M^{-1} s^{-1}.[24] Dimerization followed by dehydration of HNO (eq 2) was the major route for its elimination anaerobically, and since its rate constant has been reported to be 8×10^9 M^{-1} s^{-1},[23] this reaction competed with the fast trapping of nitroxyl by the ferric porphyrin. The value found for k_{on} seemed to be reasonable taking into account that k_{on} for the reaction with NO is 4.5×10^5 M^{-1} s^{-1} (25 °C) for $[Fe^{III}TPPS(H_2O)_2]^{3-}$ and 1.5×10^9 M^{-1} s^{-1} (25 °C) for $Fe^{II}TPPS$.[25]

Despite the fact that reaction rate (k_{on} values) is not markedly different for the reaction of ferric porphyrins with NO or NO^-/HNO donors, the ferrous nitrosyl porphyrin obtention is known to depend on the specific requirements of the reductive nitrosylation when NO is the reactant.[17] The ferric porphyrins tested are both fast and efficient traps for NO^-/HNO regardless the media, whereas the binding of NO affords labile ferric nitrosyl complexes that, in the end, may not efficiently yield the reductive nitrosylation product. On the other hand, comparison of the results obtained for $[Fe^{III}TPPS]^{3-}$ with the reaction of metmyoglobin with NO^-/HNO shows that the protein is more efficient in terms of $Fe^{II}(NO)$ product to nitroxyl donor ratios.[7a] However, the simple porphyrin systems could be used as powerful tools to gain insight into the NO^-/HNO reaction with ferriheme proteins, where NO has been shown to exhibit different performances depending on the protein environment of the hemin.[17] Furthermore, the use of soluble ferric porphyrins should be explored as direct, selective traps for NO^- in biological media.

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Supporting Information Available: Experimental details for the reaction of Fe(III) protoporphyrin IX and TSHA, FTIR, microanalyses, kinetic data, mass spectra (PDF). This material is available free of charge via the INTERNET at http://pubs.acs.org.

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