A Flavo-Diiron Protein from *Desulfovibrio vulgaris* with Oxidase and Nitric Oxide Reductase Activities. Evidence for an in Vivo Nitric Oxide Scavenging Function†

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ABSTRACT: A few members of a widespread class of bacterial and archaeal flavo-diiron proteins, dubbed FprAs, have been shown to function as either oxidases (dioxygen reductases) or scavenging nitric oxide reductases, but the questions of which of these functions dominates in vivo for a given FprA and whether all FprAs function as oxidases or nitric oxide reductases remain to be clarified. To address these questions, an FprA has been characterized from the anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris*. The gene encoding this *D. vulgaris* FprA lies downstream of an operon encoding superoxide reductase and rubredoxin, consistent with an O_2-scavenging oxidase function for this FprA. The recombinant *D. vulgaris* FprA can indeed serve as the terminal component of an NADH oxidase. However, this oxidase turnover results in irreversible inactivation of the enzyme. On the other hand, the recombinant *D. vulgaris* FprA shows robust anaerobic nitric oxide reductase activity in vitro and also protects a nitric oxide-sensitive *Escherichia coli* strain against exposure to exogenous nitric oxide. It is, therefore, proposed that this *D. vulgaris* FprA functions as a scavenging nitric oxide reductase in vivo and that this activity protects *D. vulgaris* against anaerobic exposure to nitric oxide. The location of a gene encoding a second FprA homologue in the *D. vulgaris* genome also suggests its involvement in nitrogen oxide metabolism.

Several members of an apparently widespread class of bacterial and archaeal proteins, called FprA[1] (or A-type flavoprotein), harbor a novel type of active site featuring a flavin in close contact with a non-heme, non-sulfur diiron site contributed from flavodoxin-like and metallo-β-lactamase-like domains, respectively (*1*−*3*). The diiron site is ligated by histidine, glutamate, and aspartate side chains, but FprAs otherwise bear no apparent relationship to other diiron proteins. A lactam binding region in FprA is also not apparent. An FprA homologue in the anaerobic sulfate-reducing bacterium *Desulfovibrio gigas*, named *r*ubredoxin: *o*xygen *o*xidoreductase (ROO), was proposed to function as a dioxygen reductase (O_2R) (*2*, *4*, *5*). More recently, in vivo evidence for a nitric oxide reductase (NOR) function was obtained for the FprA homologue, referred to as flavorubredoxin, from the facultative anaerobic bacterium *Escherichia coli* (*6*). This nonrespiratory, scavenging NOR activity catalyzes reduction of NO to N_2O, thereby protecting *E. coli* against "nitrosative stress" under anaerobic growth conditions. Thus, expression of flavorubredoxin was induced by anaerobic exposure of *E. coli* to nitric oxide, flavorubredoxin knockout *E. coli* strains were more sensitive than wild-type strains to anaerobic nitric oxide exposure, and expression of plasmid-borne flavorubredoxin restored nitric oxide resistance (*6*, *7*). *E. coli* flavorubredoxin was reported to show both NOR and O_2R activities in vitro (*8*, *9*). A five-gene cluster in the anaerobic, acetogenic bacterium *Moorella thermoacetica* encodes an FprA together with oxidative stress protection proteins (*10*). While this gene clustering is consistent with an O_2-scavenging function, we subsequently demonstrated efficient anaerobic scavenging NOR activity both in vitro and in vivo for *M. thermoacetica* FprA (*11*). Implication of NOR activity for an FprA in a pathogenic protist has also been reported (*12*).

The ∼100 FprA homologues in the NCBI database (www.ncbi.nlm.nih.gov) are found predominantly in air-sensitive (i.e., anaerobic or microaerophilic) microorganisms, often in multiple genomic copies. Unlike their NOR activity, aerobic O_2R turnover of both the *E. coli* flavorubredoxin and *M. thermoacetica* FprA resulted in irreversible inactivation (*6*, *11*), perhaps suggesting that NOR is the true function of FprAs. However, the generality of the anaerobic NOR function for FprAs has not been established. *D. gigas* ROO, for example, has not been reported to show NOR activity. On the basis of its homology to *D. gigas* ROO, an FprA homologue from the closely related anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris*, was proposed to function as an O_2-scavenging ROO (*13*). This *D. vulgaris* FprA gene is found ∼130 base pairs downstream of an *rbo-rub* operon, which encodes a superoxide reductase (*rbo*) and rubredoxin (*rub*) (*14*). Both of these latter proteins are known to be involved in oxidative stress defense (*15*−*17*). This situation is reminiscent of that in *M. thermoacetica*, where

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[1] Abbreviations: FprA, flavo-diiron protein; NOR, nitric oxide reductase; O_2R, dioxygen reductase; ROO, rubredoxin:oxygen oxidoreductase; Hrb, high molecular weight rubredoxin (NADH:FprA oxidoreductase); flavo-Hrb, truncated Hrb lacking the C-terminal rubredoxin domain and containing only FMN as a cofactor; MOPS, *N*-morpholinoethanesulfonic acid; IPTG, isopropyl β-D-thiogalactoside; ccNIR, cytochrome *c* nitrite reductase.

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the aforementioned five-gene cluster encoding FprA also encodes its reductase, Hrb (*h*igh molecular weight *rub*redoxin), rubredoxin, superoxide reductase, and rubrerythrin (another protein known to be involved in oxidative stress defense) (*10*, *15*, *16*). Reported here are recombinant expression, isolation, and functional characterization of the FprA that is encoded downstream of the *rbo-rub* operon in *D. vulgaris*.

MATERIALS AND METHODS

Reagents and General Procedures. All solutions were prepared in deionized water. DNA restriction and ligating enzymes were obtained from New England Biolabs, Inc. NADH (Sigma Chemical Co.) and protein molecular weight standards (Bio-Rad, Inc.) were used without further purification. Molecular biology manipulations followed standard protocols (*18*). Recombinant *D. vulgaris* rubredoxin, *M. thermoacetica* Hrb, and *M. thermoacetica* FprA were obtained as previously described (*11*, *15*). Where indicated, solutions were made anaerobic by repetitive vacuum/argon or N_2 gas exchange or by extensive purging with argon or N_2 gas via syringe needles inserted through tight-fitting rubber septa. Protein purity was judged by SDS−PAGE (15% polyacrylamide gels) and Coomassie blue staining (*19*). Western blotting followed a standard procedure (*20*). Chromosomal DNA of *D. vulgaris* and plasmid DNA were isolated and purified using a QIAamp DNA mini kit and QIAprep spin miniprep kit (Qiagen Inc.), respectively. ESI protein mass spectrometry was performed at the University of Georgia Chemical and Biological Sciences Mass Spectrometry Facility on a Perkin-Elmer Sciex APII plus quadrupole mass spectrometer. N-Terminal amino acid sequencing and DNA sequencing were carried out in the University of Georgia Integrated Biotechnology Laboratories. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Inc.) with bovine serum albumin as the standard. Metal contents of FprA were determined by inductively coupled plasma-atomic emission analysis at the University of Georgia Chemical Analysis Facility. Flavin was identified and quantitated as described previously (*11*). Antibodies against the purified recombinant *D. vulgaris* FprA were raised in rabbits at the Animal Care and Use Facility at the University of Georgia and purified from serum by a standard procedure (*21*). Ultraviolet−visible absorption spectra and absorbance time courses were measured in 1 cm path length quartz cuvettes on a Shimadzu UV-2401PC scanning spectrophotometer.

D. vulgaris FprA Cloning, Overexpression, and Purification. Anaerobic growth of *D. vulgaris* (Hildenborough) at 37 °C in Postgate medium C (*22*) and preparation of cell extracts were carried out as described previously (*16*). N- and C-terminal PCR primers, DvFprAN [5′-acgacagccatATGCATCCTATCGAAATC-3′] and DvFprAC [5′-taatggatccCTAACCCCCCGCACTTTTC-3′] (with coding regions in capital letters) incorporating *Nde*I and *Bam*HI restriction sites (underlined), respectively, were used for PCR amplification and subsequent cloning of the FprA gene [genome locus ID DVU3185 (*13*)] downstream of the *rbo-rub* operon from *D. vulgaris* genomic DNA into both pET-(21b+) (Novagen, Inc.) and pCYB1 (New England Biolabs, Inc.), generating the respective plasmids pET-DvFprA (without the His tag) and pCYB1-DvFprA. Overexpression in *E. coli* and purification of DvFprA2 were identical to that described for *M. thermoacetica* FprA (*11*). The pET-DvFprA (non-His-tagged) plasmid was typically used for overexpression. The molar absorptivity of purified *D. vulgaris* FprA at 470 nm was determined from the protein and flavin quantitations, carried out as described above, and this molar absorptivity was subsequently used to calculate FprA concentrations from measured absorbances.

Hrb and Flavo-Hrb Expression and Characterization. Recombinant *M. thermoacetica* Hrb was expressed, purified, and handled under low-temperature (0−4 °C), low-light conditions, as previously described, and Hrb concentrations were determined using a published molar absorptivity (*11*). A variant of Hrb, labeled flavo-Hrb, could be purified using the identical procedure except taking no precautions to avoid exposure to light and if all manipulations were carried out at room temperature. Flavo-Hrb was verified by SDS−PAGE, UV−vis absorption, mass spectrometry, and N-terminal amino acid sequencing to consist of a truncated Hrb, lacking the C-terminal rubredoxin domain and containing only FMN as a cofactor. The N-terminal amino acid sequence, MDTKALHTLTYGLXT, determined for the flavo-Hrb was identical to that of Hrb (cf. NCBI accession number AAG00803), and the protein molecular mass of 17828 Da (determined by ESI mass spectrometry) indicated cleavage after residue T164. Flavo-Hrb did not catalyze FprA reduction by NADH but proved to be a convenient catalyst for reduction of *D. vulgaris* rubredoxin by NADH (albeit at rates too slow to support a measurable NADH:FprA oxidoreductase activity). Nevertheless, the so-produced reduced rubredoxin, when anaerobically added in excess, served as an efficient electron donor to FprA for catalytic NOR activity.

Oxidase and NOR Assays. All assays were conducted at room temperature in 50 mM MOPS, pH 7.0. NADH-dependent oxidase (O_2-consumption) assays were monitored in air-saturated buffer by the rate of decreasing absorbance at 340 nm due to O_2-dependent NADH oxidation. For NOR assays, nitric oxide from saturated (∼1.8 mM) aqueous stock solutions was delivered via a gastight syringe to anaerobic assay mixtures. The saturated stock solutions were prepared by bubbling gaseous nitric oxide through anaerobic deionized water for 15 min. The gaseous nitric oxide (98.5%) (Aldrich) had been prepurified by bubbling through 100 mL of a 10% KOH solution. NO-dependent NADH consumption was measured anaerobically by monitoring the decrease in absorbance at 340 nm. Alternatively, the NADH-dependent NOR activity was monitored by measuring the rate of decrease in NO concentration with a commercially available Clark-type NO-sensitive electrode (2 mm diameter) and chamber (WPI, Inc.), as described previously (*11*). Further details, including component concentrations, are provided in the figure legends. All protein concentrations are expressed on a monomer basis.

FprA Complementation of E. coli Growth Sensitivity to NO. *E. coli* strain AG300, containing a disrupted flavorubredoxin gene, was kindly provided by Paul R. Gardner (*6*). The anaerobic growth and complementation studies were carried out as described previously for recombinant *M. thermoacetica FprA* (*11*) using *E. coli* AG300 that had been transformed with either pCYB1 or pCYB1-DvFprA. Reported results were obtained with 100 mL cultures maintained

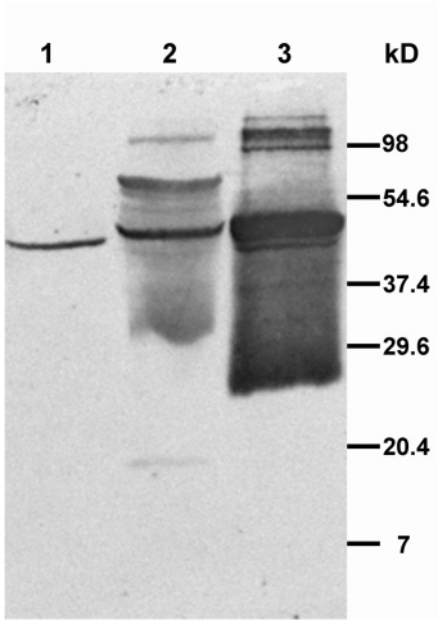

FIGURE 1: Western blot probed with antibodies raised against the recombinant *D. vulgaris* FprA. Lanes: 1, purified recombinant *D. vulgaris* FprA; 2, *D. vulgaris* cell extract; 3, purified recombinant *M. thermoacetica* FprA. Molecular mass marker positions are indicated.

at 37 °C. Isopropyl β-D-thiogalactoside (IPTG) (0.05 mg/L) was anaerobically added to the cultures at OD_{550nm} ~0.1. Where required, nitric oxide was anaerobically delivered via a gastight syringe from a saturated aqueous stock solution (prepared as described for the enzyme assays) to the 100 mL cultures 60 min after IPTG addition to give an initial nitric oxide concentration of 7.5 μM. Optical densities were measured by removing 1 mL aliquots of medium from the culture bottles, using sterile disposable 1 mL syringes.

RESULTS

Throughout this section, all protein concentrations are expressed on a monomer basis.

Expression of FprA in D. vulgaris. Western blots of *D. vulgaris* cell extracts using antibodies raised against the recombinant *D. vulgaris* FprA (cf. Figure 1) show that this protein is constitutively expressed in *D. vulgaris* under standard laboratory anaerobic growth conditions using lactate as electron donor and sulfate as electron acceptor. The effect of nitric oxide or dioxygen on the level of FprA expression in *D. vulgaris* has not been reported and was not investigated in this work.

FprA Overexpression, Purification, and Physicochemical Properties. Recombinant *D. vulgaris* FprA was readily overexpressed in and isolated from *E. coli* under the same conditions and with similar yields as for *M. thermoacetica* FprA (*11*). Also paralleling the *M. thermoacetica* FprA, the *D. vulgaris* FprA was shown by gel filtration to be a homodimer in solution, whereas SDS−PAGE showed a monomer of molecular mass ~45 kDa, consistent with that calculated from the amino acid sequence (45079 Da). The *D. vulgaris* FprA copurified with substoichiometric heme, a not uncommon occurrence for heterologously expressed proteins in *E. coli*. Extensive efforts were made to characterize what turned out to be contaminating hemes in *D. gigas* ROO (*4*, *5*, *23*); the *D. gigas* ROO crystal structure revealed

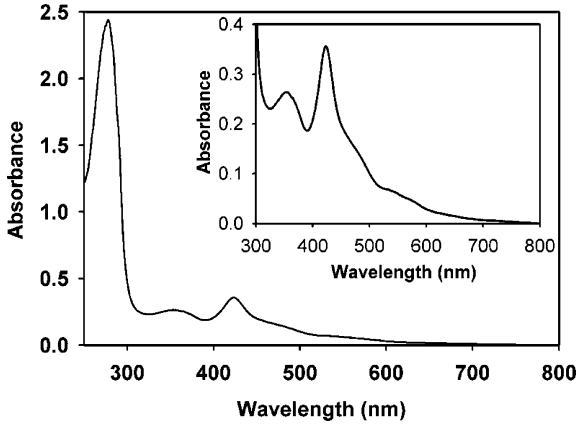

FIGURE 2: UV−vis absorption spectrum of as-isolated recombinant *D. vulgaris* FprA in aerobic 50 mM MOPS, pH 7.3.

no heme or obvious heme binding site (*2*). At least five other FprAs have been found to function as NORs and/or O_2Rs in the absence of any detectable heme content (*8*, *9*, *11*, *24*−*26*). We, therefore, conclude that the heme copurifying with recombinant *D. vulgaris* FprA is a contaminant. Attempts to remove the heme contaminant from the recombinant *D. vulgaris* FprA preparations included anion/cation-exchange and hydrophobic chromatographies, ammonium sulfate precipitation, and mild acidic or basic treatment. None of these treatments succeeded in removing all of the heme. Figure 2 shows the UV−vis absorption spectrum of as-isolated recombinant *D. vulgaris* FprA, which is dominated [as in the case of *D. gigas* ROO (*4*, *5*)] by heme and flavin absorptions. Following heat denaturation to release the protein-bound flavin, an extinction coefficient of 12 (mM FprA monomer)$^{-1}$ cm^{-1} was determined at 470 nm, where the flavin absorption predominates. This value is identical to that of *M. thermoacetica* FprA (*11*). Metal and flavin analyses confirmed a cofactor content of 0.7−0.9 FMN and 1.7−1.9 iron per *D. vulgaris* FprA monomer in multiple preparations. Assuming $\epsilon_{420} = 100$ mM^{-1} cm^{-1} for the heme contaminant, the estimated mol ratio of heme/flavin was ~0.3.

O_2R and NOR Activities. The recombinant *D. vulgaris* FprA exhibited both O_2R and anaerobic NOR activities. Panels A and B of Figure 3 show traces comparing NO- and O_2-dependent NADH consumption rates, respectively, of *D. vulgaris* FprA vs *M. thermoacetica* FprA using *M. thermoacetica* Hrb as the NADH:FprA oxidoreductase. For NOR activity (Figure 3, panel A) the NADH concentration decreased linearly with time (over periods where the NADH concentration was not rate-limiting), whereas for O_2R activity (Figure 3, panel B) this decrease was nonlinear and indicative of a progressive decrease in the rate of NADH consumption. Thus, *D. vulgaris* FprA, similarly to *M. thermoacetica* FprA (*11*), became inactivated when turning over as O_2R but not when turning over as anaerobic NOR.

The gene for the *D. vulgaris* FprA is just downstream of that encoding rubredoxin (*13*), suggesting that rubredoxin serves as a proximal electron donor to this *D. vulgaris* FprA in vivo. Consistent with this suggestion, Figure 4, panel A, shows that the reduced *D. vulgaris* rubredoxin−*D. vulgaris* FprA combination functions as an NOR with a K_m for the rubredoxin−FprA interaction of ~8 μM. For comparison, the K_m for the *M. thermoacetica* Hrb−FprA interaction [the

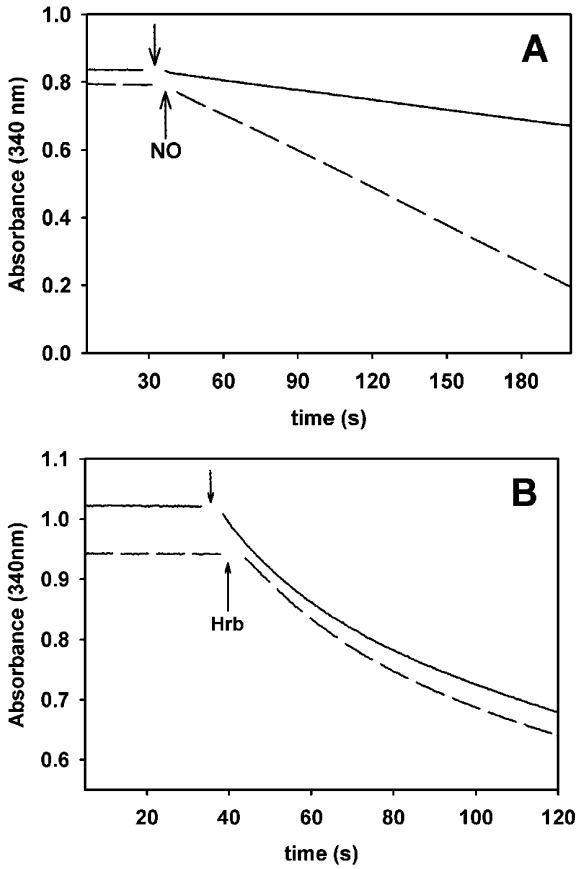

FIGURE 3: NADH:NO oxidoreductase (panel A) and NADH:O_2 oxidoreductase (panel B) activity of recombinant *D. vulgaris* FprA (solid traces) or *M. thermoacetica* FprA (dashed traces) monitored as the time courses of NADH consumption (ΔA_{340}) in 50 mM MOPS, pH 7.0 (anaerobic for panel A or air-saturated for panel B) at room temperature. Concentrations for panel A were 1 μM FprAs, 1 μM *M. thermoacetica* Hrb, and 160 μM NADH. Nitric oxide (100 μM) was anaerobically added from an aqueous stock solution at the times indicated by the arrows. Concentrations for panel B were 0.7 μM FprAs, 0.8 μM Hrb, and 160 μM NADH. Reactions were initiated by adding Hrb (which showed no O_2R activity on this scale) at the times indicated by the arrows.

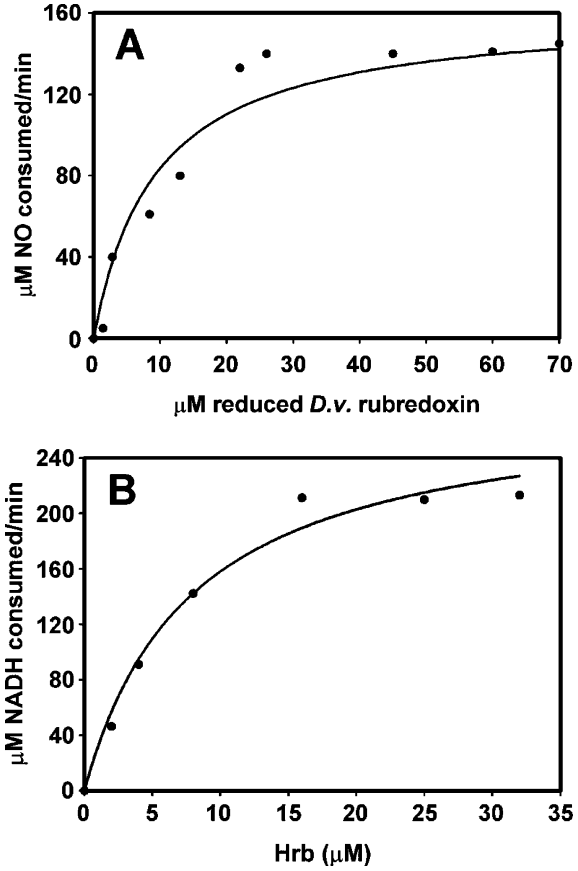

FIGURE 4: Michaelis−Menten plots of NADH:NO oxidoreductase activity illustrating the reduced *D. vulgaris* rubredoxin−*D. vulgaris* FprA interaction (panel A) and the *D. vulgaris* FprA−*M. thermoacetica* Hrb interaction (panel B) in anaerobic 50 mM MOPS, pH 7.0 at room temperature. For panel A, appropriate volumes of a 450 μM stock solution of reduced rubredoxin (prepared by anaerobic reductive titration with NADH in the presence of 1 μM flavo-Hrb) were added to an anaerobic NO chamber, which contained 120 nM recombinant *D. vulgaris* FprA and 50 μM nitric oxide. Nitric oxide consumption rates were monitored using an NO electrode and are plotted as initial rates. On the time scale of these experiments negligible nitric oxide consumption was observed when FprA was omitted from the assay mixture. The fit (solid trace) to the data (filled circles) was obtained using the parameters $K_m = 8$ μM and $k_{cat} = 22$ s^{-1}. For panel B, the reaction mixtures contained 250 nM *D. vulgaris* FprA, 160 μM NADH, 100 μM NO, and the indicated concentrations of *M. thermoacetica* Hrb. Reactions were initiated by adding FprA from a concentrated stock solution. Initial rates of NADH consumption (from ΔA_{340}) are plotted. The fit (solid trace) to the data (filled circles) was obtained using the parameters $K_m = 8$ μM and $k_{cat} = 19$ s^{-1}.

genes for which are known to be cotranscribed (*10*)] is only slightly lower, ~2 μM (*11*).

Figure 4, panel B, shows that the recombinant *D. vulgaris* FprA−*M. thermoacetica* Hrb interaction during NOR turnover features a similarly low K_m (~8 μM) as for the *D. vulgaris* FprA−rubredoxin combination. The similar K_ms presumably reflect the strong amino acid sequence and, therefore, structural similarity between the Hrb rubredoxin domain and the *D. vulgaris* rubredoxin. *M. thermoacetica* Hrb could, therefore, be conveniently used as an NADH:*D. vulgaris* FprA oxidoreductase in a *saturating* manner.

Figure 5 illustrates Michaelis−Menten plots for the O_2R and NOR activities of *D. vulgaris* FprA in the presence of saturating *M. thermoacetica* Hrb. Table 1 lists the resulting kinetic parameters, along with those previously reported for other FprAs. The *D. vulgaris* FprA K_m for dioxygen is the same within experimental uncertainty as that of *M. thermoacetica* FprA. On the other hand, the *D. vulgaris* FprA K_m for NO is ~5 times higher than that measured for *M. thermoacetica* FprA, and the k_{cat}/K_m values for the *D. vulgaris* FprA NOR and O_2 reductase activities are essentially identical to each other.

An improved fit for the *D. vulgaris* FprA NOR activity profile in Figure 5 was obtained by adopting a kinetic scheme (Scheme 1) previously used to analyze *M. thermoacetica* FprA NOR turnover kinetics (*11*), which was itself adapted from that used for bacterial respiratory NORs that contain a binuclear heme−non-heme iron active site (*27*). According to this scheme, at saturating levels of NADH and Hrb, reduction of two NO to N_2O occurs at a single reduced FprA active site (E_{red}).

The rate law applying to Scheme 1 is $v = (V_{max}[NO]^2)/(K_1K_2 + K_2[NO] + [NO]^2)$, where v is the initial velocity (*28*).[2] Fits of this rate law to the NOR data plotted in Figure

[2] This rate law was incorrectly transcribed in ref *11*, but the correct rate law, as listed here, was fitted to those results.

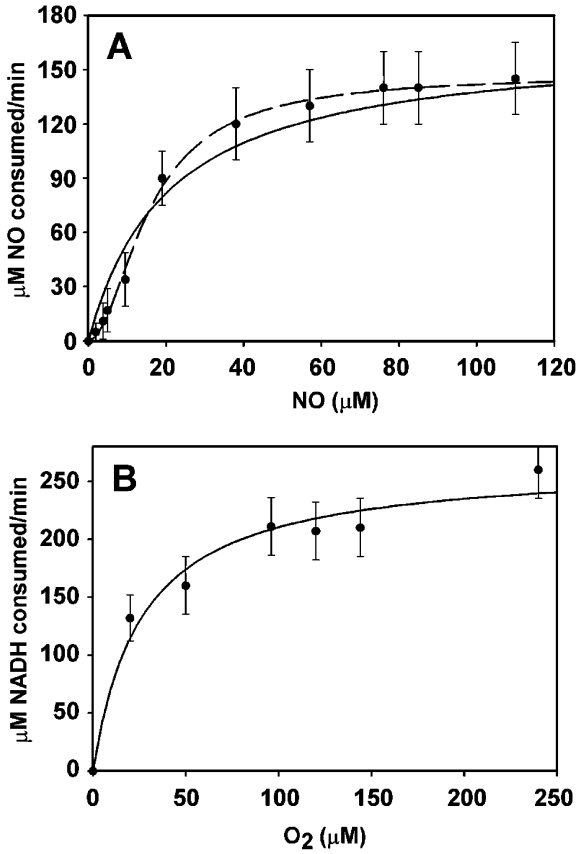

FIGURE 5: Comparison of *D. vulgaris* FprA NOR (panel A) and O_2R (panel B) activities measured as initial rates in 50 mM MOPS, pH 7.0 at room temperature. Solid traces represent fits (parameters for which are listed in Table 1) of the Michaelis−Menten equation to the data (solid circles). Error bars on data points represent the range for three determinations. Panel A: NADH:NO oxidoreductase activities in anaerobic buffer with 230 nM FprA, 170 μM NADH, and 16 μM *M. thermoacetica* Hrb. Reactions were initiated by adding FprA, and nitric oxide consumption was monitored with an NO-sensitive electrode. The dashed trace represents a fit of the rate law derived from Scheme 1 (cf. text). Panel B: NADH:O_2 oxidoreductase activities in air-saturated buffer with 170 μM NADH, 16 μM Hrb, and 250 nM FprA. Reactions were initiated by adding FprA, and O_2-dependent NADH consumption was monitored at 340 nm.

5, panel A, yielded the dashed curve with parameters $k_{cat} = 12\ s^{-1}$, $K_1 = 217\ \mu M$, and $K_2 = 3\ \mu M$. For *M. thermoacetica* FprA, K_1 and K_2 were 5 and 2 μM, respectively, indicating a modest degree of cooperativity in binding the first and second NOs (*11*). The much larger difference between K_1 and K_2 for the *D. vulgaris* FprA indicates a much larger degree of cooperativity in NO binding.

Protection of an NO-Sensitive E. coli Strain by D. vulgaris FprA. Expression of *M. thermoacetica* FprA was shown to protect an *E. coli* flavorubredoxin knockout strain from NO-induced death, thus providing in vivo evidence for NOR activity of *M. thermoacetica* FprA, even in the absence of its putative native reductase, Hrb (*11*). Figure 6 shows that plasmid-borne expression of the *D. vulgaris* FprA, presumably via its NOR activity, also restores anaerobic growth to the *E. coli* flavorubredoxin knockout strain exposed to nitric oxide. Once again, the source of electrons must be from a non-native electron donor (since *E. coli* contains no rubredoxin).

Table 1: Steady-State Kinetic Parameters for O_2R and NOR Activities of FprAs

FprA source	activity	K_m (μM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)[a]
M. thermoacetica[b]	O_2R	26	50	1.9
M. thermoacetica[b]	NOR	5	48	9.6
D. vulgaris[c]	O_2R	24	17	0.7
D. vulgaris[c]	NOR	19	12	0.6
D. gigas[d]	O_2R	NR[e]	NR[e]	NR[e]
E. coli[f]	NOR	<1	4[g]	>4
E. coli[f]	O_2R	NR[e]	NR[e]	NR[e]
Synechocystis sp.[h]	O_2R	NR[e]	NR[e]	NR[e]
M. arboriphilus[i]	O_2R	2	180[g]	90

[a] For either NO or O_2 with a saturating electron donor on a FprA monomer (i.e., active site) basis at room temperature. [b] In 50 mM phosphate, pH 7.0 (*11*). [c] In 50 mM MOPS, pH 7.0 (this work). [d] ROO; buffer and pH not specified (*4*). [e] Michaelis−Menten parameters not reported (NR). [f] Flavorubredoxin in 50 mM Tris, pH 7.6 (*9*). [g] Corrected to an active site basis (cf. ref *11*). [h] In 10 mM Tris, pH 7.6 (*25*). [i] Reduced F_{420}:O_2 oxidoreductase activity of *Methanobrevibacter arboriphilus* FprA in 50 mM Tris, pH 7.6 (*26*).

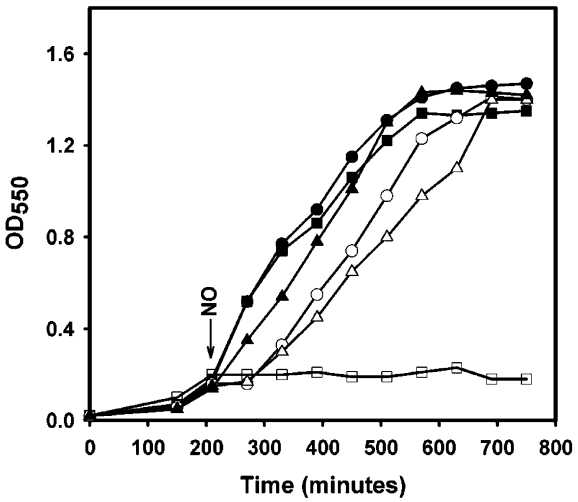

FIGURE 6: Restoration of anaerobic growth to *E. coli* AG300 (flavorubredoxin knockout strain) in the presence of exogenous nitric oxide by expression of the plasmid-borne *M. thermoacetica* FprA or *D. vulgaris* FprA genes. Cultures were grown in anaerobic minimal medium at 37 °C as described in Materials and Methods. IPTG was added at OD_{550} ~0.1. Circles: *E. coli* AG300 + pFprA-pCYB1 (expressing *M. thermoacetica* FprA). Triangles: *E. coli* AG300 + pCYB1-DvFprA (expressing *D. vulgaris* FprA). Squares: *E. coli* AG300 + pCYB1 (no FprA). Filled symbols: control (no added nitric oxide). Open symbols: nitric oxide (7.5 μM) was added from a saturated aqueous stock solution at the time indicated by the arrow.

Scheme 1

$$Fe^{II}Fe^{II} + NO \overset{K_1}{\rightleftharpoons} Fe^{II}(Fe\text{-}NO)$$

$$Fe^{II}(Fe\text{-}NO) + NO \overset{K_2}{\rightleftharpoons} (Fe\text{-}NO)_2$$

$$(Fe\text{-}NO)_2 \xrightarrow{k_{cat}} Fe^{III}Fe^{III} + N_2O$$

DISCUSSION

The recombinant *D. vulgaris* FprA characterized in this work thus appears to be a flavo-diiron enzyme which catalyzes reduction of NO or O_2 equally efficiently in vitro based on initial rates but which is irreversibly inactivated during aerobic O_2R turnover. This *D. vulgaris* FprA also

appears to function as a viable NOR in vivo, protecting an NO-sensitive *E. coli* strain even at an intracellular NO concentration well below the enzyme's Michaelis−Menten K_m for NO. The effective sub-K_m intracellular NO-scavenging ability of this *D. vulgaris* FprA may be related to the strong cooperativity in NO binding found when the in vitro NOR kinetics were analyzed according to Scheme 1. The demonstration that the *D. vulgaris* rubredoxin−FprA combination functions as an NOR is consistent with the proximity of their genes in the *D. vulgaris* genome. On the other hand, the ability of the heterologous FprAs from *D. vulgaris* and *M. thermoacetica* to function as NORs in *E. coli* in the absence of their putative native proximal electron donors, rubredoxin and Hrb, respectively, suggests that these FprAs are also effective scavengers of reducing equivalents, which are then funneled into nitric oxide. This conclusion is further supported by the observation of Gardner et al. (*6*) that disruption of the *E. coli* flavorubredoxin reductase gene (which is cotranscribed with that for flavorubredoxin) did not prevent in vivo NO scavenging by the flavorubredoxin, implying that in this mutant strain some other (unidentified) proximal electron donor(s) must be supplying reducing equivalents to flavorubredoxin at a rate sufficient to support its functional NOR turnover.

While *D. vulgaris* (Hildenborough) has never been conclusively shown to *grow* in either aerobic or subaerobic dioxygen atmospheres, it can *survive* limited exposure to air, and some of its oxidative stress defense enzymes have been identified (*13*, *16*, *29*). However, neither the *D. vulgaris* FprA characterized in this work nor any other FprA homologue has yet been demonstrated to act as an O_2R in vivo. In light of its O_2R inactivation and robust NOR activity, we suggest that this *D. vulgaris* FprA functions as a scavenging NOR in vivo. Furthermore, on the basis of the high amino acid sequence homology (57% identity, 72% similarity) of this *D. vulgaris* FprA to *D. gigas* ROO (cf. Figure 1S in Supporting Information), we predict that the *D. gigas* protein is also capable of functioning as a scavenging NOR.

The *D. vulgaris* genome encodes a second FprA homologue (NCBI accession number AAS96489, genome locus ID DVU2014), the deduced amino acid sequence of which shows 37% identity to the *rbo-rub*-associated *D. vulgaris* FprA characterized in this work and also shows conservation of the diiron ligands and flavin binding domain found in *D. gigas* ROO (cf. Supporting Information, Figure 1S). The location of the gene encoding this second FprA homologue in the *D. vulgaris* genome is also noteworthy: its stop codon lies 75 base pairs upstream of the start codon for a gene (AAS96488, DVU2013) encoding the so-called hybrid cluster protein, HCP, also known as prismane protein, the names deriving from its unusual iron−sulfur−oxygen cluster (*30*, *31*). The function of HCP has not been conclusively established. Expression of HCPs in at least four distinct bacterial species was induced by nitrate and/or nitrite under anaerobic growth conditions, and *E. coli* HCP was reported to exhibit hydroxylamine reductase activity in vitro (catalyzing reduction of NH_2OH to NH_3 and H_2O) (*32*−*35*). The proximity of HCP and FprA genes in *D. vulgaris* is consistent with the involvement of the two proteins in nitrogen oxide metabolism. This HCP was in fact isolated many years ago from the same *D. vulgaris* strain (Hildenborough) used in this work grown anaerobically on lactate and sulfate (*36*, *37*), indicating that this HCP is produced constitutively under these laboratory growth conditions. Unlike *M. thermoacetica*, this *D. vulgaris* strain has never been demonstrated to utilize nitrate or nitrite as respiratory terminal electron acceptors (*38*, *39*). *D. vulgaris* (Hildenborough) nevertheless contains a periplasmic cytochrome *c* nitrite reductase (ccNIR), which apparently serves a detoxifying role by preventing inhibition of sulfate reduction by nitrite generated by symbiotic nitrate-reducing bacteria (*38*, *40*). In fact, a cDNA macroarray analysis indicated that the genes encoding the ccNIR and a *second* HCP homologue were substantially upregulated when *D. vulgaris* (Hildenborough) was exposed to nitrite in mid-log phase of growth on lactate and sulfate (*40*). In *E. coli* the periplasmic ccNIR generates an intracellular flux of NO upon exposure to nitrite (*41*), but neither *D. vulgaris* FprA homologue was apparently upregulated upon exposure to nitrite. The *D. vulgaris* FprAs may thus provide constitutive protection against nitric oxide exposure.[3] Constitutive expression upon anaerobic growth of *D. vulgaris* (Hildenborough) on lactate and sulfate is also observed for oxidative stress-related proteins, including the superoxide reductase (also known as desulfoferrodoxin) and rubredoxin that are encoded upstream of the gene encoding the FprA examined in this work (*43*−*45*).

Despite the case made here that FprAs from at least four different sources function as scavenging NORs in vivo, it is unclear whether this function universally applies to the ∼100 known FprA homologues. A recent report has shown that, at least in vitro, two recombinant methanogenic archaeal FprAs function as reduced factor F_{420}:dioxygen oxidoreductases (cf. Table 1), whereas under the same conditions, the recombinant *M. thermoacetica* FprA (provided by us) did not show this activity (*26*). Conversely, under in vitro conditions where the *M. thermoacetica* FprA showed NOR activity, the two methanogenic archaeal FprAs did not. *Clostridium acetobutylicum* is classified as a strictly anaerobic bacterium; however, shifting it to microaerobic growth conditions (5 vol % headspace O_2) was reported to cause induction of an FprA (*46*). Both in vitro and in vivo studies on many more FprAs are needed in order to determine the extent to which an O_2R vs NOR dichotomy exists within the widespread FprA family.

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SUPPORTING INFORMATION AVAILABLE

Figure 1S showing alignment of amino acid sequences of FprAs from *M. thermoacetica*, *D. vulgaris*, and *D. gigas*. This material is available free of charge via the Internet at http://pubs.acs.org.

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[3] It is noteworthy in this regard that the *D. vulgaris* (Hildenborough) genome does not encode a recognizable homologue of flavohemoglobin, the only other bacterial enzyme that has been clearly implicated as a detoxifying nitric oxide scavenger (*7*, *41*, *42*).

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