The Millisecond Intermediate in the Reaction of Nitric Oxide with Oxymyoglobin is an iron(III)-Nitrato Complex, not a Peroxynitrite

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Supplementary Information

Experimental details

NO and Mb-O_2 Solutions. Lyophilized horse heart myoglobin was purchased from Sigma. The protein was dissolved in 200 mM CHES pH 9.5 to a final concentration of 1 to 2 mM and purged with argon before being brought into a glovebox with a controlled atmosphere of less than 1 ppm O_2 (Omni-Lab System; Vacuum Atmospheres Co.). The protein was reduced by addition of a 5-fold excess of sodium dithionite followed by dialysis against 500 mL anaerobic buffer for 2 h or passage through a Sephadex G-25 column (GE Healthcare). Complete heme reduction and dithionite removal were confirmed by UV-vis spectroscopy with a Cary 50 spectrometer. Solutions were then transferred to sealed serum bottles and exposed to $^{16}O_2$ (Airgas) or $^{18}O_2$ (99% ^{18}O; ICON Stable Isotopes) gas. Formation of Mb-O_2 was confirmed by UV-vis spectroscopy and the final Mb concentration was determined (typically 1.2 to 1.8 mM). Before drawing the solution in 2-mL RFQ syringes, the serum bottles were exposed to the glovebox to release excess O_2. Saturated $^{14}N^{16}O$ solutions were made by bubbling $^{14}N^{16}O$ gas (Airgas) (scrubbed with 1 M KOH) into argon-purged buffer for 10 to 15 min. Alternatively, serial additions of NO gas into a sealed vial of anaerobic buffer was used for the preparation of $^{14}N^{16}O$ (Airgas), $^{15}N^{16}O$ (98% ^{15}N, Aldrich), and $^{15}N^{18}O$ (98% ^{15}N, 95% ^{18}O, Aldrich) solutions. Solutions in sealed serum bottles were drawn in RFQ syringes using a needle attachment. To rule out any concerns with contaminant gases, experiments were also carried out using an NO donor and produced identical results and conclusions. Specifically, a concentrated solution of diethylamine NONOate (Cayman Chemical) (25 mM) was diluted to 1.4 mM in anaerobic phosphate buffer (20mM, pH 7.0), and incubating for 1 h at room temperature in the RFQ syringes. Concentration of NO solutions was determined by titration with deoxy-Mb and ranged from 1.4 to 2.0 mM. Contamination of NO solutions with nitrite was determined using an $\varepsilon_{354} = 22.5\ M^{-1}cm^{-1}$ (Strickler S. J. & Kasha M.*, J. Am. Chem. Soc.*, **1963**, *85*, 2899-2901) and was found to never exceed 3 mM.

Rapid Freeze Quench (RFQ) Experiments. RFQ syringes were mounted in the System 1000 Chemical / Freeze Quench Apparatus (Update Instruments, Inc.) equipped with a water bath maintained at 3° C. Reaction times were controlled by varying either the ejection speed or the volume of the reactor hose. 5 ms are added to the calculated reaction time to account for time of flight and freezing in liquid ethane. Samples of 125 – 250 μL were ejected into a glass funnel attached to an NMR tube and containing liquid ethane at < -120° C. The frozen sample was packed into the tube as the assembly sat within a Teflon block cooled with liquid nitrogen to -100° C. Once packed the frozen samples were stored in liquid nitrogen before analysis by resonance Raman (RR). Successful mixing and NO dioxygenation reaction was confirmed by UV-vis analysis of samples ejected into a microcentrifuge tube, and by RR analysis of samples shot into a room temperature NMR tube and frozen manually.

Resonance Raman and UV-vis Spectroscopy. Low temperature RR spectra (~105 K) were obtained using a backscattering geometry on RFQ samples in NMR tubes. All spectra were collected on a custom McPherson 2061/207 spectrograph (0.67 m with variable gratings) equipped with a Princeton Instruments liquid N_2-cooled CCD detector (LN-1100PB). Excitation at 413, 351, and 637 nm was provided by a krypton laser (Innova 302, Coherent) while excitation at 514 and 458 nm were provided by an Innova I90C-3 argon ion laser. A Kaiser Optical supernotch filter was used to attenuate Rayleigh scattering at 413 nm while long-pass filters (RazorEdge, Semrock) were used for other excitation wavelengths.

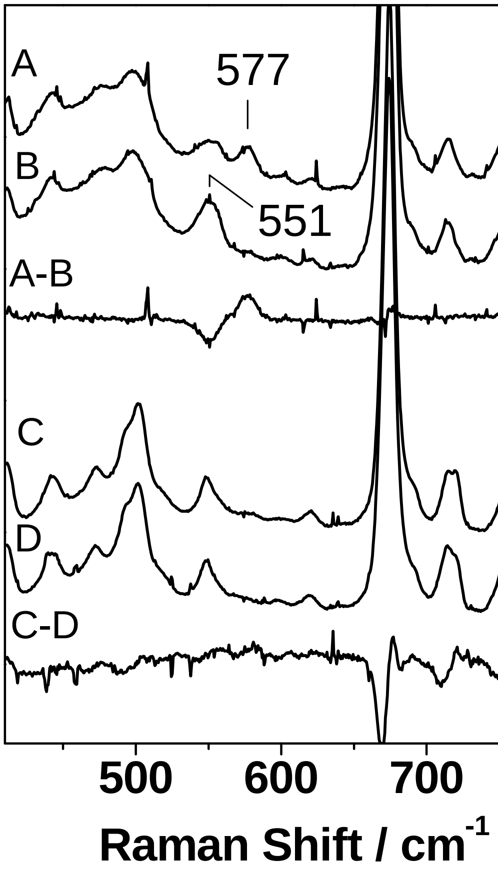

Figure S1. Low-frequency RR spectra of Mb-$^{16}O_2$ (A), Mb-$^{18}O_2$ (B), Mb-$^{16}O_2$ + $^{14}N^{16}O$ at 6 ms (C) and Mb-$^{18}O_2$ + $^{14}N^{16}O$ at 6 ms. λ_{exc} = 413 nm, 20 mW.

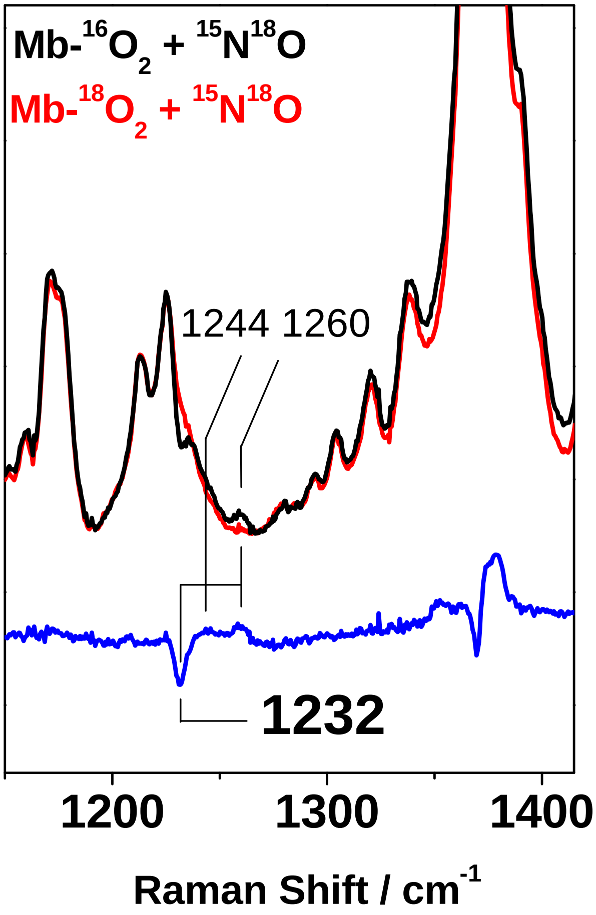

Figure S2. Mid-frequency RR spectra of the 6-ms RFQ samples for the reaction of $^{15}N^{18}O$ with Mb-$^{16}O_2$ (black) versus Mb-$^{18}O_2$ (red). The difference spectrum is shown in blue.