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Formation of Nitric Oxide from Nitrite by the Ferriheme *b* Protein Nitrophorin 7

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The formation of the central signaling molecule NO[1] from NO_2^- occurring in the blood has recently been identified as an exceedingly important physiological reaction in the human organism.[2] The conversion is, at least in part, accomplished at the heme iron of hemoglobin (Hb). However, whereas deoxyHb exhibits nitrite reductase and anhydrase activities,[2c] reaction of NO_2^- with metHb and also met-myoglobin (metMb) is not observed.[3] Moreover, in the presence of certain base molecules functioning as oxygen atom acceptors, the conversion of NO_2^- into NO was described for the model heme Fe^{III}(TPPS) [TPPS = tetra(sulfonatophenyl)porphyrinato anion] in aqueous solution at pH 5.8 and 6.3.[4]

The nitrophorins (NPs) constitute a group of ferriheme proteins from the saliva of the blood-feeding insect *Rhodnius prolixus* that coordinate the heme via a His residue.[5] Their biological function is to transport NO as a vasodilator into the host's tissue (pH ~7.4). Contrary to metHb and metMb, recombinant nitrophorin 7 (NP7) shows a marked spectral change upon incubation with $NaNO_2$ as the sole substrate, as shown in Figure 1, even at pH 7.5.[6] The spectral change clearly exhibits isosbestic behavior and reaches saturation after some time, indicating the full conversion into a compound **1** that is EPR-silent. The absorption spectrum of **1** is similar to that of the $\{FeNO\}^6$ species[7] of NP7,[8] but the resolution of UV−vis spectroscopy is not high enough for unambiguous identification.

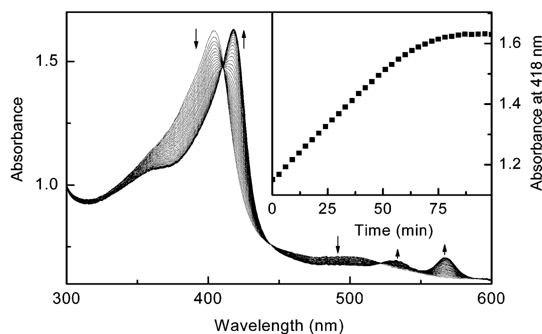

Figure 1. Spectral change observed for NP7 in 100 mM MOPS/NaOH, 50 mM NaCl (pH 7.5) at 37 °C in the presence of 1 mM $NaNO_2$.

For further characterization of **1**, resonance Raman (RR) spectroscopy of **1** in comparison to NP7[Fe^{III}] and NP7$\{FeNO\}^6$ was performed. The high-frequency region of each of the resulting spectra is presented in Figure 2. In general, the spectra of NP7[Fe^{III}] and NP7$\{FeNO\}^6$ compare well with the corresponding RR spectra reported for NP1 and NP4.[9] Moreover, the spectral traces of NP7$\{FeNO\}^6$ and **1** are identical. As in the case of the NP1 and NP4 nitrosyls,[9] the so-called oxidation state marker $\nu_4 = 1376$ cm^{-1} for both NP7 complexes indicates significant ferriheme character. The ν_3 mode in case of NP7[Fe^{III}] (1484 cm^{-1}) exhibits the typical frequency of a six-coordinate high-spin (water on) heme, whereas the shift toward 1509 cm^{-1} upon NO binding is characteristic for six-coordinate low-spin hemes.[10]

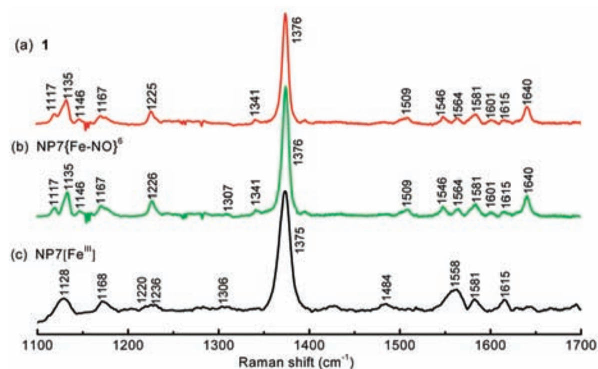

Figure 2. Resonance Raman spectra of (a) **1**, (b) NP7$\{FeNO\}^6$, and (c) NP7[Fe^{III}]. All samples were prepared in 100 mM MOPS/NaOH, 50 mM NaCl (pH 7.5). Spectra were recorded at 77 K with $\lambda_{ex} = 406.7$ nm.

The identification of **1** as an $\{FeNO\}^6$ entity by RR spectroscopy is well-supported by FT-IR spectroscopy, which shows the vibrational mode of the Fe−N−O fragment at 1903 cm^{-1} and a shoulder at 1911 cm^{-1} (see Figure S1 in the Supporting Information). The comparison with other heme proteins in Table 1 shows that this mode is characteristic of ferriheme nitrosyl complexes.

Table 1. Frequency of the N−O Stretching Vibration of **1** in Comparison with Those in Nitrosyl Complexes of Some Representatives of Histidine-Coordinating Ferriheme Proteins

ferric heme protein	ν_{N-O} (cm^{-1})	reference
1 (this work)	1903, 1911 (shoulder)	this work
R. prolixus NP1	1904 (shoulder), 1917	11
R. prolixus NP4	1908, 1922 (weak)	12
human Hb	1925	13
horse Mb	1927	14
P. stutzeri cd$_1$ nitrite reductase	1910	13

2-(4-Trimethylammonio)phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (TMA-PTIO) was applied as a spin trap for NO quantification.[15] As Figure 3a shows, addition of TMA-PTIO to **1** leads to the loss of NO from the iron and concomitant formation of the initial high-spin NP7[Fe^{III}]. Upon completion of the reaction, the amount of NO was determined by EPR spectroscopy of TMA-PTIO (Figure S2 in the Supporting Information). An equimolar protein/NO ratio of 1:(1.1 ± 0.1) was determined. As a control, NO was not detected when TMA-PTIO was added to a solution void of NP7. Furthermore, initiation of the reaction in the presence of 15 μM TMA-PTIO did not change the Soret band absorption until TMA-PTIO was completely consumed (Figure 3b). This reflects the fact that the protein is fully recovered upon turnover and that it is generally capable of catalytically producing NO in the presence of an NO acceptor, as is the case in vivo. The integrity of the protein mass was confirmed by ESI-Q-TOF MS.

The only potential electron source is one or more other nitrite molecules. To test this hypothesis, the amounts of NO_2^- consumed

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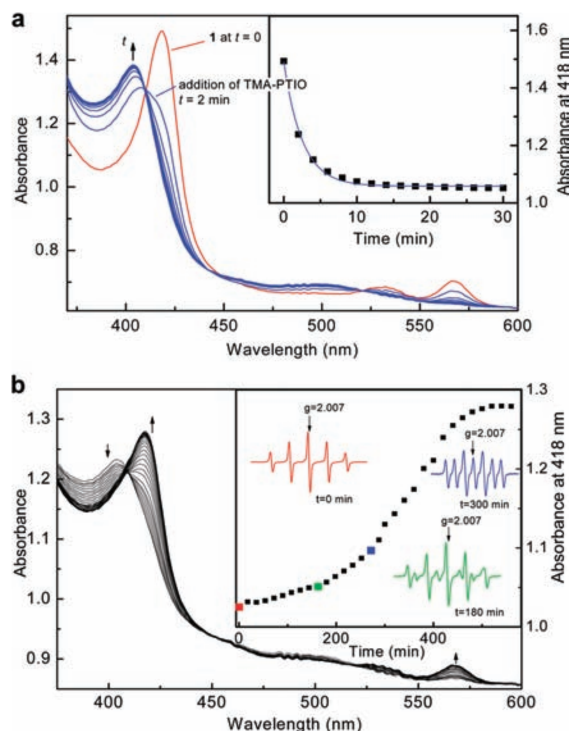

Figure 3. (a) Absorption spectrum of **1** in 100 mM MOPS/NaOH, 50 mM NaCl (pH 7.5) (red trace). The spectrum changes upon addition of TMA-PTIO (blue traces), leading to the initial species in a first-order process with $t_{1/2}$ = 1.7 min (R = 0.9981) (inset). (b) Absorption spectra for the reaction of NP7 (10.6 μM) with 10 mM $NaNO_2$ in the presence of 15 μM TMA-PTIO at 37 °C. The inset shows the change of the Soret absorption vs time and the EPR spectra taken at room temperature at the indicated time points.

and NO_3^- produced were determined by an adapted Griess reaction.[16] In comparison with the amount of NP7, 1.41 ± 0.03 molar equiv of NO_2^- was consumed while 0.51 ± 0.01 molar equiv of NO_3^- was simultaneously produced.[17] Furthermore, the formation of **1** is strongly pH dependent: at pH 6.0, the conversion is rapidly accomplished (Figure S3 in the Supporting Information), in good agreement with the disproportionation

$$3NO_2^- + 2H^+ \rightarrow 2NO + NO_3^- + H_2O$$

By analogy to the nitrite anhydrase reaction catalyzed by metHb,[2c] N_2O_3, which homolyzes to NO and NO_2, is a considerable product. In water, NO_2 further decomposes, yielding NO_3^-. However, excess 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO)[18] in the NP7 reaction did not affect the yield of NO_3^- (Figure S4 in the Supporting Information). Furthermore, the expected nitroxide radical was not detected by EPR spectroscopy. Notably, NO is required as a trigger for metHb nitrite anhydrase reaction.[2] Altogether, the results suggest a significantly different reactivity of NP compared to metHb.

A reaction between the heme d_1 center of heme cd_1 nitrite reductase and NO_2^- was recently described with both hemes in Fe^{III}; however, the reductant is not clear.[19] The disproportionation of NO_2^- readily occurs in strongly acidic solution, but the rate is extremely low at pH ≥7.[20] Although the reaction is not promoted by heme proteins,[3] it was observed in a few model ferrihemes with exclusion of water.[21] Similarly, Fe^{III}salen promotes the NO_2^-disproportionation noncatalytically in dry CH_3CN, yielding $(Fe^{III}salen)_2O$, but water strongly decreases the yield.[22]

In conclusion, the catalytic NO_2^- disproportionation supported by the ferriheme of NP is an unprecedented case in an aqueous environment. This process suggests that in vivo NP may not only be a NO transporter but also can act as a NO producer.

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Supporting Information Available: Complete refs 2b and 2c, experimental details, the FT-IR spectrum of **1**, NO quantification by EPR, UV-vis spectra for the reaction of NP7 with NO_2^- at pH 6.0 and the reaction in the presence of DMPO, and the stoichiometry of the reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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