SUPPORTING INFORMATION FOR

A Diferrous-Dinitrosyl Intermediate in the N₂O-Generating Pathway of a Deflavinated Flavo-Diiron Protein

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Stopped-Flow Spectrophotometry

Anaerobic protein and NO solutions in buffer were used for all experiments. Spectral time courses for reactions of reduced deflavo-FDP with substoichiometric NO were obtained using 10:1 (v/v) mixing of 210 - 250 µM anaerobic solution of reduced deflavo-FDP with a ~1.8 mM NO solution. Spectral time courses with excess NO were obtained using 1:1 (v/v) mixing of a 360 µM anaerobic solution of reduced deflavo-FDP with a 1.8 mM NO solution. The solutions in both drive syringes and the optical cell were cooled to 2-3 °C prior to stopped-flow mixing. The NO concentrations immediately after stopped-flow mixing were quantified by stopped-flow mixing the same drive syringe-loaded solution of NO with 1-5 mM Fe^{II}EDTA in place of the protein.\(^1\) The concentrations of FDP and NO immediately after mixing are listed in the figure captions. Spectra of the starting reduced deflavo-FDP under these conditions were obtained by stopped-flow mixing with anaerobic buffer in place of the NO solutions.

Preparation of RFQ EPR and Mössbauer Samples

Five-milliliter drive syringes were loaded with either anaerobic reduced deflavo-FDP solutions in buffer or anaerobic NO solutions in buffer and rapid-mixed 1:1 (v/v). To prepare an unreacted sample, the reduced deflavo-FDP solution was rapid-mixed with deoxygenated buffer in place of the NO solution. For reactions with substoichiometric NO a ~1 mM anaerobic reduced deflavo-FDP solution was rapid-mixed with a 1.8 mM buffered NO solution (prepared at room temperature). For reactions with excess NO, 200 to 300 µM reduced deflavo-FDP was rapid-mixed with a 3 mM NO solution in buffer (prepared at 4 °C). After the appropriate aging times, 350 microliters of the reaction mixtures were sprayed into liquid isopentane at -150 to -160 °C. The resulting snow was collected in a funnel immersed in the cold isopentane and
packed into the bottom of a 4-mm O.D. quartz EPR tube attached to the bottom of the funnel with 1/8” inner diameter silicon tubing (Cole-Parmer). The spin concentrations were corrected for the packing factor as previously described.¹

For RFQ Mössbauer samples, a ~1 mM reduced deflavo-FDP solution at room temperature was loaded into a 5 mL drive syringe and rapid-mixed 1:1 (v/v) with the 3 mM buffered NO solution prepared at 4 °C loaded into another 5 mL drive syringe. After the appropriate aging times, 700 microliters of the reaction mixture was sprayed into a 50 mL Falcon tube (Fisher Scientific) containing ~40 mL of liquid ethane at -160 to -170 °C. Excess liquid ethane was removed by decanting into a waste container followed by evacuation of the sample cooled in a dry ice/methanol bath.¹ PLEASE NOTE: liquid ethane is highly flammable. Do not expose liquid ethane to an open flame and perform all manipulations in a well-ventilated area. Approximately 400 microliters of the resulting snow was transferred with a liquid N₂-cooled spatula to a liquid N₂-cooled Mössbauer cup equipped with a screw cap lid. The remaining 300 microliters of snow was resuspended in ~20 mL of liquid ethane that had been cooled to -170 °C. A glass funnel was connected to an EPR tube and the assembly chilled to -130 to -160 °C using the isopentane bath, as described above. The resuspended snow in liquid ethane was poured into the chilled EPR funnel, and the snow was packed into the EPR tube. All of the reported quench times are the sums of the aging time (minimum ~5 ms) plus an estimated 15-ms freezing time.² Samples were stored in a liquid N₂ until data collection.
Figure S1. Mössbauer spectrum after thawing and five-minutes exposure to air of the RFQ sample used to obtain the spectrum in Figure 5C. The red trace is a simulation of the data (black vertical bars) using the parameters listed in Table 1 for the species diFe$^{III}$ (69%) and Fe$^{II}$ (23%).
The spectrum was collected at 4.2 K and 45-mT magnetic field applied parallel to the γ-ray direction.

References

1. Caranto, J. D., Weitz, A., Hendrich, M. P., and Kurtz, D. M., Jr. (2014) The nitric oxide reductase mechanism of a flavo-diiron protein: Identification of active-site intermediates and products. *J. Am. Chem. Soc.* 136, 7981-7992.

2. Bollinger, J. M., and Krebs, C. (2006) Stalking intermediates in oxygen activation by iron enzymes: Motivation and method. *J. Inorg. Biochem.* 100, 586-605.