Conversion of NO_2 to NO by reduced coenzyme F_{420} protects mycobacteria from nitrosative damage

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In mycobacteria, F_{420}, a deazaflavin derivative, acts as a hydride transfer coenzyme for an F_{420}-specific glucose-6-phosphate dehydrogenase (Fgd). Physiologically relevant reactions in the mycobacteria that use Fgd-generated reduced F_{420} ($F_{420}H_2$) are unknown. In this work, $F_{420}H_2$ was found to be oxidized by NO only in the presence of oxygen. Further analysis demonstrated that NO_2, produced from NO and O_2, was the oxidant. UV-visible spectroscopic and NO-sensor-based analyses proved that $F_{420}H_2$ reduced NO_2 to NO. This reaction could serve as a defense system for *Mycobacterium tuberculosis*, which is more sensitive to NO_2 than NO under aerobic conditions. Activated macrophages produce NO, which in acidified phagosomes is converted to NO_2. Hence, by converting NO_2 back to NO with $F_{420}H_2$, *M. tuberculosis* could decrease the effectiveness of antibacterial action of macrophages; such defense would correspond to active tuberculosis conditions where the bacterium grows aerobically. This hypothesis was consistent with the observation that a mutant strain of *Mycobacterium smegmatis*, a nonpathogenic relative of *M. tuberculosis*, which either did not produce or could not reduce F_{420}, was ≈4-fold more sensitive to NO_2 than the wild-type strain. The phenomenon is reminiscent of the anticancer activity of γ-tocopherol, which reduces NO_2 to NO and protects human cells from NO_2-induced carcinogenesis.

macrophage | deazaflavin | nitrogen dioxide | nitric oxide | tuberculosis

Coenzyme F_{420}, a 7,8-didemethyl-8-hydroxy-5-deazaflavin derivative (Fig. 1), is a 2-electron or hydride transfer restricted redox catalyst ($E^{o\prime} = -360$ mV) similar to the nicotinamides ($E^{o\prime} = -320$ mV) (1, 2). F_{420} is found in all methanogenic and certain nonmethanogenic archaea, where it participates in energy metabolism, NADP reduction, oxygen detoxification, and sulfite reduction (3–6). In the bacterial domain, F_{420} is found in certain members of the *Actinobacteria* phylum, such as *Mycobacterium* species (7). These organisms express an F_{420}-dependent glucose-6-phosphate dehydrogenase (Fgd, Reaction **1**) (7, 8).

$$\text{Glucose 6-phosphate} + F_{420} \rightarrow \text{6-phosphogluconate} + F_{420}H_2$$

[Reaction 1]

The physiological fate of $F_{420}H_2$ produced by Fgd is unknown. An insertional inactivation of *fbi*C, an essential gene for the synthesis of the deazaflavin chromophore or catalytic unit of F_{420} (9), renders *Mycobacterium tuberculosis* hypersusceptible to acidified nitrite (10). This in vitro treatment simulates an environment inside the phagosomes of an infected-activated macrophage, which produces nitric oxide (NO) by the action of inducible nitric oxide synthase (iNOS or NOS2) (11). Upon acidification of a phagosome, nitrite, a major product of NO oxidation, is converted to nitrous acid (HNO_2; $pK_a = 3.16$) (12), which in turn dismutates to NO and NO_2 (10, 13); NO_2 arises also from a reaction of NO with O_2 (14). These observations suggest that the pathogenic mycobacteria could use $F_{420}H_2$ to combat an attack of reactive nitrogen intermediates generated by the activated macrophage. We have tested this hypothesis. The resulting data point toward a mechanism that *M. tuberculosis* may use to counter the bactericidal actions of macrophages.

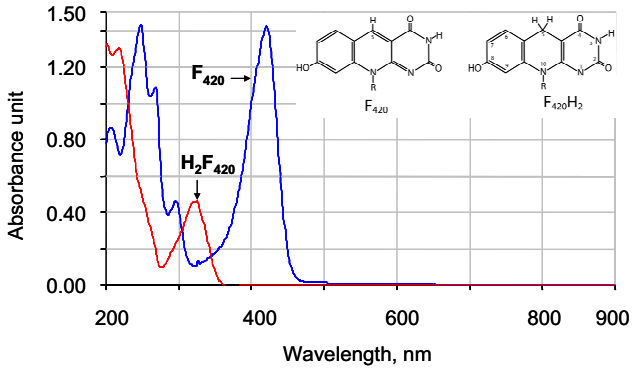

Fig. 1. UV-visible spectra of oxidized and reduced forms of coenzyme F_{420}. F_{420} was reduced with sodium borohydride.

Results and Discussion

In a phosphate-buffered aqueous solution, $F_{420}H_2$ was rapidly oxidized by the NO-releasing compound diethylamine-NONOate (DEA-NONOate) and oxygen (Fig. 2).

$$(C_2H_5)_2N{-}N(O^-){=}N{-}O^- + H^+ \rightarrow (C_2H_5)_2NH + 2NO$$

[Reaction 2]

Neither DEA-NONOate nor O_2 alone was able to perform this reaction (Fig. 2). To ascertain that NO was responsible for oxidizing $F_{420}H_2$, we examined whether the oxidation rate of $F_{420}H_2$ can be controlled by the NO release rate. For this purpose, two other derivatives of diazeniumdiolates, spermine-NONOate and DETA-NONOate, which release NO slowly, were used. The half-lives of DEA-, spermine-, and DETA-NONOate to release NO are 2 min, 39 min, and 20 h, respectively (15). Indeed, with the last two compounds, the $F_{420}H_2$ oxidation was much slower than that with DEA-NONOate. With 80 μM dissolved O_2 and 25 μM $F_{420}H_2$, DEA-NONOate (0.5 mM), spermine-NONOate (0.5 mM), and DETA-NONOate (2.5 mM) oxidized $F_{420}H_2$ at the rates of 17.3, 0.94, and 0.22 μmol min^{-1}

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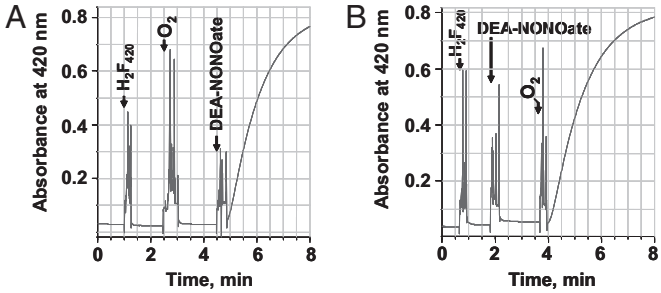

Fig. 2. Oxidation of $F_{420}H_2$ by nitric oxide in the presence of oxygen. Nitric oxide was generated in situ from NONOate derivatives. Into a 1-mL anaerobic solution of 50 mM potassium phosphate buffer (pH 7.0) in a stoppered cuvette with N_2 in the headspace, the following were added to the final concentrations and in the sequence indicated: (*A*) Reduced F_{420} (20 μM), oxygen (80 μM), and DEA-NONOate (320 μM). (*B*) Reduced F_{420} or $F_{420}H_2$ (20 μM), DEA-NONOate (320 μM), and oxygen (80 μM). The time of each addition is shown by an arrow. Oxidation of $F_{420}H_2$ was monitored spectrophotometrically at 420 nm. DEA-NONOate, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide sodium salt; DETA-NONOate, 2,2′-(hydroxynitrosohydrazino)bisethanamine; spermine-NONOate, *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine.

L^{-1}, respectively. These data established that NO was one of the entities involved in the oxidation of $F_{420}H_2$.

To understand the role of O_2 in the NO-dependent $F_{420}H_2$ oxidation, the effect of O_2 concentration on the reaction rate and amount of F_{420} produced at fixed concentrations of DEA-NONOate and $F_{420}H_2$ was studied. The results presented in Fig. 3 show that when DEA-NONOate was present in excess, the rate of $F_{420}H_2$ oxidation and the amount of F_{420} formed increased with the dissolved O_2 concentration.

The above results led to the question of how a combination of NO and O_2 oxidized $F_{420}H_2$. In an aqueous solution, nitric oxide reacts rapidly and spontaneously with oxygen to produce mainly

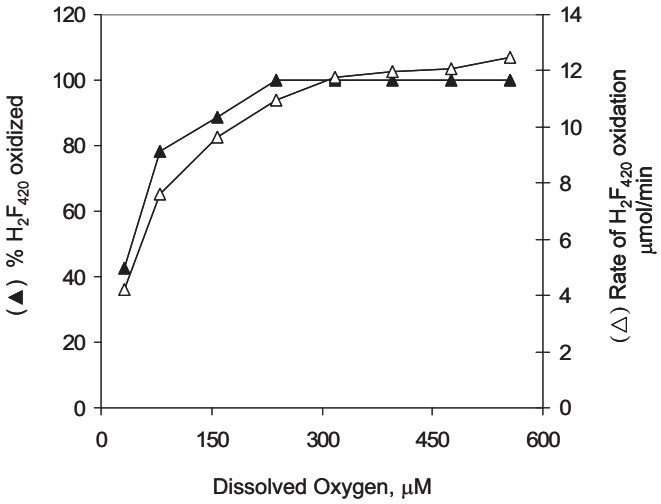

Fig. 3. Reaction rate and amount of F_{420} produced at various dissolved oxygen concentrations and at fixed levels of DEA-NONOate and $F_{420}H_2$. The assay mixture contained 1 mL of anaerobic 100 mM potassium phosphate buffer (pH 7.0), with 20 μM $F_{420}H_2$ and varied volumes of O_2 added to the nitrogen headspace. Before the assay, the mixture was held at room temperature for 15 min to ensure equilibration with the added oxygen. The reaction was initiated by the addition of DEA-NONOate (final concentration, 320 μM) and was followed spectrophotometrically at 420 nm. The dissolved oxygen concentration was calculated by using a value of 43,800 atm/mol fraction for the Henry's constant for oxygen (40).

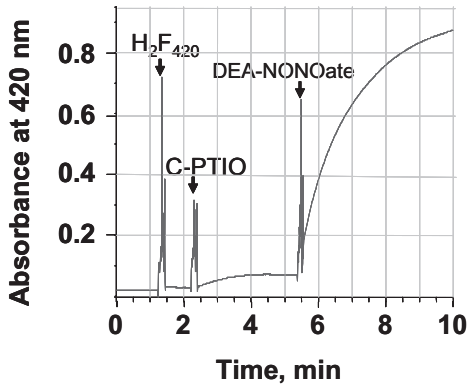

Fig. 4. Oxidation of $F_{420}H_2$ under anaerobic condition by nitrogen dioxide generated from an in situ reaction between carboxy-PTIO and NO (from DEA-NONOate). Into 1 mL of anaerobic 50 mM potassium phosphate buffer (pH 7.0) in a stoppered cuvette with N_2 in the headspace, the following were added: 25 μM $F_{420}H_2$, 100 μM C-PTIO, and 250 μM DEA-NONOate. The point of each addition is shown by an arrow and the name of the compound. Oxidation of $F_{420}H_2$ was monitored spectrophotometrically at 420 nm. Carboxy-PTIO or C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt. Other abbreviations are shown in the legend for Fig. 2.

nitrite, via the intermediate formation of nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) (Reactions **3–5**) (14).

$$2NO + O_2 \rightarrow 2NO_2 \qquad \textbf{[Reaction 3]}$$

$$NO_2 + NO \rightarrow N_2O_3 \qquad \textbf{[Reaction 4]}$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \qquad \textbf{[Reaction 5]}$$

To test whether nitrite was the $F_{420}H_2$-oxidizing agent, in an experiment similar to that represented in Fig. 2, DEA-NONOate was incubated in an aerobic potassium phosphate buffer for 60 min at 25 °C (allowing dissipation of NO, NO_2, and N_2O_3 via Reactions **3–5**), and then this solution was mixed with $F_{420}H_2$. This treatment did not result in $F_{420}H_2$ oxidation. Also, as determined from the UV-visible spectral characteristics, 1 mM sodium nitrite or 1 mM sodium nitrate did not oxidize $F_{420}H_2$. However, the subsequent addition of DEA-NONOate to these aerobic mixtures caused the oxidation of $F_{420}H_2$. Therefore, nitrite or nitrate was neither an $F_{420}H_2$-oxidizing agent nor an inhibitor of $F_{420}H_2$ oxidation. It is unlikely that N_2O_3 was responsible for $F_{420}H_2$ oxidation because this compound is not an oxidizing agent (16). Thus, it was hypothesized that NO_2, a fairly potent oxidant (16), was the compound that oxidized $F_{420}H_2$. This hypothesis was tested as described below.

In the first assay, NO_2 was generated in situ via a reaction between C-PTIO, a nitronyl nitroxide, and NO produced from DEA-NONOate in an anaerobic solution (17). The data in Fig. 4 show that the oxidation of $F_{420}H_2$ was observed only if both DEA-NONOate and C-PTIO were present in the reaction mixture, suggesting that $F_{420}H_2$ was oxidized by NO_2. Because of an overlap between the UV-visible spectra of C-PTIO and $F_{420}H_2$ (17), this system was unsuitable for spectroscopic studies. Therefore, further experiments were performed using pure NO_2 gas. The resulting data demonstrated that NO_2 did indeed oxidize $F_{420}H_2$ directly (Fig. 5). The UV-visible spectra of the reaction mixture (Fig. 5*A*) that was recorded immediately after each addition of NO_2 into an anaerobic solution of $F_{420}H_2$ in 100 mM potassium phosphate buffer (pH 7.0) showed the appearance of the oxidized form of F_{420}. The amount of F_{420} produced was proportional to the amount of NO_2 added into the reaction mixture (Fig. 5*B*); because NO_2 would rapidly be converted to

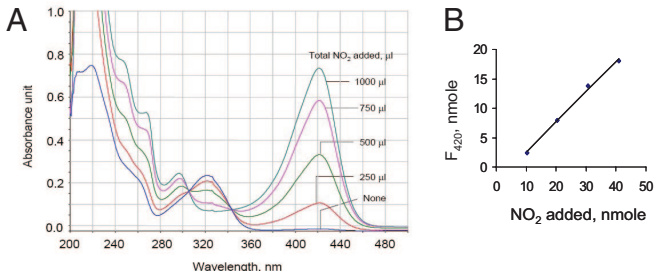

Fig. 5. Oxidation of $F_{420}H_2$ by nitrogen dioxide gas under anaerobic condition. (*A*) To 1 mL of an anaerobic 25 μM $F_{420}H_2$ solution in 50 mM potassium phosphate buffer (pH 7.0) in a 3-mL quartz cuvette, nitrogen dioxide gas was added by using a gas-tight syringe. The UV-visible spectrum of the solution was recorded before NO_2 addition and then after each addition of 250 μL of (10.22 nmol) NO_2. (*B*) Relationship between NO_2 added and F_{420} generated. Note: Most of the NO_2 added was likely converted into NO_2^- and NO_3^-.

nitrite and nitrate in the phosphate-buffered solution we used (Reactions **6** and **7**) (14), the ratio of NO_2 added and F_{420} formed was not 1:1.

$$2NO_2 \rightarrow N_2O_4 \qquad \textbf{[Reaction 6]}$$

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \qquad \textbf{[Reaction 7]}$$

The UV-visible spectral changes shown in Fig. 5*A* clearly indicated that $F_{420}H_2$ was oxidized to F_{420}. This oxidation is known to be a 2-electron or hydride transfer process (2, 18). The observation leads to the hypothesis that $F_{420}H_2$ oxidation was coupled to 2-electron reduction of NO_2 that generated NO (Reaction **8**).

$$F_{420}H_2 + NO_2 \rightarrow F_{420} + NO + H_2O \qquad \textbf{[Reaction 8]}$$

This possibility was tested via measurements with an ISO-NOPF100H NO probe from World Precision Instruments. The addition of NO_2 in the nitrogen headspace of a sealed serum bottle containing anaerobic 100 mM potassium phosphate buffer (pH 7.0) created a high electrical current (Fig. 6) because the NO sensor detects NO_2, albeit with much less sensitivity than that for NO (Michael McIntosh and Xueji Zhang, World Precision Instruments, personal communication). However, the current dissipated quickly because of the conversion of NO_2 to nitrite and nitrate (Reactions **6** and **7**). The overall result was a current spike (Fig. 6). When $F_{420}H_2$ was present in the reaction mixture, a similar current spike was also seen, but the residual current was higher than that recorded with NO_2 alone (Fig. 6). This residual current represented some of the NO generated from NO_2 by $F_{420}H_2$. NO is relatively stable in an anaerobic aqueous solution, but it can be dissipated via Reactions **4** and **5**, if NO_2 is present. Because added NO_2 was lost quickly via Reactions **6** and **7**, only a small amount of NO was generated. However, because of the lack of residual NO_2, this NO was not sequestered and provided a higher residual current. The amount of F_{420} formed in this experiment was proportional to the amount of NO_2 added. Therefore, it was clear that $F_{420}H_2$ reduced NO_2 to NO. Based on the well-established ground-state chemistry of F_{420} (2, 18), we hypothesize that this reduction occurs via a hydride transfer mechanism; this possibility is currently under investigation.

Because F_{420} catalyzes an NAD(P)H-type hydride transfer, the reactivity of the latter toward NO_2 was examined. NADH reacts with NO_2 in the presence of oxygen (19). In this process, NO_2 is reduced to nitrite, and an NAD· radical is produced as an intermediate, and the latter reacts with oxygen to generate superoxide (O_2^-). We found that in an anaerobic assay system of the type shown in Fig. 5, NO_2 does not oxidize NADH or

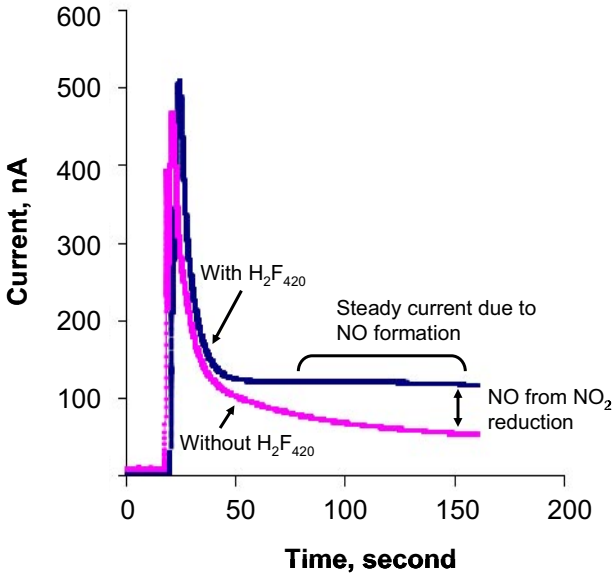

Fig. 6. Nitric oxide production from the reaction of nitrogen dioxide and $F_{420}H_2$. One milliliter of NO_2 (1 atm) was added to the nitrogen headspace of a stoppered, 10-mL serum bottle containing 7.5 mL of anaerobic 100 mM potassium phosphate buffer (pH 7.0) with 20 μM $F_{420}H_2$ (blue) or without $F_{420}H_2$ (pink). NO production was measured by using an NO sensor (ISO-NOPF100H) (World Precision Instruments).

NADPH; in each case the concentration of the reduced cofactor was 160 μM. Therefore, in its ability to reduce NO_2 to NO, $F_{420}H_2$ was distinct from NAD(P)H.

To examine whether nitrogen dioxide caused nitrosation of the phenol moiety of F_{420}, the products from a reaction similar to that shown in Fig. 2*A*, but with $F_{420}H_2$ and DEA-NONOate at 50 and 500 μM concentrations, respectively, were characterized. In a C_{18}-silica column-based reversed-phase HPLC analysis employing a diode array detector (20), the deazaflavin product from the reaction and authentic F_{420} exhibited the same retention time and UV-visible spectrum [supporting information (SI) Fig. S1]. A nitrophenol generated from a reaction between a phenol and NO_2 binds more tightly to C_{18}-silica than the corresponding phenol (21, 22). These results ruled out the possibility of nitrosation of $F_{420}H_2$ with NO_2 or NO.

The ability to use $F_{420}H_2$ in reducing NO_2 to NO would be useful to *M. tuberculosis* in combating the host defense system. Although both NO and NO_2 exhibit antimycobacterial activities, NO_2 is more potent in killing *M. tuberculosis* (23). Therefore, because an activated macrophage produces NO and converts this compound in its acidified phagosomes to NO_2 either via the formation and dismutation of nitrous acid (13) or a reaction of NO with O_2 (14) for bringing about a more aggressive attack, *M. tuberculosis* could use Fgd-generated $F_{420}H_2$ to reduce NO_2 back to NO and lower the effectiveness of the antibacterial action of macrophages. This hypothesis is consistent with the observed hypersensitivity of *M. tuberculosis fbi*C strain to acidified nitrite (10). A mycobacterial cell contains a substrate level of glucose 6-phosphate (24). Therefore, $F_{420}H_2$ produced via the Fgd reaction (Reaction **1**) is an effective vehicle for delivering substantial reducing power for NO_2 detoxification. To provide an experimental validation for this deduction, the effect of NO_2 on the viability of *Mycobacterium smegmatis*, a close relative of *M. tuberculosis* and a nonpathogen, was studied. In this experiment, actively growing cultures of wild-type, *fgd*::*aph*, and Δ*fbic*::*aph* strains of *M. smegmatis* were exposed to NO_2, and then the numbers of the living cells in these cultures were

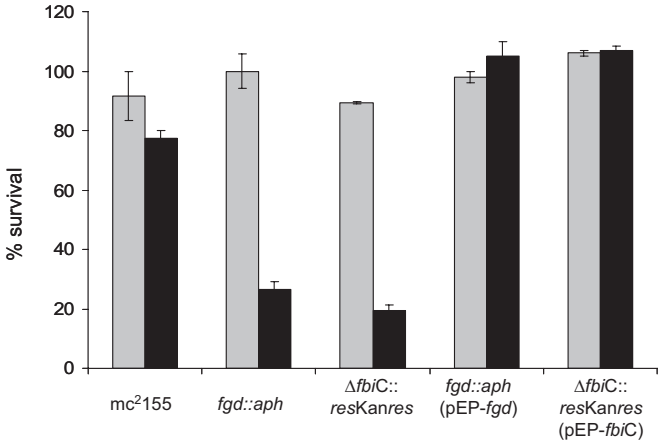

Fig. 7. Requirement for reduced F_{420} for NO_2 detoxification in *M. smegmatis*. The sensitivities of wild-type, *fgd*::*aph*, Δ*fbic*::*aph*, *fgd*::*aph* (pEP-*fgd*), and Δ*fbic*::*aph* (pEP-*fbiC*) strains to NO_2 were studied. In each case, the cells were exposed to NO_2 by incubating a corresponding diluted suspension in growth medium with spermine-NONOate under air; a control culture received water in place of spermine-NONOate solution. In each case, including for the control, the number of surviving cells per milliliter in a culture is presented as a percentage of the value recorded for a sample drawn before incubation with spermine-NONOate (or water); as a result, the value even for the control culture was <100%. Each error bar was calculated from measurements in triplicate. Gray bar, control culture; black bar, NO_2-exposed culture.

determined (Fig. 7). NO_2 was generated in situ through a reaction between dissolved O_2 and NO (derived from spermine-NONOate; half-life, 39 min); an exposure time of 6 h was selected because it provided an observable yet mild effect on the viability of the wild-type cells. The survivability of a strain was expressed in terms of the ratio of the colony-forming units from an NO_2-exposed culture and that from a control culture (without NO_2 exposure). The values for these ratios for the wild-type, *fgd*::*aph*, and Δ*fbic*::*aph* strains were 0.8, 0.25, and 0.2, respectively (Fig. 7). Therefore, an inability to synthesize F_{420} or to produce $F_{420}H_2$ made the organism ≈4-fold more sensitive to NO_2. When the *fgd*::*aph* and Δ*fbic*::*aph* were complemented with Fgd and Fbic expression vectors, respectively, they were able to tolerate NO_2 even better than the wild-type cells (Fig. 7); the increased tolerance was likely caused by above-normal level of $F_{420}H_2$ generated by the elevated levels of FbiC and Fgd proteins. These results established a requirement for $F_{420}H_2$ in the reduction of NO_2. However, it remains possible that the observed phenotypes of the mutant strains were not solely the result of a loss of the chemical reaction described in this report but also in part caused by the lack of the activity of a yet to be identified NO_2 detoxification enzyme that requires $F_{420}H_2$. The overall phenomenon is similar to the inhibition of neoplastic transformation of human cells by γ-tocopherol (25). It has been suggested that the nitrosation of primary amines of DNA bases by NO, which leads to cancer-causing mutations, requires oxidation of this oxide to NO_2 (25). Because γ-tocopherol efficiently reduces NO_2 back to NO, it protects cells from NO_2-induced carcinogenesis (25).

It has been known that PA-824, a bicyclic nitroimidazole and a promising candidate drug for the treatment of tuberculosis, acts as an antimycobacterial agent only when the organism produces F_{420} and reduces it to $F_{420}H_2$ (9, 20, 26). A recent report shows that the basis for this activity is the $F_{420}H_2$-dependent production of NO from PA-824 (27); NO kills *M. tuberculosis* anaerobically. The imidazole ring of PA-824 is reduced with $F_{420}H_2$ by a nitroreductase called Ddn (27). This reaction is followed by the release of the nitro group of the compound as nitrous acid that breaks down to NO (27). Therefore, F_{420}-dependent reactions not only provide a protection to the mycobacteria from nitrosative stresses, but also can be exploited to impose such a stress on these organisms.

In summary, $F_{420}H_2$-dependent NO_2 reduction reaction is a defense tool for the mycobacteria against NO_2 stress. It should be noted that often NO has been cited as the agent that kills *M. tuberculosis* under both aerobic and low-oxygen or hypoxic conditions (28, 29), although, as mentioned above, NO_2 has been shown to be a more potent antimycobacterial agent (23), and it is produced under aerobic conditions (10, 13, 14). Therefore, the $F_{420}H_2$-dependent defense against nitrosative stress would be useful to *M. tuberculosis* when it grows aerobically and causes active tuberculosis.

Materials and Methods

Chemicals. Coenzyme F_{420} was purified from *Methanothermobacter thermautotrophicus* as described (30, 31). 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide sodium salt (DEA-NONOate), *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (spermine-NONOate), 2,2′-(hydroxynitrosohydrazino)bisethanamine (DETA-NONOate), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (carboxy-PTIO or C-PTIO) were from Cayman Chemicals. Sodium borohydride was from Sigma–Aldrich. $F_{420}H_2$ was generated by reducing F_{420} with sodium borohydride in an anaerobic water solution under nitrogen atmosphere in a rubber stopper-sealed glass tube followed by neutralization with hydrochloric acid. Only freshly made $F_{420}H_2$ was used in the experiments. Nitrogen dioxide gas was prepared in microscale (32). The anaerobic techniques used in this work have been described in ref. 30.

$F_{420}H_2$ Oxidation Assays. The assays were carried out as described in ref. 30. A 1-mL anaerobic aqueous solution containing 50 mM potassium phosphate buffer (pH 7.0) and 20 or 25 μM $F_{420}H_2$ under a N_2 headspace in a 12-mm × 75-mm (8.5 mL) KIMAX borosilicate tube (Kimble/Kontes) sealed with a cutoff black butyl rubber stopper no. 00 was used for each assay. To this solution, DEA-NONOates, spermine-NONOate, and DETA-NONOate were added from respective 50 mM anaerobic stock solutions in 10 mM sodium hydroxide. For C-PTIO, a 50 mM stock was prepared in water. $NaNO_2$ or $NaNO_3$ was added from an anaerobic 100 mM stock solution in water. NO_2 was added by using a gas-tight syringe. Oxidation of $F_{420}H_2$ was followed spectrophotometrically at 420 nm (at 25 °C), and the rates were calculated by using a value of 41.4 mM^{-1} cm^{-1} for the extinction coefficient of oxidized F_{420} at this wavelength (31).

NO Measurement Assay. NO produced in an anaerobic $F_{420}H_2$ oxidation–NO_2 reduction assay was measured with an Apollo 1000 single-channel, free radical detector employing an amperometric-type NO probe (ISO-NOPF100H; World Precision Instruments). The data were collected and analyzed by using a WPI DataTrax. The measurement was performed under anaerobic conditions. For this purpose, a 10-mL serum bottle containing 7.5 mL of 100 mM potassium phosphate buffer (pH 7.0), with 25 μM $F_{420}H_2$ was sealed with a 20-mm septum stopper (Bellco Glass), and the content was made anaerobic via 3 cycles of evacuation and pressurization with N_2 (30); a control vial was prepared without $F_{420}H_2$. The final headspace gas was N_2 at 1.3 atm. Then a 1-inch 18-gauge hypodermic needle with a Luer hub (Precision Glide needle; Becton Dickinson), which was shortened to 0.5 inches by cutting part of its tip end, was inserted into the bottle through the rubber stopper. Through this needle the probe was lowered in a manner where 2 mm of the probe tip was inside the buffer; before use, the probe was polarized according to the manufacturer's instructions. After the current output stabilized, NO_2 gas was added to the headspace of the bottle through the rubber stopper by using a syringe, and the probe output was measured. To avoid contamination of air, the syringe was flushed with N_2 before use.

Determining the Sensitivities of M. *smegmatis* to NO_2. A strain to be tested was grown in 7H9-glycerol medium with 0.05% Tween 80 at 37 °C until the early-logarithmic stage of growth. Then a sample of this culture was diluted with fresh medium to a 600-nm optical density of 0.5 [as measured with a Lambda 25 UV-visible spectrophotometer (PerkinElmer)]. One hundred microliters of this diluted culture was added to 900 μL of fresh medium, and this mixture was supplemented with 10 μL of 100 mM spermine-NONOate right after the latter was prepared in water; a control culture received water in place of the spermine-NONOate solution. The tubes with dilute cell suspen-

sions (±spermine-NONOate) were incubated aerobically in 13-mm × 100-mm borosilicate culture tubes (catalog no. 73750-13100; Kimble/Kontes) for 6 h at 37 °C in a C25 incubator shaker (New Brunswick Scientific) operating at 240 rpm. Then the number of surviving cells in each such suspension was estimated via plating on 7H9-glycerol-agar medium and determining the number of colony-forming units.

Construction of *M. smegmatis* Δ*fbiC*::*aph* and *fgd*::*aph* Strains. A Δ*fbiC* strain was generated by replacing the corresponding coding sequence with a kanamycin resistance cassette (*aph*) via homologous recombination (33). For this purpose, a 926-bp upstream region (UP) and 920-bp downstream region (DN) of *M. smegmatis fbiC* coding sequence (Msmeg_5126) were PCR-amplified with the following primer pairs: for the UP element, ccgaggatcctcgagaaggcaagggtctcggtcggcgtggtgag (forward) and gctcgaattccaaagccactcccgaataactccacgag (reverse) (underlined sequences, BamHI and EcoRI sites, respectively); for the DN element, cggccgaattccaccacatccagaccagctggg (forward) and gcccggctcgaggccgtcgaacacgagattca (reverse) (underlined sequences, EcoRI and XhoI sites, respectively). The UP and DN amplicons were digested with the above listed restriction enzymes. These restricted DNA fragments and a *res-Km-res* element that was excised from pCG122 (34) as an EcoRI fragment were cloned into BamHI and XhoI sites of pBluescript to obtain pEPfbiCKO1. Then the *fbiC* knockout construct was excised from pEPfbiCKO1 with XhoI and was cloned into the XhoI site of pJQ200KS (35) to obtain the suicide plasmid pEPfbiCKO2; pJQ200KS does not replicate in mycobacteria, but in an integrated form it allows a *sacB*-mediated sucrose counterselection and confers resistance to gentamicin in mycobacteria. In pEPfbiCKO2, the cloned fragments formed the following arrangement: 5′UP3′…5′*res-Km-res*3′…5′DN3′. Transformation of *M. smegmatis* mc^{2}155 (wild-type strain) with pEPfbiCKO2 via electroporation (36) and selection of the transformants on gentamicin and kanamycin provided a merodiploid strain. By counterselecting segregants derived from the merodiploid strain on sucrose in the presence of kanamycin, a *M. smegmatis* Δ*fbiC*::*res-Km-res* strain was obtained; we call this strain Δ*fbiC*::*aph*. A Southern blot with EcoRI-digested chromosomal DNA and PshAI-digested pEPfbiCKO2 as the probe confirmed this genotype and showed that the mutant did not carry a remnant of pJQ200KS. An *fgd*::*aph* strain was constructed by inserting a kanamycin resistance cassette (Amersham Pharmacia Biotech) as a BamHI fragment at the BglII site of the gene; the details of this construction will be presented elsewhere. A Southern blot analysis confirmed the *fgd*::*aph* genotype of this strain. Assays for F_{420} and Fgd in cell lysates as described (24, 37) showed that the *fgd*::*aph* and Δ*fbiC*::*aph* strains lacked Fgd activity and F_{420}, respectively.

Construction of Complementing of Plasmids for *M. smegmatis fbiC* and *fgd*. The constructs were based on pSMT3, a mycobacteria–*Escherichia coli* shuttle vector that allows selection for hygromycin resistance and expression of cloned genes constitutively from the hsp60 promoter in mycobacteria (38). To generate the *fbiC* complementing plasmid (pEP-*fbiC*), the corresponding coding sequence along with a 222-bp upstream region (bearing the *fbiC* promoter and ribosome-binding site) was PCR-amplified from *M. smegmatis* chromosomal DNA with primers GATATCCGCGGACAGAGACAG (forward) and AAGCTTGACCTCGCGGTGAAG (reverse) (underlined sequence: EcoRV and HindIII sites, respectively) and cloned into EcoRV and HindIII sites of pSMT3; the selection of the upstream element was based on an earlier report (39). Similarly, the Fgd expression plasmid (pEP-*fgd*) was constructed by amplifying the corresponding coding sequence along with the 264-bp upstream region with the primers GGTTTTCGGGATCCAGATGGCAGTGCCGAAG (forward, with a BamHI site) and GAGCACTGCAGCTCGCGCGAGCACCCGCACG (reverse, with a PstI site) and cloning the amplicon into BamHI- and PstI-digested pSMT3.

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