Supporting Information

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SI Text

Enzyme Expression and Purification. Metal-substituted quercetin dioxygenases (QDOs) were prepared by growing *Escherichia coli* BL21 (DE3) carrying plasmid pQuer4, as previously described (1). Fe-QDO was prepared from *E. coli* cells grown in Luria Bertani medium, and Co- and Mn-QDOs were prepared from *E. coli* grown in M9 minimal medium. Each culture was induced to express protein by the addition of 50 mg/L isopropyl-ß-D-thiogalactopyranoside. The cultures for Co- and Mn-QDOs were supplemented with 1 mM $MnCl_2$ and $CoCl_2$ salt, respectively. Cells were harvested and the protein purified as previously described (2), except the protein was eluted from the DEAE-Sephacel column stepwise with 125 mM NaCl in 50 mM Tris buffer, pH 7.5 (wash buffer) and 175 mM NaCl in 50 mM Tris buffer, pH 7.5 (elution buffer). Also, the DEAE-Sepharose column was omitted from the purification procedure. Enzymatic activity was assayed at 380 nm in a standard spectroscopic assay using quercetin as a substrate as described in ref. 3). Active samples were analyzed for purity by SDS-PAGE electrophoresis. The purest, most active samples were pooled and concentrated and the buffer was exchanged to 50 mM phosphate buffer (pH 7.0) by centrifugation in a Vivaspin 15R centrifugal filter unit (10,000 molecular weight cutoff). Protein concentration was determined by a Bradford assay, and enzyme was stored in 10% glycerol at −20 °C.

Nitroxygenase Assay and Control Experiments. In a typical experiment, quercetin and enzyme were added to the immunoprecipitation (IP) buffer at pH 6.5. The reaction was initiated by adding oxygen-saturated solution (130 μM). The reaction was followed by loss of quercetin absorbance at 380 nm (Fig. S1). Control experiments with other nitrogenous reagents show no similar reactivity (Fig. S2).

Liquid Chromatography-Electrospray Ionization MS Analysis of Assay Products. Extracts were analyzed on an Accela liquid chromatograph coupled to a Linear Trap Quadrupole Orbitrap Discovery mass spectrometer (Thermo Electron) using positive and negative electrospray ionization (+ESI/ − ESI). Final extracts were diluted 10-fold into mobile phase and then injected (10 μL) into the liquid chromatography (LC) system consisting of a 15 cm × 2.1 mm (5 μm, 80 Å) extended-C18 column (Agilent Technologies). A binary mobile phase gradient containing 0.1 % (vol/vol) formic acid in water (A) and acetonitrile (B) was applied as follow: 97% A for 5 min, to 98% B in 30 min, held for 5 min, back to 97% A in 1 min, and equilibrated for 5 min at 97% A. Additional chromatographic parameters were as follows: column temperature, 30 °C; flow rate, 350 μL/ min. Full-scan accurate mass spectra (m/z range 50–700) of eluting compounds were obtained at high resolution (30,000 FWHM) on the Orbitrap mass analyzer using internal calibration (accuracy of measurements <2 ppm) and processed using Xcalibur v.2.0.7 software. Electrospray source conditions were: sheath and auxiliary gas flow 50 and 5 arbitrary units (a.u.), respectively; heated capillary temperature 300 °C; electrospray voltage 4.5 kV for +ESI and 5.0 kV for −ESI; capillary voltage 43 V for +ESI and −43 V for −ESI; tube lens voltage 205 V for +ESI and −148 for −ESI.

Synthesis and Characterization of ^{15}N Angeli's Salt. The synthesis of ^{15}N-labeled Angeli's salt (AS) followed previously published procedures (3). Proof of purity of the ^{15}N-labeled AS was assessed by reaction of the compound with deoxymyoglobin to form the H^{15}NO-Mb adduct, which has previously been characterized by its unique ^{1}H-^{15}N coupling in the ^{1}H NMR (4).

A 0.5-mM sample of deoxy myoglobin (Fe^{II}Mb) in IP buffer (pH 7) was added to ^{15}N AS in an NMR tube and allowed to stand for 45 min. ^{1}H NMR was then recorded using WET1D pulse sequence. The appearance of a doublet with N-H coupling of 71 Hz for the proton of nitrosyl hydride (HNO) confirmed the presence of ^{15}N-labeled Angeli's salt (Fig. S3).

Confirmation of CO Formation by Trapping with Deoxy-Myoglobin. A home-built UV-visible (UV-vis) long-necked cuvette with a side arm attached to a 25-mL round bottom flask was used to trap the CO by deoxy-myoglobin, as shown in Fig. S4. In the UV cuvette was added deoxy myoglobin (11 μM) in IP buffer, pH 7.0. Quercetin (33 μM) in IP buffer, pH 8 was added to the round-bottom flask. The reaction was initiated by adding Angeli's salt (327 μM) to the quercetin. The progress of the reaction was monitored by the shift in Soret absorbance from 434 to 423 nm, confirming formation of CO-Fe^{II}Mb.

LC-MS of Products of Nitroxygenase Mn-QDO Assay with Modified Quercetin Derivatives Quercetin-5-Sulfonic Acid and Quercetin-5-Methoxy The enzymatic reaction of Mn-QDO (2.1 μM) with quercetin derivatives **2** (23 μM) and **3** (25 μM) with Angeli's salt (180 μM) were carried out in IP buffer at pH 7 for an hour. After the completion of reaction, which was followed by loss of respective quercetin absorbance at 380 nm, the reaction mixture was analyzed in LC-MS for products **7** and **8** (Fig. S5).

Rates of Kinetic Runs of Nitroxygenase Activity Under Varying Conditions. Assay reactions with varying amounts of quercetin, Mn-QDO, and the HNO-precursor AS were carried out in a screw-capped UV cuvette and monitored by following the decrease of the substrate absorbance at 380 nm. In a typical experiment, to a 2 mL of IP buffer at pH 7, appropriate concentrations of enzyme and substrate were added. The reaction was initiated by gently shaking the cuvette before placing it in the spectrometer. Concentrations and derived rates are given in Table S1.

Kinetic Modeling of Nitroxygenase Reaction Sequence. The following reaction sequence was used to estimate the rate of the nitroxygenase reaction, Eq. **3** below:

$$\mathrm{AS} \rightarrow \mathrm{HNO} + \mathrm{NO_2^-} \qquad k = 6.45 \times 10^{-4}\ \mathrm{M^{-1}\,S^{-1}}, \qquad \textbf{[S1]}$$

$$\mathrm{HNO} + \mathrm{HNO} \rightarrow \mathrm{N_2O} + \mathrm{H_2O} \qquad k = 8.0 \times 10^{6}\ \mathrm{M^{-1}\,S^{-1}}, \qquad \textbf{[S2]}$$

$$\mathrm{Q} + \mathrm{HNO} \rightarrow \mathrm{P} \qquad k = 3.3 \times 10^{4}\ \mathrm{M^{-1}\,S^{-1}}. \qquad \textbf{[S3]}$$

Because of the relatively slow release of HNO by AS, as well as its rapid rate of dimerization rate constants, the reaction HNO with Mn-QDO could only be obtained by kinetic modeling. Because Mn-QDO itself has no observable absorbance in the UV-vis spectral range, the rate of the reaction was simulated using the loss of absorbance of the quercetin. Concentration changes of quercetin over the course of reaction were simulated using the REACT for Windows (5) program utilizing the reaction sequence (Eqs. **1**–**3**), as has previously been applied to HNO trapping by heme globins (6). The concentration vs. time profiles thus obtained were compared to experimental kinetic traces of quercetin after conversion of ΔOD to concentrations. These simulations were repeated by

varying the rate constant for Eq. **3** until a best fit was achieved, shown in Fig. S6.

Nonenzymatic Reaction of HNO with Quercetin. The pH dependence of the nonenzymatic reaction of HNO with quercetin was determined by determining rate of loss of absorbance at 380 nm during the anaerobic reaction, as shown in Fig. S7.

Analysis of Metal Content in Metallo-QDOs by Inductively Coupled Plasma and Atomic Absorption Spectrometry. The metals cobalt and manganese were analyzed on an AS 93Plus autosampler coupled to an Elan 9000 inductively coupled plasma (ICP)-MS (Perkin-Elmer-SCIEX). ICP-MS parameters were as follows: rf power 1,300 W, lens voltage 6.75 V, analog stage voltage −2,000 V, pulse stage voltage 900 V, and nebulizer gas flow 0.89 L/min. Isotopes monitored were ^{55}Mn and ^{59}Co. Data were acquired and processed using Elan software version 3.4. Samples were diluted 1,000- and 2,500-fold for cobalt and manganese, respectively, into nitric acid (1%). Cobalt and manganese concentrations were determined using external calibration. Six calibration standards (0.5–10 μg/L) were prepared by performing serial dilutions of 1,000 mg/L calibration solutions containing cobalt or manganese in 1% nitric acid (Perkin-Elmer). Calibration curves were prepared for Co and Mn by plotting linear regressions ($r^2 > 0.998$) of analyte responses versus analyte concentrations.

Iron was analyzed using a GCS furnace autosampler coupled to a graphite furnace atomic absorption spectrometer iCE 3000 Series AA (Thermo Scientific). To determine the best atomization temperature program, a 5 μg/L iron solution in 1% nitric acid was prepared by diluting an iron calibration solution (1,000 mg/L, Perkin-Elmer). Optimized atomization temperature program was as follows: from room temperature to 100 °C (10 °C/s), then held for 30 s, to 700 °C (150 °C/s), then held for 20 s, to 1,900 °C (0 °C/s), then held for 3 s, and to 2,900 °C (0 °C/s), then held for 3 s. The furnace was purged with argon at a flow rate of 200 mL/min at all temperature steps except for the atomization step. Additional atomic absorption parameters were as follows: iron hollow cathode lamp wavelength 248.3 nm; lamp current 75%; band pass 0.2 nm; background correction Zeeman. Absorbance data were acquired and processed using Thermo SOLAAR software. Samples were diluted 150-fold into nitric acid (1%) and placed by the autosampler into an electrographite cuvette (20 μL) for analysis. Iron concentrations were determined using external calibration curve. Calibration standards were prepared by the autosampler by performing serial dilutions of a stock solution containing 10 μg/L of Fe in 1% nitric acid, resulting in six standards ranging from 0.5 to 10 μg/L. A calibration curve was prepared by plotting a linear regression ($r^2 > 0.998$) of the analyte response versus the analyte concentration.

During ICP-MS and AA analyses calibration was monitored through use of blanks and continuous calibration verification standards with an acceptability criterion of ±15%. Two identical matrix spikes [i.e., one matrix spike/matrix spike duplicate pair (MS/MSD)] were prepared by adding a known amount of metals, Fe, Co, and Mn, to analyzed samples, increasing total concentration by approximately a factor of 2. Recovery percentage of spiked amounts (criteria 85–115%) as well as MS/MSD relative percentage difference (criteria 15%) were evaluated for quality-assurance purposes. The resulting concentrations are given in Table S2, which were used to derive percent contents shown in Table 1 of main text.

1. Schaab MR, Barney BM, Francisco WA (2006) Kinetic and spectroscopic studies on the quercetin dioxygenase from *Bacillus subtilis*. *Biochemistry* 45:1009–1016.
2. Barney BM, Schaab MR, LoBrutto R, Francisco WA (2004) Evidence for a new metal in a known active site: Purification and characterization of an iron-containing quercetin dioxygenase from *Bacillus subtilis*. *Protein Expr Purif* 35:131–141.
3. Francis TB, Baruch R (1975) Thermal decomposition of oxyhyponitrite (Sodium Trioxodinitrate (II)) in aqueous solution. *Inorg Chem* 14:558–563.
4. Sulc F, Immoos C, Pervitsky D, Farmer PJ (2004) Efficient trapping of HNO by deoxymyoglobin. *J Am Chem Soc* 12:1096–1101.
5. Manka MJ, ed. (2001) REACT for Windows, Ver. 1.2 (Alchemy Software, Wesley, Chapel, FL).
6. Kumar MR, et al. (2009) Nitrosyl hydride (HNO) as an O_2 analogue: Long-lived HNO-adducts of ferrous globins. *Biochemistry* 48:5018–5025.

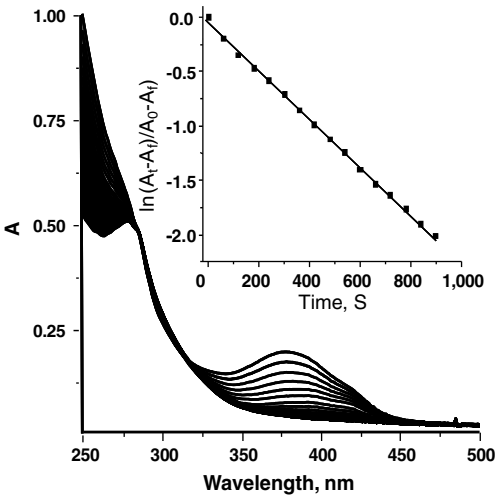

Fig. S1. UV spectrum of reaction of HNO (137 μM) with Mn-QDO (8.5 μM) showing decrease of quercetin (13.8 μM) absorbance at 380 nm with time and (*Inset*) a plot of $\ln(A_t - A_f)/(A_0 - A_f)$ versus time (■) with best fit (*Right*).

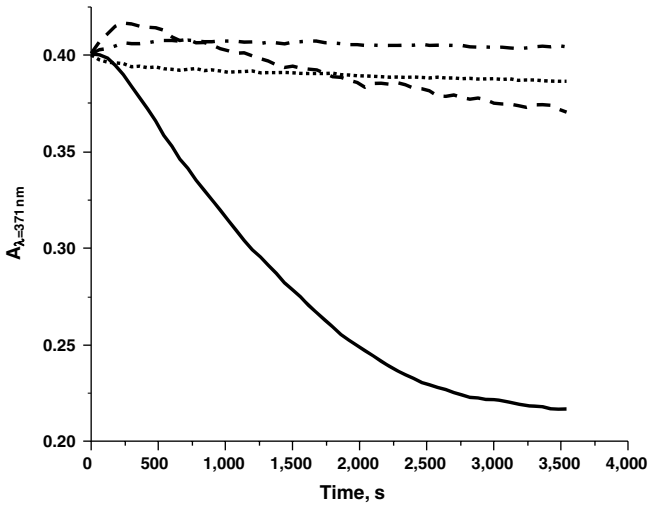

Fig. S2. Normalized UV-vis spectral absorbance versus time plots of anaerobic reactions in IP buffer at pH 7 of Mn-QDO and quercetin mixtures with nitrogenous reagents. Experimental concentrations for traces shown are as follows: (solid line) 68 μM AS, 15.4 μM quercetin, 8.5 μM Mn-QDO; (dash-dot line) 67 μM $NaNO_2$, 15.5 μM quercetin, 8.5 μM Mn-QDO; (dotted line) 94.5 μM NH_2OH, 21 μM quercetin, 10.5 μM Mn-QDO; (dashed line) control experiment using 123 μM AS and 15.5 μM quercetin, with no Mn-QDO added.

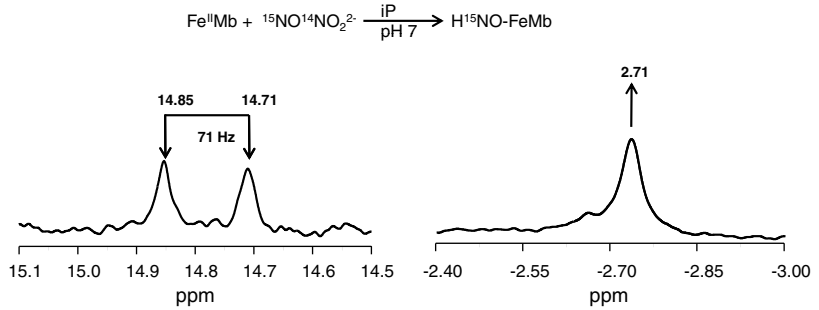

Fig. S3. ^1H NMR of $H^{15}NO$-FeMb showing characteristic splitting of nitrosyl hydride resonance due to ^{15}N coupling.

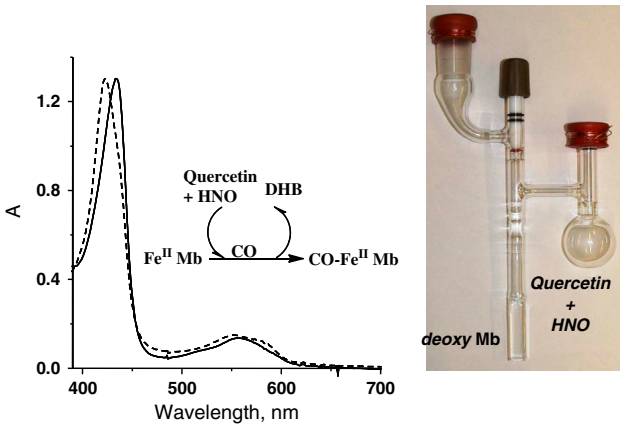

Fig. S4. UV-vis spectra of formation of CO – Fe^{II}Mb (dotted line) by trapping of CO by deoxy-myoglobin (11 μM, line) released in the reaction of quercetin (33 μM) with AS (327 μM). DHB, 3, 4-dihydroxybenzonitrile.

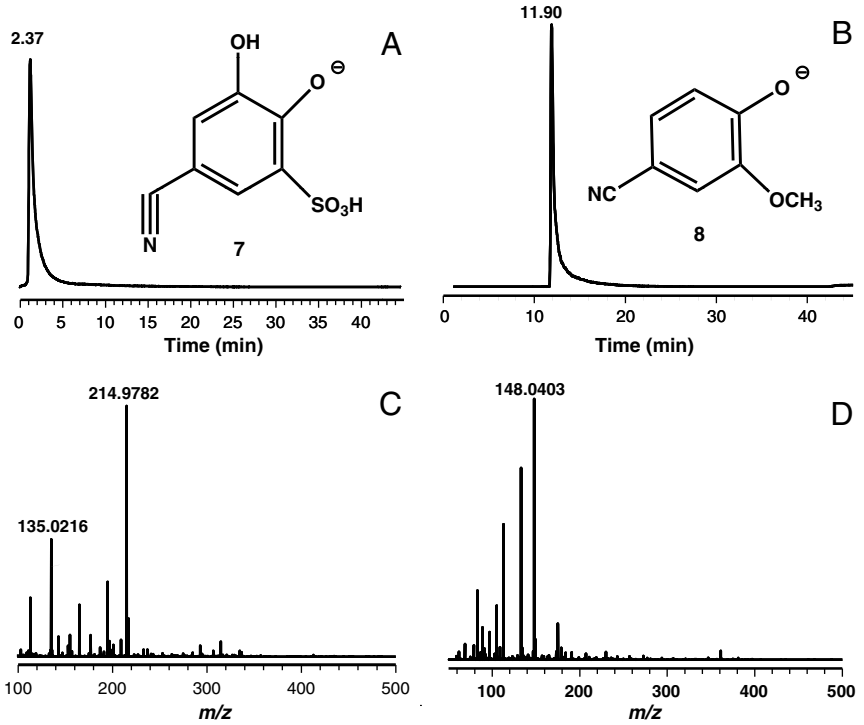

Fig. S5. LC-MS of reaction of HNO from decomposition of AS (180 μM) with Mn-QDO (2.1 μM) in 50 mM IP buffer at pH 7 with compounds **2** (23 μM) and **3** (25 μM); *A* and *B* show chromatograms of **7** and **8**, and *C* and *D* show mass spectra of LC peak, respectively.

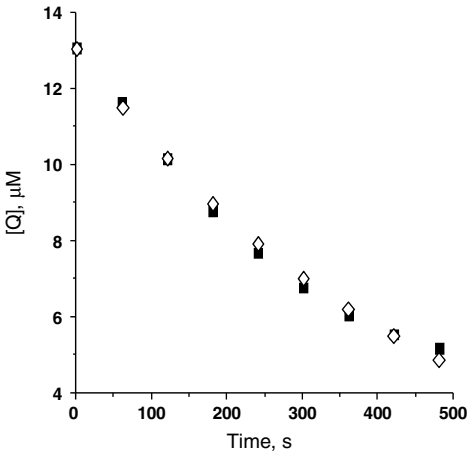

Fig. S6. Rate of loss of quercetin (13 μM) on reaction with Mn-QDO (1.3 μM) and AS (130 μM) in IP buffer at pH 7. Experimental data points (■) and simulated data (♦).

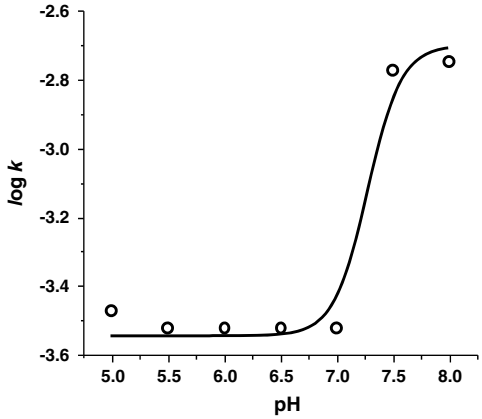

Fig. S7. Effect of pH on the rate of loss of absorbance at 380 nm during the anaerobic reaction quercetin (15.7 μM) with Angeli's salt (158 μM) in 100 mM phosphate buffer.

Table S1. Rates of kinetic runs

Run	[Q], 10^{-5} M	[Mn-QDO], μM	[AS], 10^{-4} M	k_{decay},* 10^{-3} s^{-1}
1	1.38	8.51	1.37	6.40
2	1.54	8.51	0.68	2.30
3	1.80	4.26	0.68	0.60
4†	1.50	4.26	1.37	1.10
5†	1.61	4.26	1.37	1.00

*Calculated by plotting $\ln(A_t - A_f)/A_0 - A_f)$ vs. second.
†Enzyme and substrate added last, respectively.

Table S2. Analysis of cobalt, manganese (ICP-MS), and iron (AA)

Metal ion content			
Sample	Fe (AA), μM	Co (ICP-MS)	Mn (ICP-MS), μM
Fe-QDO	92.29	ND	0.768
CoQDO	7.14	308 μM	32.02
Mn-QDO	22.05	ND	473

ND, not detected.