Nitrosyl hydride (HNO) replaces dioxygen in nitroxygenase activity of manganese quercetin dioxygenase

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Quercetin dioxygenase (QDO) catalyzes the oxidation of the flavonol quercetin with dioxygen, cleaving the central heterocyclic ring and releasing CO. The QDO from *Bacillus subtilis* is unusual in that it has been shown to be active with several divalent metal cofactors such as Fe, Mn, and Co. Previous comparison of the catalytic activities suggest that Mn(II) is the preferred cofactor for this enzyme. We herein report the unprecedented substitution of nitrosyl hydride (HNO) for dioxygen in the activity of Mn-QDO, resulting in the incorporation of both N and O atoms into the product. Turnover is demonstrated by consumption of quercetin and other related substrates under anaerobic conditions in the presence of HNO-releasing compounds and the enzyme. As with dioxygenase activity, a nonenzymatic base-catalyzed reaction of quercetin with HNO is observed above pH 7, but no enhancement of this basal reactivity is found upon addition of divalent metal salts. Unique and regioselective N-containing products ($^{14}N/^{15}N$) have been characterized by MS analysis for both the enzymatic and nonenzymatic reactions. Of the several metallo-QDO enzymes examined for nitroxygenase activity under anaerobic condition, only the Mn(II) is active; the Fe(II) and Co(II) substituted enzymes show little or no activity. This result represents an enzymatic catalysis which we denote nitroxygenase activity; the unique reactivity of the Mn-QDO suggests a metal-mediated electron transfer mechanism rather than metal activation of the substrate's inherent base-catalyzed reactivity.

nitroxyl | quercetinase

Dioxygenases are enzymes that incorporate both atoms of dioxygen into a targeted substrate, e.g., the enzyme catechol 1,2-dioxygenase cleaves the C–C bond between catechol's two hydroxyl groups forming the dicarboxylate *cis,cis*-muconic acid (1). Although highly divergent, there are two broadly defined classes of nonheme dioxygenases: those that directly bind dioxygen at the metal center or those in which dioxygen binds to the substrate that has been activated by interaction with the metal center (2). Such questions also apply to quercetin dioxygenase (QDO), which catalyzes the oxidation of the flavonol quercetin (**1**) with dioxygen (Fig. 1). During turnover, QDO cleaves the central heterocyclic ring of quercetin, releasing CO, and yielding the corresponding depside. The QDO from *Bacillus subtilis* is unusual in that it has been shown to be active with several divalent metal cofactors such as Cu, Fe, Mn, and Co. Comparison of the catalytic activities suggest that Mn(II) is the preferred cofactor for this enzyme (3).

The simple molecule nitrosyl hydride (HNO) is the singly reduced and protonated form of nitric oxide, NO. HNO has garnered much interest because it demonstrates a physiological reactivity distinctly different from its congener nitric oxide (4, 5). In a series of papers, we have reported the preparation and characterization of the HNO adduct of myoglobin (HNO-Mb) by reduction of NO-Mb (6, 7) and by trapping of free HNO by deoxymyoglobin (Mb-Fe^{II}) (8) or the reaction of metmyoglobin with nitrite and borohydride (9). Like the oxyadduct of myoglobin, HNO-Mb is diamagnetic and is uniquely characterized by a ^{1}H NMR signal due to the N-bound hydride found at *ca.* 15 ppm, well away from other protein resonances, which allowed structural characterization of the heme pocket by 2D NOE and COSY experiments (10). Other ferrous globins also rapidly trap free HNO to directly yield stable HNO adducts, identifiable by changes in electronic absorbances as well as unique ^{1}H and ^{15}N NMR resonances due to the nitrosyl hydride (11).

The anionic form of nitroxyl is isoelectronic with dioxygen and exists as a triplet, $^3NO^-$, in aqueous solutions above pH 12; at neutral pH, the protonated singlet ^{1}HNO dominates (12). HNO does not accumulate in aqueous solutions due its rapid dimerization producing N_2O between pH 2 to 11 (Eq. **1**) (13). Thus HNO cannot be stored as reactant nor can it be identified by direct methods; it is typically generated in situ via precursor compounds that decompose to release HNO (14). By far the most widely used precursor is Angeli's salt (sodium trioxodinitrate/$Na_2N_2O_3$), AS, which decomposes to give HNO at pH 4–8 (Eq. **2**). Other widely used precursors are the various alkylsulfohydroxamic acids, e.g., benzylsulfohydroxamic acid or Piloty's acid, which generate HNO upon deprotonation at a high pH (Eq. **3**) (15).

$$2HNO \rightarrow [H_2N_2O_2] \rightarrow N_2O_{(g)} + H_2O, \qquad [1]$$

$$HN_2O_3^- \leftrightarrows HNO + NO_2^- \qquad \text{pH 4–8}, \qquad [2]$$

$$RSO_2NHO^- \leftrightarrows RSO_2^- + HNO \qquad \text{pH 8–13}. \qquad [3]$$

Much recent interest in HNO-releasing compounds results from their potential pharmacological use for cardiovascular disorders (16–18), cancer (19), and alcoholism (20, 21). But the significance of "free" HNO in nature is as yet uncertain (22). Although several biological pathways for HNO formation have been suggested, e.g., via nitrosothiol decomposition (23, 24) or enzymatic malfunction (25–27), no true physiological role for endogenous HNO has been accepted.

Following our previous characterization of the trapping of free HNO by various O_2-binding globins (11), we hypothesized that HNO might similarly interact with O_2-dependent oxygenases; we herein report the unprecedented substitution of HNO for O_2 in the activity of Mn-QDO, resulting in the incorporation of the

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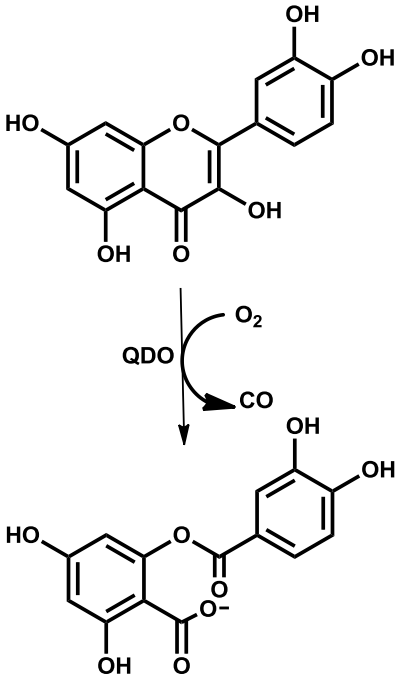

Fig. 1. Schematic of QDO dioxygenase activity.

nitroxyl-derived N atom into the product, which we denote as nitroxygenase activity.

Results

In QDO oxygenase activity assays, *ca.* 50 μL of 1.1 mM substrate quercetin in DMSO is mixed with 950 μL of aerated 50 mM Tris buffer at pH 7.5; the assay is initiated by the addition of *ca.* 10 μM of enzyme (*SI Text, Enzyme Expression and Purification*), and the loss of substrate concentration is followed at its absorbance at 380 nm. In analogous anaerobic mixtures containing quercetin and QDO, there is no observable change in the substrate concentration. But addition of the HNO-donor AS to such solution leads to a sequential loss of the substrate absorbance due to nitroxygenase activity at a rate close to that of HNO release by AS (*SI Text, Nitroxygenase Assay and Control Experiments*). Analogous experiments using other nitrogenous reagents such as nitrite and hydroxylamine show no comparable reactivity.

In a typical nitroxygenase assay, solutions of substrate and enzyme are mixed in deaerated pH 7 inorganic phosphate (IP) buffer to give desired concentrations (e.g., 13.7 μM quercetin and 8.5 μM Mn-QDO); the reaction is initiated by the addition of a concentrated stock solution of HNO precursor (137 μM AS) and the loss of substrate concentration is followed by the loss of its absorbance at 380 nm. The quercetin is consumed within an hour, and the resulting reaction mixture was analyzed by liquid chromatography (LC)-MS to identify products (*SI Text, Liquid Chromatography-Electrospray Ionization MS Analysis of Assay Products*). Negative ion LC-MS analysis of the resulting product mixtures shows complete conversion of quercetin (301 m/z) to a new dominant product ion (134 m/z) that matches a $C_7H_4O_2N^-$ anion; LC-MS retention time and fragmentation pattern is consistent with 3,4-dihydroxybenzonitrile, as confirmed with an authentic sample of the product. The use of ^{15}N-labeled AS (specifically $[O^{15}N^{14}NO_2]^{-2}$, *SI Text, Synthesis and characterization of ^{15}N-labeled Angeli's Salt*) (28) in analogous anaerobic assays results in ^{15}N-labeled product with 135 m/z peak observable in the LC-MS (Fig. 2). Generation of CO during these reactions was confirmed by the conversion of a solution of deoxymyoglobin to its ferrous CO adduct upon exposure to the head gas above the

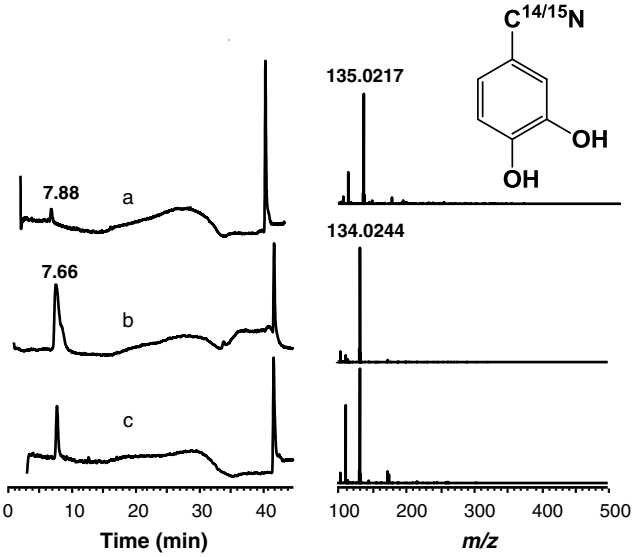

Fig. 2. Total ion chromatogram (*Left*) and mass spectra (*Right*) corresponding to the initial LC peak obtained in analysis of product mixtures from the reactions of (*A*) labeled $Na_2{}^{15}NONO_2$ and (*B*) natural abundance $Na_2N_2O_3$ with quercetin (**1**). (*C*) Chromatogram and mass spectrum obtained using authentic 3,4 dihydroxy benzonitrile (**6**).

assay mixture (*SI Text, Confirmation of CO Formation by Trapping with Deoxy-Myoglobin*).

Further confirmation of the nitroxygenase activity was obtained by assays utilizing the analogous flavonol substrates quercetin-5-methoxy and quercetin-5-sulfonic acid, compounds 2 and 3 in Fig. 3. Analysis of the resulting product mixtures by LC-MS showed similar major ion peaks consistent with 3,4-dihydroxy-5-methoxy benzonitrile and 3,4-dihydroxy-5-sulfonic benzonitrile products (*SI Text, LC-MS of Products of Nitroxygenase Mn-QDO Assay with Modified Quercetin Derivatives Quercetin-5-Sulfonic Acid and Quercetin-5-Methoxy*), which were not seen in control experiments of anaerobic reactivity of these 5-substituted quercetin derivatives with AS in the absence of Mn-QDO at pH 7.

Initial kinetic analysis was performed and analyzed using the sequential reactions depicted in Eqs. **4**–**7**. Rate analysis of the nitroxygenase reactivity is complicated by the slow decomposition rate of the HNO-donor AS and the competitive dimerization of free HNO (29).* The slow release of HNO from AS would be predicted as the rate-limiting step, but at relatively low substrate concentrations, the initial rates of reaction show dependence on both [QDO] and [AS], implying the catalytic turnover and dimerization are competitive in this regime (*SI Text, Rates of Kinetic Runs of Nitroxygenase Activity Under Varying Conditions*). Previous mechanistic studies of Mn-QDO have suggested that formation of a quercetin/QDO complex, $K_M = 4$ μM, precedes reaction with dioxygen (3). If quantitative quercetin/QDO complex formation is assumed at 100-fold excess substrate to enzyme, modeling of the rate of reaction under these conditions gives a rate constant of 3.3×10^4 M^{-1} s^{-1} for this pseudobimolecular reaction (Eq. **7**) (*SI Text, Kinetic Modeling of Nitroxygenase Reaction Sequence*).

$$\mathrm{AS} \rightarrow \mathrm{HNO} \qquad k_1 = 6.75 \times 10^{-4}\ \mathrm{s}^{-1}, \qquad \textbf{[4]}$$

$$\mathrm{HNO} + \mathrm{HNO} \rightarrow \mathrm{N_2O} + \mathrm{H_2O} \qquad k_d = 8.0 \times 10^6\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}, \qquad \textbf{[5]}$$

*A reviewer has pointed out that reliance on the decomposition rates of AS as a measure of HNO production is a major assumption in our kinetic analysis, as certain reports suggest that HNO production may not necessarily be constant in the pH 4–8 range.

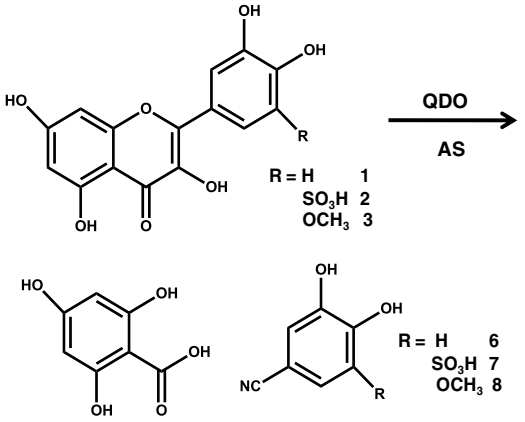

Fig. 3. Schematic of Mn-QDO nitroxygenase activity substrates and products.

$$\text{quercetin} + \text{QDO} \leftrightarrows \text{quercetin/QDO complex} \qquad K_M = 4\ \mu\text{M}, \quad \textbf{[6]}$$

$$\text{quercetin/QDO complex} + \text{HNO} \rightarrow \text{product} + \text{QDO}$$
$$k = 3.3 \times 10^4\ \text{M}^{-1}\,\text{s}^{-1}. \quad \textbf{[7]}$$

The dioxygenase activity of Mn-QDO has been shown to have distinctive pH dependence between pH 5.5 and 8, with a maximum rate of *ca.* pH 7.0 (3). The pH dependence of the nitroxygenase activity was investigated from pH 5 to 8, shown as the plot in Fig. 4. For comparison, the decomposition rate of AS under the experimental conditions is also plotted, essentially constant from pH 4 to 8 (30). Above pH 7, a nonenzymatic consumption of quercetin is observed; correcting for this background reactivity demonstrates that the pH dependence of the Mn-QDO nitroxygenase catalysis mimics that of its oxygenase activity.

A nonenzymatic, basal reactivity of AS with quercetin initiates above pH 7 (*SI Text, Nonenzymatic Reaction of HNO With Quercetin*). LC-MS analysis of the product mixtures obtained a large product peak consistent with 3,4 dihydroxybenzonitrile (compound 6 in Fig. 3), therefore retaining the regioselectivity of the enzymatic reaction. The observed rates of the nonenzymatic reaction become competitive to the enzymatic rates above pH 7.5.

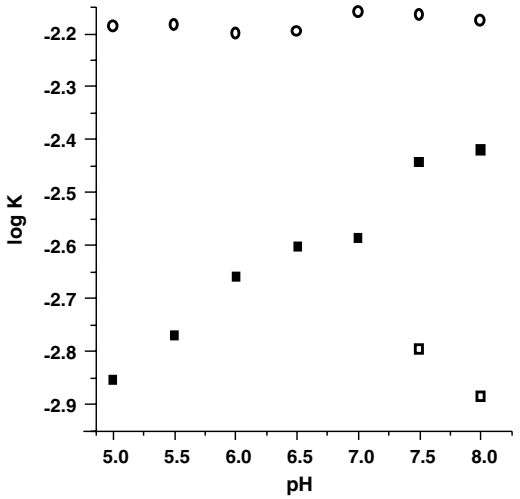

Fig. 4. A comparative plot of effect of pH on rate of decomposition of Angeli's salt (○); loss of quercetin absorbance at 380 nm during reaction with Angeli's salt in the presence of Mn-QDO (■) at 1:10:100, Mn-QDO/AS/quercetin; and same after subtraction of nonenzymatic rate (□).

The analogous nonenzymatic reaction of quercetin with dioxygen has long been known to proceed at similar pH range (31). The pH and oxygen dependence of the rate of reaction supports a direct reaction of the monoanionic form of quercetin (pK_a 7.03) (32) with dioxygen, originally postulated as a simple nucleophilic attack of a carbon-centered anion on dioxygen (33). Subsequent reports have suggested an outersphere single electron transfer (SET) generates a quercetin-based C radical which may then react rapidly with dioxygen or superoxide. Both the base-catalyzed (34) and SET (35) mechanisms have been suggested in aqueous/organic mixtures. The observed nonenzymatic nitroxygenase reactivity is also consistent with a base-catalyzed mechanism; HNO is well known to undergo facile and rapid nucleophilic attack by anions such as thiolates and amides (36).

A unique feature of the Mn-QDO nitroxygenase reactivity is the regioselectivity of N-atom incorporation into the product (Fig. 5). The presumed initial product, 2-((3,4-dihydroxyphenyl)(imino)methoxy)-4,6-dihydroxybenzoate, undergoes a 1,3 proton transfer to generate the observed nitrile and phenolic products (37); this is analogous to the hydration of the initial phenolic ester seen at basic pH, resulting in carboxylic and phenolic products (33). For the observed nitroxygenase products, the N-atom addition occurs selectively at the site corresponding to that suggested for initial dioxygen reaction in previously proposed dioxygenase mechanisms—i.e., of initial nucleophilic attack by a substrate anion or coupling of the substrate and diatom radicals. The role of the metal cofactor in enhancing this reactivity may be attributed to either substrate activation (i.e., promotion of the reactive tautomeric anion of the flavonol) or to a promotion of electron transfer between the substrate and diatom, generating and orienting the radicals needed.

As previously noted, flavonol dioxygenases have been identified that act with various metal-ion cofactors (38, 39). For instance, several species of *Aspergillus* have flavonol dioxygenases with Cu cofactors (40–42); a QDO from *Streptomyces* sp. designated strain FLA (flavonol utilization) was characterized with divalent Fe, Ni, and Co cofactors (43). The QDO from *B. subtilis*, has been characterized with divalent Fe, Mn, and Co cofactors (44) and tested for activity with divalent Mg, Ni, and Cd (45). It has been suggested that exchange of the active-site metal ion may allow biological modulation of enzyme activity (45).

To test the metal-ion dependence of nitroxygenase activity, samples of Fe-QDO were expressed in *Escherichia coli* cells grown in LB medium, and Co-QDO was expressed in M9 mini-

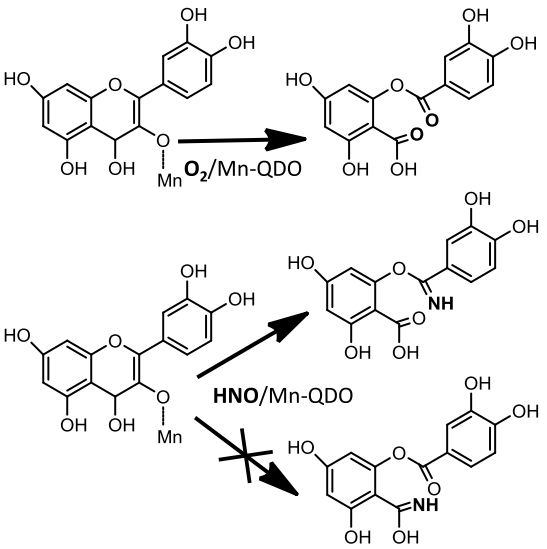

Fig. 5. Comparison of regioselectivity of QDO oxygenase and nitroxygenase reactivity.

mal medium in the presence of added Co(II) salts, analogous to the expression of Mn-QDO (44). Metal-ion incorporation in each batch was corroborated by inductively coupled plasma (ICP)-MS and atomic absorption spectrometric analysis of the reconstituted enzymes (*SI Text, Analysis of Metal Content in Metallo-QDOs by Inductively Coupled Plasma and Atomic Absorption Spectrometry*), which showed only minor odd-metal contamination of the Mn and Fe-QDO samples, with the Co-QDO sample containing *ca.* 10% Mn (Table 1).

The dioxygenase activities of the metal-substituted QDO samples were examined (Fig. 6*Upper*), which confirms the comparative values previously determined (i.e., Mn > Fe > Co) (3). In contrast, only the Mn-QDO demonstrates nitroxygenase activity under standard assay conditions at pH 5.5 (Fig. 6*Lower*) (the minor activity seen for the Co-QDO sample may be due to the *ca.* 10% Mn-QDO content).

Discussion

A continuing question regarding the native dioxygenase activity of QDO is whether dioxygen binds to the metal cofactor prior to insertion into the substrate. The unique reactivity of the Mn-QDO in the nitroxygenase activity argues against simple substrate activation by metal coordination, as the Lewis acidity of divalent Mn, Fe, and Co may be assumed to be roughly comparable (46). Under the SET hypothesis, the metal ion acts as a conduit for an internal electron transfer between the metal-bound flavonol and HNO, also orienting the resultant organic radicals to facilitate coupling.

Similar tertiary complexes have recently been crystallographically characterized for site-specific mutants of homoprotocatechuate 2,3-dioxygenase (2,3-HPCD): The substrate 4-nitrocatechol and dioxygen were found bound to the active-site Fe ion in several distinct intermediate states (47). EPR and Mossbauer spectroscopy characterization of both the Fe- and Mn-substituted 2,3-HPCD provide evidence of M^{III}-radical intermediates, which suggests sequential oxidation and reduction of the metal cofactor may take place during such dioxygenase activity (48, 49).

Fig. 7 display possible reaction pathways for both the dioxygenase and nitroxygenase activities of Mn-QDO. By analogy to the 2,3-HPCD mechanistic studies (47–49), the Mn-QDO dioxygenase reaction is proposed to go through an initial tertiary complex, *A*, in which an initial SET has yielded an Mn^{III}-superoxide species. A second SET from substrate to metal allows subsequent radical coupling between the metal-bound superoxide and substrate. Nucleophilic attack on the ketone by the resulting alkyl-peroxide, *C*, generates a bridging dioxalane, *D*, which then decomposes with the release of CO to depside product, *E*.

The corresponding sequence for nitroxygenase activity is given below, with an initial Mn^{III}-aminoxyl radical adduct, *A′*, and subsequent intracomplex SET to generate the substrate radical, *B′*. Radical coupling would then form an alkylhydroxamate intermediate, *C′*, and nucleophilic attack would generate an isoxazolidine bridge, *D′*, which then decomposes to the parent carboximidic ester, *E′*.

The unique reactivity of the Mn-QDO suggests a metal-mediated electron transfer mechanism rather than metal-activation of the substrate's inherent base-catalyzed reactivity. There has been much discussion over the electronic nature of bonding in transition metal HNO complexes (50), for instance in the putative catalytic intermediate in nitric oxide reductase P450nor (51). An HNO-Mn^{II} species with a d^5 electronic configuration is unprecedented, as all isolable HNO-metal complexes are diamagnetic with low-spin d^6 metal ions. But there exists precedence for reduction of a metal-bound HNO in the catalytic cycles of various nitrite reductases (52–54). The singly reduced form of HNO, the aminoxyl radical anion (HNO^-), is generated by photolysis of the stable HNO adduct of deoxymyoglobin (55); and published theoretical calculations suggest that a proton-coupled oxidation HNO/Fe^{II} to H_2NO/Fe^{III} is enthalpically favorable (56, 57).

A second related issue is the radical coupling step. Previously proposed dioxygenase mechanisms contend that the pendant beta-oxygen atom of the metal-bound superoxide (47), *B* in Fig. 7, couples to the organic radical. But the observed regioselectivity of the nitroxygenase reactivity demands that an N radical, the alpha-atom attached to the metal in *B′*, couples to the substrate radical. The HNO adduct is depicted as N bound, as found in characterized end-on HNO-metal complexes (58). There is much recent evidence for O-bound linkage isomers of metal-nitrosyls, termed isonitrosyls, but these are typically only observed after photolysis at low temperature within a solid or crystalline material (59–62). The analogous O-bound HNO, isonitroxyl, has never been characterized to our knowledge; theorectical calculations have predicted the O-bound isonitroxyl adduct of Fe porphyrinates would be over 50 kJ/mol higher in energy than the corresponding N-bound nitroxyl (63).

Table 1. ICP-MS and atomic absorption spectrometric metal analysis of metallo-QDOs

QDO	% metal content		
	Mn	Fe	Co
Mn	95.34	4.66	ND
Fe	0.83	99.17	ND
Co	10.40	2.32	87.28

ND, not detected.

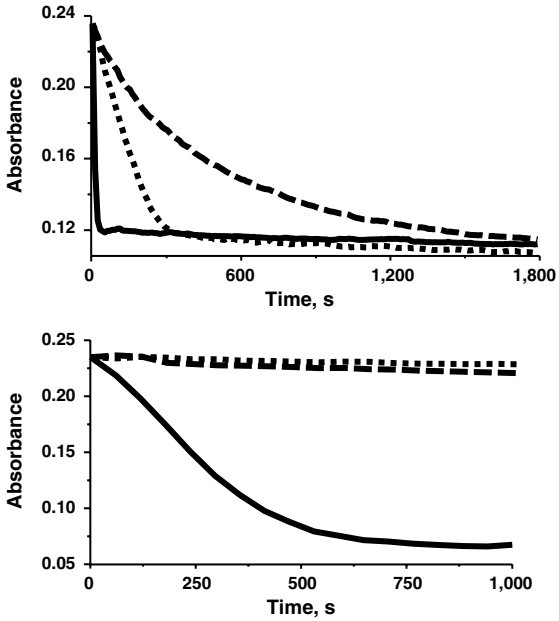

Fig. 6. Normalized time course absorbance plots of catalytic reaction of metal-substituted QDOs, as followed by loss of quercetin absorbance at 380 nm in IP buffer pH 6.5. (*Upper*) Dioxygenase assays in aerobic solutions (130 μM O_2): Co-QDO (2.85 μM, [Q]: 14.25 μM, dashed), Fe-QDO (2.48 μM, [Q]: 12.42 μM, dotted), and Mn-QDO (2.31 μM, [Q]: 11.55 μM, solid). (*Lower*) Nitroxygenase assays in anaerobic solutions: Co-QDO (2.98 μM, [Q]: 14.90 μM, [AS]: 148 μM, dashed), Fe-QDO (2.85 μM, [Q]: 14.2 μM, [AS]: 140 μM, dotted), and Mn-QDO (3.73 μM, [Q]: 18.63 μM, [AS]: 184 μM, solid).

Materials and Methods

AS was purchased from Cayman chemicals and used as received. Quercetin and 3,4-dihydroxy benzonitrile were purchased from Tokyo Chemical Industry America; quercetin-5-sulfonic acid and quercetin-5-methoxy were purchased from Indofine Chemical Company. Metal-substituted QDOs were prepared by growing *E. coli* BL21 (DE3) carrying plasmid pQuer4, as

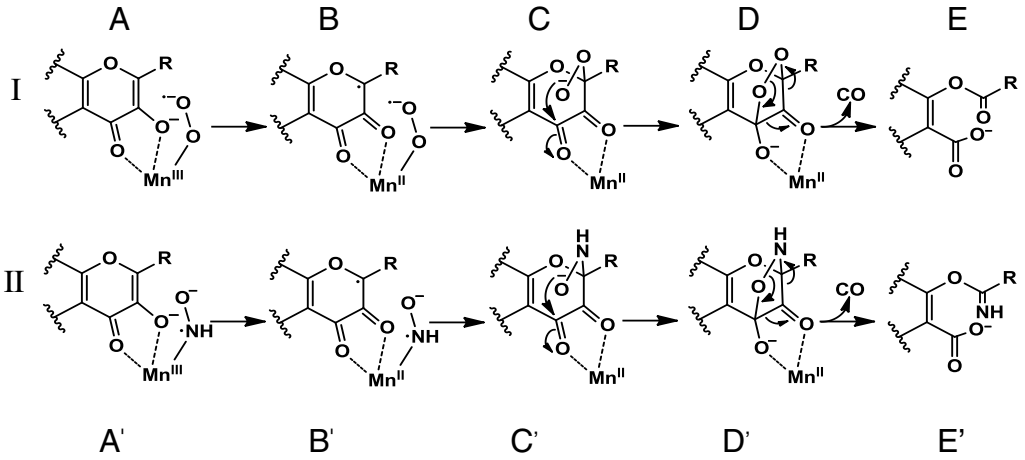

Fig. 7. Proposed reaction pathway for Mn-QDO catalyzed dioxygenase (I) and nitroxygenase (II) reactivities, as described in the text.

previously described (3). Specific data on the metal analysis of metal-substituted QDOs by ICP and atomic absorption spectrometry is available in *SI Text, Analysis of Metal Content in Metallo-QDOs by Inductively Coupled Plasma and Atomic Absorption Spectrometry*. LC-electrospray ionization MS analysis of assay products were analyzed on an Accela liquid chromatograph coupled to a Linear Trap Quadrupole Orbitrap Discovery mass spectrometer, procedures given in *SI Text, Liquid Chromatography-Electrospray Ionization MS Analysis of Assay Products*. Unless otherwise stated, all reaction solutions were prepared inside an anaerobic glove box.

Assays of Mn-QDO Nitroxygenase Activity. 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 assay, 13.7 μM quercetin is mixed with 8.5 μM of enzyme in deareated IP buffer at pH 7; the reaction is initiated by the addition of a stock AS solution to give final concentration of 137 μM. The reaction was initiated by gently shaking the cuvette before placing it in the spectrometer, followed by observing the loss of absorbance at 380 nm.

The Nonenzymatic Reaction of HNO with Quercetin. HNO-precursor AS (1.3 μM) was added to IP buffer at appropriate pH containing quercetin (0.13 μM). The reaction was followed by observing the loss of absorbance at 380 nm.

Dioxygenase Assays Using Metal-Substituted QDOs. In a typical experiment, quercetin and enzyme was added to the 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.

Nitroxygenase Assays Using Metal-Substituted QDOs. The nitroxygenase assays utilized of AS with the enzyme Co- and Fe-QDOs and quercetin were carried out in IP buffer at a range of pH. No significant losses of quercetin absorbance were observed under conditions studied.

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