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Dioxygen and nitric oxide scavenging by *Treponema denticola* flavodiiron protein: a mechanistic paradigm for catalysis

Rosanne E. Frederick,

Department of Chemistry, University of Texas at San Antonio, San Antonio, TX 78249, USA

Jonathan D. Caranto,

Department of Chemistry, University of Texas at San Antonio, San Antonio, TX 78249, USA

Cesar A. Masitas,

Department of Chemistry, University of Texas at San Antonio, San Antonio, TX 78249, USA

Linda L. Gebhardt,

Wadsworth Center, New York State Department of Health, Albany, NY 12201, USA

Charles E. MacGowan,

Wadsworth Center, New York State Department of Health, Albany, NY 12201, USA

Ronald J. Limberger, and

Wadsworth Center, New York State Department of Health, Albany, NY 12201, USA

Donald M. Kurtz Jr.

Department of Chemistry, University of Texas at San Antonio, San Antonio, TX 78249, USA

Donald M. Kurtz: donald.kurtz@utsa.edu

Abstract

Flavodiiron proteins (FDPs) contain a unique active site consisting of a non-heme diiron carboxylate site proximal to a flavin mononucleotide (FMN). FDPs serve as the terminal components for reductive scavenging of dioxygen (to water) or nitric oxide (to nitrous oxide), which combats oxidative or nitrosative stress in many bacteria. Characterizations of FDPs from spirochetes or from any oral microbes have not been previously reported. Here, we report characterization of an FDP from the anaerobic spirochete, *Treponema (T.) denticola*, which is associated with chronic periodontitis. The isolated *T. denticola* FDP exhibited efficient four-electron dioxygen reductase activity and lower but significant anaerobic nitric oxide reductase activity. A mutant *T. denticola* strain containing the inactivated FDP-encoding gene was significantly more air-sensitive than the wild-type strain. Single turnover reactions of the four-electron-reduced FDP ($\text{FMNH}_2\text{-Fe}^{\text{II}}\text{Fe}^{\text{II}}$) (FDP_{red}) with O_2 monitored on the milliseconds to seconds time scale indicated initial rapid formation of a spectral feature consistent with a *cis*- μ -1,2-peroxo-diferric intermediate, which triggered two-electron oxidation of FMNH_2 . Reaction of FDP_{red} with NO showed apparent cooperativity between binding of the first and second NO to

Correspondence to: Donald M. Kurtz, Jr., donald.kurtz@utsa.edu.

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the diferrous site. The resulting diferrous dinitrosyl complex triggered two-electron oxidation of the FMNH₂. Our cumulative results on this and other FDPs indicate that smooth two-electron FMNH₂ oxidation triggered by the FDP_{red}/substrate complex and overall four-electron oxidation of FDP_{red} to FDP_{ox} constitutes a mechanistic paradigm for both dioxygen and nitric oxide reductase activities of FDPs. Four-electron reductive O₂ scavenging by FDPs could contribute to oxidative stress protection in many other oral bacteria.

Keywords

Flavodiiron protein; Nitric oxide reductase; Dioxygen reductase; Spirochete; *Treponema denticola*

Introduction

Treponema denticola (Td) is a Gram-negative, motile, anaerobic spirochete associated with dental biofilms and chronic periodontitis in humans [1–5]. Td is able to survive the redox stressful periodontal environment despite the absence of genes encoding classical superoxide dismutases or catalases in its genome [6, 7]. The Td genome does, however, encode homologs of peroxiredoxin and superoxide reductase [7, 8], and the transcripts for these two oxidative stress protection enzymes were shown to be upregulated upon exposure of Td to air [9]. Some of the authors recently showed that the superoxide reductase does indeed protect Td against intracellular superoxide generation [10]. Td may also be exposed to nitrosative stress from nitric oxide (NO) generated either by reduction of nitrite in the oral cavity [11] or from the inflammatory response [12, 13]. While the air sensitivity of Td has been well documented [14], we have found no previous reports of the effects of NO exposure on Td. The Td genome lacks a homolog of flavohemoglobin, which provides aerobic nitrosative stress protection in many bacteria by oxidatively scavenging nitric oxide (NO) [15, 16].

The Td genome encodes a putative flavodiiron protein (FDP), which has been implicated in protection against either oxidative stress or anaerobic nitrosative stress in many air-sensitive bacteria, archaea and a few protists [17–32]. This protection is due to FDP's ability to reductively scavenge either O₂ (to H₂O) or NO (to N₂O) using NADH as the electron donor, and redox-mediating proteins, usually an NADH rubredoxin:oxidoreductase (NROR) and rubredoxin (Rd). These FDP activities are referred to as O₂ reductase (O₂R) and NO reductase (NOR). In vivo functions of FDPs are usually ascribed to either O₂R or NOR activities, but not to both.

The active sites of nearly all characterized FDPs contain a unique and characteristic combination of non-heme diiron carboxylate and flavin mononucleotide (FMN) cofactors, as shown in Scheme 1. Under aerobic conditions, FDPs typically contain a FMN_{ox}-Fe^{III}Fe^{III} active site (FDP_{ox}). Both O₂R and NOR turnover proceed via four-electron-reduced FDP, which contains an FMNH₂-Fe^{II}Fe^{II} active site (FDP_{red}). This site can reduce up to four NO to two N₂O or one O₂ to 2H₂O, thereby regenerating FDP_{ox} [21]. Here, we show that Td FDP exhibits both O₂R and NOR activities in vitro and provide evidence supporting an O₂-scavenging function in vivo. Single turnover reactions of FDP_{red} with O₂ and NO were used

to chronologically resolve reactions at the diiron site from those at the FMN. Our results represent the first characterization of an FDP from any spirochete or from any member of the large consortia of oral bacteria. They also support a paradigm for the cofactor/substrate reaction chronologies in FDPs.

Materials and methods

Reagents and general procedures

Reagents and buffers were of the highest quality commercially available. All solutions were prepared in water that had been passed through a Milli-Q® ultrapurification system (Merck Millipore, Inc.) to achieve a resistivity of 18 MΩ. *T. denticola* (ATCC 35405) was grown in new oral spirochete medium (NOS) with 10 % heat-inactivated rabbit serum, 10 mg of cocarboxylase per mL, at 36 °C in an anaerobic chamber (Coy Laboratory Products) containing an atmosphere of 85 % N₂/5 % CO₂/10 % H₂ [10]. For growth on semi-solid media, 0.5 % agarose was included (NOS agarose). Unless otherwise noted, the buffer for all reactions of the isolated Td FDP was 50 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) pH 7.3, hereafter referred to as buffer. NROR [24] and Rd [33] were prepared as previously described. Nucleotide sequencing was performed at the Wadsworth Center Molecular Genetics Core Facility.

Insertional inactivation of the Td FDP gene

The FDP-encoding gene, TDE1078, in *T. denticola* 35405 was inactivated by insertion of an erythromycin resistance (*erm*) cassette using a procedure completely analogous to that previously described by us for production of a mutant strain containing *erm*-disrupted TDE1754 [1, 10]. The strategy for insertion of the *erm* cassette into the genomic TDE1078 is described in the Supplementary Material and results confirming this insertion are shown in Figs. S1–S4. The mutant Td strain containing the *erm*-disrupted TDE1078 is hereafter referred to as 1078 M. Other than erythromycin resistance, the anaerobic growth and propagation of the 1078 M strain in NOS medium were qualitatively indistinguishable from those of the wild-type strain.

Oxidative and nitrosative stress phenotypes

All culture manipulations were conducted in the Coy anaerobic chamber containing the atmosphere listed above. Oxidative stress phenotypes upon exposure of the wild-type and 1078 M strains to air were determined using the liquid culture “single-dose” method, as previously described [10]. Anaerobic NOS cultures were grown to an OD₅₀₀ ~0.2, then diluted tenfold with air-saturated NOS minus cysteine and thioglycollate, and layered with mineral oil in the anaerobic chamber and incubated at 36 °C. Samples of these treated cultures were removed at various exposure times, serially diluted, and plated on NOS agarose to determine survivals as colony-forming units per milliliter of culture (CFU/mL) after 9 days of anaerobic incubation. For nitric oxide exposure, 10 µL of log-phase culture (OD₆₀₀ ~0.3) was diluted in 5 mL of NOS and incubated for 1–4 h, after which small volumes of a freshly prepared concentrated stock solution of the nitric oxide donor, diethylammonium (Z)-1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate), in 0.01 M NaOH were added to achieve initial concentrations in the range of 0.2–200 µM.

Growth curves were obtained (OD_{600}) for up to 5 days at 36 °C. Control experiments showed no effect on growth of the same added volumes of 0.01 M NaOH.

Recombinant FDP expression and purification

The plasmid used to express Td FDP for the in vitro spectroscopic and kinetic studies contained a synthetic FDP gene, the nucleotide sequence of which had been codon-optimized for *E. coli* expression. This codon-optimized gene was synthesized and subcloned into pAG8H [34] by GenScript (Piscataway, NJ, USA). The plasmid encodes an 8×His-tag at the N-terminal end of the protein. *E. coli* BL21 (DE3) competent cells (Invitrogen) were transformed with this plasmid. The transformed cells were grown at 37 °C in Luria–Bertani broth supplemented with 100 mg/L ampicillin. Overexpression of the FDP was induced in shaking 1-L cultures when the OD_{600} reached ~0.8 by the addition of isopropyl β -D-1-thiogalactopyranoside to a concentration of 0.8 mM. 100 mg of ferrous ammonium sulfate was then immediately added to each liter of culture. After 4 h at 37 °C, the cells were harvested by centrifugation. The cell pastes were frozen at –80 °C overnight and then suspended in 50 mM MOPS, 20 mM imidazole, 500 mM NaCl, pH 7.3 (buffer A). The cells were lysed by pulsed sonication, and cell debris was removed by centrifugation. The supernatants were applied to a column containing 10 mL of Ni Sepharose® Fast Flow affinity resin (GE Healthcare) equilibrated with buffer A, and the protein was eluted with buffer A containing 500 mM imidazole. The eluted FDP was washed with 50 mM MOPS, 100 mM NaCl pH 7.3 (buffer B) by several concentration/redilution cycles in 30-kilodalton molecular weight cutoff Amicon centrifugal filters (Millipore). The purified protein, referred to as as-isolated Td FDP, was concentrated to 1–2 mM (active site basis) and stored at –80 °C.

Protein, FMN, and iron analysis

Protein concentrations were determined by the bicinchoninic acid assay (Pierce). Protein iron and FMN contents were determined using a standard ferrozine assay [35], and $\epsilon_{461\text{ nm}} = 12,000\text{ M}^{-1}\text{ cm}^{-1}$, respectively, the latter of which was also used to determine protein active site concentrations in the FMN-enriched FDP.

FMN enrichment

The as-isolated Td FDP contained 70–80 % of the expected FMN content of 1 per protein monomer. A procedure similar to that previously described for another FDP [36] was used to achieve full FMN occupancy. One milliliter of ~1 mM Td FDP (monomer basis) in buffer B was incubated with ~2 mol FMN:mol FDP monomer at 4 °C overnight. Excess FMN was removed by repetitive concentration/dilution cycles in buffer B until the filtrate was colorless. The FMN:iron mol ratio using this method was reproducibly ~1:2, and protein containing this ratio was used for all in vitro experiments.

Steady-state O_2R and NOR activities

Assays were conducted at room temperature (~22 °C) in rubber septum-sealed cuvettes made anaerobic via successive vacuum/ N_2 exchanges. The one-milliliter assay solutions contained 0.3 μM NROR, 4 μM Rd and 30-nM (active sites) Td FDP in buffer. For O_2R

assays, various initial concentrations of O₂ were achieved by syringe injections of small volumes of air-saturated buffer. Reactions were initiated by the addition of small volumes of NADH from a concentrated stock solution to achieve an initial concentration of 130–200 μM NADH. Rates of NADH consumption were measured spectrophotometrically as

$A_{340\text{ nm}} (\epsilon_{340\text{ nm}} = 6220\text{ M}^{-1}\text{ cm}^{-1})$ from linear regression fits to the initial linear portion of the time course. NADH consumption rates were converted to O₂ consumption rates assuming a molar consumption ratio of 2NADH:O₂. This stoichiometry was determined by repeated injections of small volumes of air-saturated buffer into a septum-sealed-cuvette containing 1 mL of anaerobic assay solutions plus excess NADH and measuring the magnitude of NADH consumption upon each O₂ injection. The parameters k_{cat} and K_{m} were determined from unweighted non-linear least squares regression fits of the Michaelis–Menten equation to the set of initial rates versus initial O₂ concentrations. NOR activities and kinetics parameters were determined analogously using 1 μM FDP in the assay mixtures. NO was injected into the assay mixture from an anaerobic buffered aqueous solution of saturated NO (~1.8 mM NO). Rates of NO consumption assumed a 1NADH:2NO stoichiometry [30].

Preparation of FDP_{red}

Td FDP_{red} was prepared from the FMN-enriched FDP in 50 mM MOPS pH 7.3 as described for another FDP [37]. An anaerobic solution containing 180–300 μM of Td FDP in buffer containing 2.2 molar equivalents of NADH, Rd (1–5 μM) and NROR (0.5–1 μM) was incubated overnight at room temperature in an anaerobic glove box under an N₂ atmosphere. The NADH:FDP mol ratio amounts to a small stoichiometric excess of NADH-reducing equivalents over [FMN + diiron sites].

Stopped-flow spectrophotometry

Stopped-flow UV–Vis absorption spectrophotometry for reactions of Td FDP_{red} with either NO or O₂ was performed using an SX20 stopped-flow spectrometer (Applied Photophysics, Ltd.), essentially as described previously for another FDP [36, 37]. All stopped-flow reactions were carried out at 2 °C in buffer. Anaerobic Td FDP_{red} solutions were mixed in the stopped-flow apparatus 1:1 (v/v) with air-saturated buffer, anaerobic NO-saturated buffer or NO delivered from the decay of a buffered solution of disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazene-1-ium-1,2-diolate (PROLI-NONOate) at 2 °C. Preparation of the PROLI-NONOate solutions and quantification of their NO-delivery equivalents have been described previously [37]. Protein, O₂ and NO concentrations immediately after mixing are listed in the figure legends. Spectral time courses were obtained using a photodiode array detector and 1-cm sample cell pathlength. Rate constants were obtained by fitting single-wavelength absorbance time courses to exponential functions using the KaleidaGraph program (Synergy Software). More details are provided in the figure legends.

EPR of FDP_{red} reaction with sub-stoichiometric NO

In the N₂-filled glove box, 3.8 μL of a 20-mM PROLI-NONOate solution pre-calibrated for NO delivery was added to a buffered anaerobic solution of 300 μM Td FDP_{red} at room

temperature to deliver ~0.5 eq NO per diiron site. The sample was incubated in the glove box at room temperature for ~5 min. ~300 μ L of this sample was transferred to a 4-mm O.D. quartz EPR tube and sealed with a septum. The sample was then removed from the glove box and immediately frozen by immersion of the EPR tube in liquid N₂. The EPR tube was stored in liquid N₂ until spectral collection. EPR spectra were collected on a Bruker ESP 300E X-band spectrometer equipped with a helium continuous flow cryostat model ESR 900 (Oxford Instruments, Inc.).

Results and discussion

Oxidative stress phenotype

The Td genome contains only one gene, TDE1078, encoding a FDP. Western blotting (Fig. S3) of Td cell extracts showed that the Td FDP was expressed in the wild-type strain under standard anaerobic growth conditions, whereas the corresponding mutant strain (1078 M) containing the in-frame *erm*-disrupted TDE1078 gene did not detectably express the FDP. Using our previously described method [10], we compared the effect of exposure to air-saturated media on the Td wild-type strain with that on the 1078 M strain. The results are shown in Fig. 1. Based on this comparison, the 1078 M strain is clearly more air-sensitive than the wild-type strain. This result is consistent with a reductive scavenging O₂R role for Td FDP in vivo.

O₂R steady-state activity

O₂ reductase activity of the isolated Td FDP was measured by monitoring NADH consumption upon addition of O₂ to anaerobic assay mixtures containing a non-native NROR and Rd as electron transfer mediators. Rds and Rd-like proteins can serve as efficient proximal electron donor to FDPs [24, 30, 37]. The Td genome encodes a single Rd gene, and its transcription was shown to be upregulated upon exposure of Td to dioxygen [9]. Although a Td NROR has not been characterized, a gene (TDE0096) encoding a homologous putative NADH oxidoreductase has been identified [7]. Rd or Rd-like protein domains are known to be fairly promiscuous electron acceptors [22, 30, 31]. We found empirically that, under steady-state conditions, the concentration of Rd rather than that of the NROR limits the rate of electron transfer from NADH to the FDP, and saturating Rd was used for the assays. Little or no O₂R activity was observed when Rd was omitted from the activity assay mixtures. A Michaelis–Menten plot of the O₂R activity is shown in Fig. 2. Both O₂ delivery and initial rates became insufficiently precise below ~10 μ M O₂. The lowest initial O₂ concentration used for the activity data in Fig. 2 was ~12 μ M, which gave an initial rate well above half-maximal velocity and, therefore, represents an experimental upper limit on K_{m,O_2} . The fitted K_{m,O_2} value, 7 μ M, is consistent with this observation. The fitted k_{cat}/K_m value, $\sim 4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, is within the range reported for FDPs in other organisms where transcriptional and phenotypic evidence indicates an O₂ scavenging function (see Table S1) [22, 24, 38–40].

Single turnover reactions with O₂

The in vivo oxidative stress protection and the substantial steady-state O₂R activity led us to examine the reaction pathway of Td FDP_{red} with O₂ under single turnover conditions. The

results are analyzed according to the four-electron O₂ reduction pathway shown in Scheme 2. An O₂ reduction pathway consisting of two-electron reductions of 2 O₂ to 2 H₂O₂ is ruled out by the stoichiometry of 2 mol NADH consumed per mol O₂ added. We determined this stoichiometry from a titration of anaerobic Td FDP_{red} with sub-stoichiometric O₂ in the presence of excess NADH and the catalytic electron-transfer enzymes. This stoichiometry and the four-electron reduction of O₂ to 2H₂O are commonly observed for FDPs [21, 22].¹

A UV–Vis absorption stopped-flow spectral time course for the reaction of Td FDP_{red} with O₂ is shown in the top panel of Fig. 3. An absorption feature centered at ~650 nm forms within the mixing dead time (<2 ms), then decreases in intensity concomitantly with an increase in an absorption feature with λ_{max} at 462 nm. The developing 462-nm chromophore is due to the two-electron oxidation of FMNH₂ to FMN_{ox} ($\epsilon_{462 \text{ nm}} = 12,000 \text{ M}^{-1} \text{ cm}^{-1}$), which occurs quantitatively in the presence of excess O₂.² Features attributable to the distinctive absorptions of the one-electron-oxidized FMN semiquinone (FMN_{sq}), either neutral or anionic [41], were not apparent at any point in the time course. (features attributable to a peroxy adduct of FMN were also not evident [42]). The bottom panel in Fig. 3 shows that the vast majority of this two-electron FMNH₂ oxidation occurred within ~120 ms, which we refer to as Phase I. A very minor portion of the FMNH₂ oxidized over the course of ~1 s (Phase II). The fitted k_{obs} for Phase I of the FMNH₂ oxidation (462 nm in Table 1) is essentially identical to that for the decrease in absorption of the 650-nm chromophore. This observation together with the tight isosbestic point at ~565 nm implies that the decay of the 650-nm chromophore is directly connected to the smooth conversion of the FMNH₂ to FMN_{ox}. Difference absorption spectra (O₂-reacted minus FDP_{red}, not shown) did not distinctly resolve the 650-nm feature and suggested that some FMN_{ox} absorption is present even in the initial mixing dead-time spectrum. We know of no FMN species exhibiting a 650-nm absorption feature resembling that in Fig. 3. We, therefore, assign the 650-nm chromophore to a rapidly formed product of the reaction of O₂ with the diferrous site. This product is formulated as a peroxo-diferriic species [Fe^{III}–(O₂²⁻)–Fe^{III} in Scheme 2]. The 650-nm chromophore is characteristic of *cis*- μ -1,2 (μ - η^1 : η^1) peroxo-diferriic complexes with weak ligand field O-/N-atom coordination spheres [43]. Other coordination modes, such as non-bridging end-on hydroperoxo [44] or a recently proposed μ - η^1 : η^2 peroxo [45], show absorptions centered near 500 nm and are clearly distinct from that observed in Fig. 3. Bridging carboxylate or solvent ligands could also be present. The peroxo-diferriic could then be two-electron reduced by the proximal FMNH₂, perhaps facilitated by protonation, thereby generating 2H₂O and FDP_{ox}.³ While the two-electron transfer would presumably occur in two one-electron steps, the second must be relatively rapid, since we observed no accumulation of FMN_{sq}. Rate-determining rearrangement of the initial peroxo-diferriic to a more oxidizing species is also conceivable [46, 47].

¹Td FDP showed a modest level of NADH:H₂O₂ oxidoreductase activity [$\sim 3 \mu\text{M H}_2\text{O}_2$ consumed·s⁻¹·($\mu\text{M FDP}$)⁻¹, assuming a 1NADH:1H₂O₂ stoichiometry] in anaerobic assay solutions containing 200 μM added H₂O₂.

²For the four-electron process, the initial concentrations of O₂ and FDP_{red} used for the time course in Fig. 3 is sufficient to completely oxidize the FDP_{red} to FDP_{ox}.

³We found no significant differences in k_{obs} at pH 9 from those at pH 7 listed in Table 1.

NOR steady-state activity

Td FDP showed lower NOR steady-state activity (Fig. 4) compared to its O₂R activity [NOR ($k_{\text{cat}}/K_m = 4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$)]. Unlike that for the O₂R activity, the Michaelis–Menten plot for the NOR activity showed sigmoidal character. Since two NOs are required for production of one N₂O, we have previously attributed sigmoidal behavior for steady-state NOR activities of other FDPs to positively cooperative binding of two NOs to the diiron site (presumably one to each iron) [24, 30, 31]. These successive interactions can be described by apparent dissociation constants, $K_{\text{d}(1)}$ and $K_{\text{d}(2)}$, according to Eq. 1 (where v is initial NO ($[\text{NO}]_{\text{init}}$) consumption velocity).

$$v = \frac{k_{\text{cat}}([\text{NO}]_{\text{init}})^2}{K_{\text{d}(1)}K_{\text{d}(2)} + K_{\text{d}(2)}[\text{NO}]_{\text{init}} + ([\text{NO}]_{\text{init}})^2} \quad (1)$$

As shown in Fig. 4, this apparent cooperativity model provided an improved fit to the NO concentration dependence of the NO consumption rates and indicated an approximately 15 times higher apparent affinity for the second NO than the first.

The NO reaction pathway of Td FDP_{red}

The apparently higher affinity of the second NO for Td FDP_{red} is opposite to that reported for *T. maritima* FDP_{red}, for which the NOR reaction mechanism has recently been characterized in some detail [37, 48]. We, therefore, investigated whether these reversed NO relative affinities indicated a different NO reaction pathway for Td FDP. The previously proposed reaction pathway for *T. maritima* FDP [37] is shown in Scheme 3 (with H⁺ and H₂O omitted to avoid crowding).

Stopped-flow UV–Vis absorption spectral time courses for the reaction of Td FDP_{red} with ~0.5 eq NO per diiron site monitored over either 100 ms or 100 s are shown in Fig. 5. Comparison of these time courses shows that the spectral changes are essentially complete within ~100 ms after mixing. At this sub-stoichiometric NO-to-diiron site mol ratio, little or no FMNH₂ oxidation is observed even over 100 s. The slight increases in absorbance at ~390 and 490 nm in the 100 s time course (bottom panel of Fig. 5) indicate very minor formation of anionic FMN_{sq}. The lack of significant FMNH₂ oxidation justifies subtraction of the FDP_{red} spectrum from those of the NO-treated enzyme, presuming NO reacts only with the diferrous site (which has negligible visible absorption). The 1.3- and 100-ms difference absorption spectra (Fig. 5 top panel, inset) are nearly superimposable with features at ~420, 450 and 630 nm. These difference spectra and their extinction coefficients (based on NO delivered) are characteristic of an $S = 3/2$ {FeNO}⁷ chromophore with an O,N coordination sphere (where {FeNO}⁷ is the Enemark–Feltham notation for ferrous nitrosyl [49]) and closely resemble those for the diferrous mononitrosyl complex (Fe^{II}{FeNO}⁷ in Scheme 3) previously reported for *Thermotoga* (*T.*) *maritima* FDP [37, 50]. These difference spectra are thus consistent with essentially quantitative formation of one {FeNO}⁷ per NO delivered within the mixing dead time and with the stability of the FMNH₂–diferrous mononitrosyl over at least 100 s with 0.5 eq added NO. Analogous stopped-flow spectral time courses (not shown) using higher stoichiometries, but still <1 eq NO per diiron site,

indicated some two-electron FMNH₂ oxidation. This behavior would be expected if the FMNH₂ oxidation required binding of a second NO, and if the affinity of this second NO for the diiron site were significantly higher than that of the first. This expectation is consistent with the cooperativity model used to analyze the steady-state activity data in Fig. 4.

The identification and stability of the diferrous mononitrosyl complex were verified by the EPR spectrum of a manually mixed sample of Td FDP_{red} with ~0.5 eq NO as shown in Fig. 6. Antiferromagnetic coupling of the high spin Fe^{II} with $S = 3/2$ {FeNO}⁷ within the diiron site leads to a $S = 1/2$ ground state with a characteristic EPR signal [37, 50]. The g values of 2.29 and ~2.08 are close to those in the corresponding *T. maritima* FDP complex. The sharp $g = 2.0$ signal is assigned to a very minor amount of FMN_{sq}.

Stopped-flow UV–Vis absorption spectral time courses for reaction of Td FDP_{red} with excess NO over either 130 ms or 10 s are shown in Fig. 7. The bottom panel of Fig. 7 shows that, unlike the case with 0.5 eq NO, reaction with a tenfold molar excess NO resulted in smooth and essentially quantitative two-electron oxidation of FMNH₂ to FMN_{ox} over the course of a few seconds. However, a comparison of the time courses in the top and bottom panels of Fig. 7 indicated that at most, only a very minor portion of the FMNH₂ oxidation occurred over the initial 130 ms of reaction, which once again justifies subtraction of the FDP_{red} spectrum from those of the NO-treated enzyme obtained during this initial reaction time period. The difference absorption spectral time course (Fig. 7, top panel, inset) revealed that an {FeNO}⁷ chromophore accumulated within the mixing dead time (red trace). This dead-time difference absorption closely resembles the spectrum assigned to the diferrous mononitrosyl in Fig. 5. The subsequent difference spectra show development of a more intensely absorbing {FeNO}⁷ chromophore, which accumulates maximally at ~100 ms. This intensification is assigned to essentially quantitative accumulation of a diferrous dinitrosyl ([{FeNO}⁷]₂ in Scheme 3), which is consistent with its estimated extinction coefficient of ~3000 M⁻¹ cm⁻¹ (diiron site basis) at 130 ms [37, 38]. The subsequent FMNH₂ oxidation (Fig. 7, bottom panel) is chronologically separable from formation of the diferrous dinitrosyl complex. This two-electron FMNH₂ oxidation occurs on the same time scale as the steady state $k_{cat} \sim 1 \text{ s}^{-1}$. This correlation is consistent with NOR turnover being rate-limited by a process that triggers two-electron FMNH₂ oxidation. Our detailed investigations of the NO reaction with *T. maritima* FDP_{red} [37, 48] indicated that the rate-limiting process for NOR turnover is the “spontaneous” conversion of the diferrous dinitrosyl to diferric with release of N₂O generating a transient FMNH₂/Fe^{III}Fe^{III} species (Scheme 3). The proximal FMNH₂ then rapidly re-reduces the diferric site, resulting in FMN_{ox}/Fe^{II}Fe^{II}. With excess NO, the diferrous site then undergoes a second analogous reaction sequence resulting in a second N₂O and FDP_{ox}. The UV–Vis absorption spectral features of ferrous nitrosyl intermediates accumulating during this second turnover are obscured by the much more intense FMN_{ox} absorption.

In vitro/in vivo correlations

To our knowledge, these results constitute the first reported characterization of an FDP from any spirochete or from any of the numerous oral bacterial phyla [4, 5]. The increased sensitivity of the Td 1078 M strain to air-saturated medium relative to the wild-type strain

together with the efficient steady-state O₂R activity for the isolated enzyme supports an O₂-scavenging function for Td FDP in the likely sub-aerobic but fluctuating O₂ levels of the periodontal environment [5, 9]. A previous study did not observe upregulation of the FDP gene (TDE1078) transcription upon exposure of Td to air [9]. Our Western blotting (Fig. S3) and a previous proteomics study [51] instead showed constitutive expression of the FDP in *T. denticola* under anaerobic growth conditions. This constitutive expression could function as a pre-emptive defense in contrast to other oxidative stress protection enzymes, which are upregulated upon exposure of *T. denticola* to air [9]. FDPs could provide oxidative stress protection in many other oral bacteria. Our search of the National Center for Biotechnology Information database found FDP homologs encoded in the genomes of at least five other oral treponemal species as well as in genomes of other bacterial phyla associated with periodontitis [3, 4, 52]. Td is a member of the so-called “red complex”, a consortium of three bacterial species associated with severe and chronic periodontitis [2, 3, 53]. The genomes of all three members encode FDP homologs.

The steady-state kinetics of Td FDP showed significant NOR activity albeit less efficient than the O₂R activity. We did not observe a reproducible differential sensitivity between the wild-type and 1078 M strains to a commonly used NO-delivery agent at levels spanning the purported range of NO concentrations encountered in the periodontal pocket [54]. Under an anaerobic atmosphere, growths of both strains were typically unaffected at up to 2 μM added DEA-NONOate, significantly inhibited between 20 and 100 μM DEA-NONOate, and completely suppressed at 200 μM DEA-NONOate (data not shown). However, manifestation of an NO-scavenging role for Td FDP may depend on growth conditions or the source and rate of NO production in the complex environment of the periodontal pocket [23, 55–61].

Relative O₂R vs NOR activities of FDPs

Most characterized FDPs exhibit both O₂R and NOR activities to varying levels in vitro [22, 24, 30, 31, 37, 62]. Evidence for bifunctional O₂ and NO scavenging of individual FDPs in vivo is less common [20, 63]. Up to now, the relatively higher O₂R vs NOR activity found for Td FDP in this work has been more commonly observed for FDPs than has the reverse (see Table S1). However, some FDPs have relatively similar NOR and O₂R activities. In the case of NO, our results establish that Td and *T. maritima* FDPs have similar k_{cat} values (Table S1) and the same single turnover reaction sequence despite their reversed relative affinities for the first and second NO. The structural factors that modulate the relative O₂R vs NOR activities and NO affinities have not been elucidated. Modeling of NO occupancy indicates that two NO molecules can be accommodated in the active site pockets of FDPs without significant rearrangement of amino acid residues lining the pockets [21, 64]. The crystal structure of *Desulfovibrio gigas* FDP showed that a conserved His residue, which serves as an iron ligand in all other structurally characterized FDPs, was replaced by solvent [65]. The reported O₂R and NOR k_{cat} values for this FDP, however, are both near the high end of the range for FDPs and differ by a factor of only ~3 (see Table S1) [20]. Furthermore, substitutions of the analogous His ligand residue in *T. maritima* FDP with either asparagine or alanine resulted in only very minor effects on both the diiron site structure and activities

[36]. Modulation of the relative O₂R vs NOR activities by substitutions of more distant residues in another FDP has been claimed [62].

A mechanistic paradigm for flavodiiron proteins

Our kinetics results support the generality of both the O₂ and NO reaction pathways of FDPs, which are diagrammed in Schemes 2 and 3, respectively. With excess NO, the stopped-flow single turnover time courses for both Td FDP_{red} (Fig. 7) and *T. maritima* FDP_{red} [37] showed that the diferrous mononitrosyl to dinitrosyl conversion occurs prior to the rate-determining step for turnover. Quantitative formation of the diferrous dinitrosyl species occurred more than 10 times faster than FMNH₂ oxidation in Td FDP, and a similar chronological separation of these two processes was observed for *T. maritima* FDP [37]. The rate-determining step for NOR turnover at saturating NO must, therefore, occur *after* diferrous dinitrosyl formation in both Td and *T. maritima* FDPs. We previously formulated the rate-determining step in the *T. maritima* FDP_{red} reaction with excess NO as “spontaneous” decay of the diferrous dinitrosyl to diferric + N₂O (r.d.s. in Scheme 3) [37]. This rate-determining “spontaneous” decay is supported by the observation that deflavinated *T. maritima* diferrous FDP reacted with NO to form the same diferrous dinitrosyl species, which decayed to diferric with formation of N₂O on the same time scale as the FMNH₂ oxidation in the flavinated FDP [38, 48]. In our proposed mechanism, the r.d.s. is followed by rapid reduction of the diferric back to the diferrous oxidation level by the proximal FMNH₂ (Scheme 3). A much less resolvable lag between formation of the diiron substrate complex and two-electron FMNH₂ oxidation appears to occur for the O₂R single turnover reactions of both Td (Fig. 3) and *T. maritima* FDP_{red} [36]. In this case, rapid reduction of the initially formed peroxo-diferric by the proximal FMNH₂ could minimize formation or side reactions of subsequent, more oxidizing diiron–O₂ intermediates [46, 47, 66]. Our cumulative results on Td and *T. maritima* FDPs thus provide a paradigm for FDP catalysis. Reaction with FDP_{red} leads to a catalytically committed diiron/substrate complex (containing either one O₂ or two NO) and an oxidizing diiron site, either peroxo-diferric (for O₂R) or diferric (the decay product of a diferrous dinitrosyl for NOR). The oxidizing diiron site triggers two-electron oxidation of the proximal FMNH₂ [22, 36, 37]. Turnover of FDP_{red} via either one O₂ or four NO results in a four-electron-oxidized active site (FDP_{ox}) according to Schemes 2 or 3, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Air sensitivities measured as survivals (CFU/mL) of Td wild-type (WT) and 1078 M strains in liquid cultures after dilution with air-saturated media followed by incubation for the indicated times, serial dilutions and plating. *Error bars* represent the standard deviations for three trials

Fig. 2.

Plot of initial rates vs initial O_2 concentrations for O_2R activity of Td FDP. Each data point (*circles*) represents the average of at least three determinations with standard deviations indicated by the *vertical bars*. The *curve* through the data points represents the best fit of the Michaelis–Menten equation, which yielded $K_{m,O_2} = 7 \mu\text{M}$ and $k_{\text{cat},O_2} = 28 \text{ s}^{-1}$

Fig. 3.

Top panel stopped-flow UV–Vis absorption spectral time course for reaction of Td FDP_{red} (80 μM active sites) with air-saturated buffer [initially $\sim 112 \mu\text{M}$ O_2 after 1:1 (v/v) mixing] at 2 °C. The *red trace* was recorded at 1.3 ms and the *blue trace* at 1 s after the mixing dead time (~ 2 ms). The *black traces* were recorded in between these limiting times. The *inset* shows an expanded view of the 500–700 nm region and the isosbestic at 565 nm. *Vertical arrows* indicate directions of absorbance changes with time. *Bottom panel* absorbance vs time at 462 nm (filled circle), 565 nm (filled square) (isosbestic), and 650 nm (filled diamond) from the spectral time course shown in the top panel. The traces through the $A_{462 \text{ nm}}$ and $A_{650 \text{ nm}}$ time courses represent the best-fit exponentials yielding the observed rate constants listed in Table 1

Fig. 4.

Initial rates versus initial NO concentration plot for NOR activity of Td FDP. Each data point (*circles*) represents the average of at least three determinations with standard deviations indicated by the *vertical bars*. The *curves* represent unweighted non-linear least squares regression fits of either the Michaelis–Menten equation (*solid*) or the “cooperativity” model described by Eq. 1 (*dashed*) [31]. The Michaelis–Menten fit yielded $K_m \text{ NO} = 280 \text{ } \mu\text{M}$ and $k_{\text{cat}} \text{ NO} = 1 \text{ s}^{-1}$. The “cooperativity” equation fit yielded apparent dissociation constants, $K_{d(1)} = 290 \text{ } \mu\text{M}$, $K_{d(2)} = 20 \text{ } \mu\text{M}$, and $k_{\text{cat}} = 1.3 \text{ s}^{-1}$.

Fig. 5.

Stopped-flow absorption spectral time courses obtained over either 100 ms (*top panel*) or 100 s (*bottom panel*) for reaction of 230 μM Td FDP_{red} with 130 μM NO (concentrations immediately after mixing) anaerobically in buffer at 2 °C. The spectrum labeled FDP_{red} was obtained from the same FDP_{red} solution stopped-flow mixed with deoxygenated buffer in place of the NO solution. *Inset* in the *top panel* shows the difference absorption spectra obtained by subtracting the FDP_{red} spectrum from either the 1.3 ms (*red trace*) or the 100 ms spectrum (*blue trace*) in the main panel. Indicated times are after the mixing dead time (~ 2 ms). The extinction coefficient axis in the *inset* is based on the delivered NO concentration

Fig. 6.

X-band EPR spectra of an anaerobic solution of $300 \mu\text{M}$ Td FDP_{red} in buffer (*black trace*) and of the same sample after the addition of 0.5 eq NO, incubation of the mixture for ~ 5 min at room temperature, and then freezing in liquid N_2 (*red trace*). Spectral collection parameters were: temperature 4 K, modulation frequency 100 kHz, microwave power 21 mW, modulation amplitude 10.5 G, conversion time 81.9 ms, time constant 5.1 ms

Fig. 7.

Stopped-flow absorption spectral time courses obtained over either 130 ms (*top panel*) or 1 s (*bottom panel*) for reaction of 90 μM Td FDP_{red} with 900 μM NO (concentrations immediately after mixing) anaerobically in buffer at 2 $^{\circ}\text{C}$. Indicated times are after the mixing dead time (~ 2 ms). The spectrum labeled FDP_{red} was obtained upon stopped-flow mixing the same FDP_{red} solution with deoxygenated buffer in place of the NO solution. The *inset* in the *top panel* shows the difference absorption spectra obtained by subtracting the FDP_{red} (*dashed*) spectrum from either the 1.3 ms (*red trace*) or the 100 ms spectrum (*blue trace*) in the main panel. Extinction coefficient axes are on an active site basis

Scheme 1.
Diagram of FDP active site

Scheme 2. Proposed $\text{FDP}_{\text{red}} + \text{O}_2$ reaction pathway

Scheme 3.FDP_{red} + NO reaction pathway

Table 1

Observed rate constants (k_{obs}) for the formation of the 462-nm chromophore and decay of the 650-nm chromophore during oxidation of Td FDP_{red} by O₂

Wavelength (nm)	Phase I (s ⁻¹)	Phase II (s ⁻¹)
462	113 (1) ^a	6.6 (0.7) ^a
~650	116 (1) ^a	–

Obtained from exponential fits to the absorbance time courses shown in the bottom panel of Fig. 3

^aThe average from three time courses with standard deviations in parentheses