Flavohemoglobin Hmp Affords Inducible Protection for Escherichia coli Respiration, Catalyzed by Cytochromes bo' or bd, from Nitric Oxide*

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Respiration of Escherichia coli catalyzed either by cytochrome bo' or bd is sensitive to micromolar extra-cellular NO; extensive, transient inhibition of respiration increases as dissolved oxygen tension in the medium decreases. At low oxygen concentrations (25–33 μM), the duration of inhibition of respiration by 9 μM NO is increased by mutation of either oxidase. Respiration of an hmp mutant defective in flavohemoglobin (Hmp) synthesis is extremely NO-sensitive (I50 about 0.8 μM); conversely, cells pre-grown with sodium nitroprusside or overexpressing plasmid-borne hmp produce NO and have elevated levels of immunologically detectable Hmp. Purified Hmp consumes O2 at a rate that is instantaneously and extensively (>10-fold) stimulated by NO due to NO oxygenase activity but, in the absence of NO, Hmp does not contribute measurably to cell oxygen consumption. Cyanide binds to Hmp (Kd 3 μM). Concentrations of KCN (100 μM) that do not significantly inhibit cell respiration markedly suppress the protection of respiration from NO afforded by Hmp and abolish NO oxygenase activity of purified Hmp. The results demonstrate the role of Hmp in protecting respiration from NO stress and are discussed in relation to the energy metabolism of E. coli in natural O2-depleted environments.

The pervasive importance in biology of nitric oxide, i.e. the nitrogen monoxide radical, NO, written here as NO, (1, 2) is now widely appreciated. NO plays key roles in vasodilation and intracellular signaling, yet is toxic (3). Microbiologically, NO is important as an intermediate in denitrification (4) and as a component of the antibacterial arsenal of reactive oxygen and nitrogen species generated in phagolysosomes (5). NO and redox-related species such as the nitrosium cation (NO+) and the nitroxy1 ion (NO•−) (6) are reactive with many key biomolecules including iron proteins (7), copper proteins (8), and thiol groups (9). NO is a well established and experimentally useful ligand for heme proteins; its reactions with globins and oxidases from diverse higher organisms have been extensively investigated but assumed to have little physiological significance (10). Recently, however, the hemoglobin-NO reaction has been shown to regulate the chemistry of NO and maintain it in a circulating bioactive state (11).

Reaction of NO with the mitochondrial terminal oxidase, cytochrome aa3, has been shown to involve not only the O2-binding heme (cytochrome aa3) but also the copper atom (CuA) (8, 12) which together constitute the O2-reducing binuclear active site of the enzyme. E. coli does not have cytochrome aa3 but synthesizes two other major respiratory quinol oxidases, cytochromes bo’ and bd (13, 14). Cytochrome bo’ is structurally and functionally homologous to mitochondrial cytochrome aa3 and binds two molecules of NO at CuA (15). Cytochrome bd reacts with nitrite and trioxodinitrate to form a spectrally distinctive nitrosyl complex (16–18). Since cytochromes bo’ and bd have distinct structures and catalytic properties, including sensitivity to cyanide (14), and are differentially regulated (13, 14), they might be expected to be differentially sensitive to NO, particularly since cytochrome bd lacks the NO-reactive copper site (13, 14). Indeed, such a discrimination has been reported in plant mitochondria (19), in which NO inhibits oxygen consumption by cytochrome aa3 but not by the cyanide-insensitive, non-phosphorylating, alternative oxidase. To date only the respiration of Escherichia coli possessing both oxidases has been shown to be inhibited by NO (20).

E. coli responds at the level of gene expression to NO, NO-releasing agents, and nitrosating compounds in at least two ways. Nunoshiba et al. (21) have demonstrated that NO activates the ssoRS two-component global regulatory system which results in increased synthesis of superoxide dismutase and other enzymes that provide protection from oxidative stress. However, the physiological function(s) of this system in resisting NO is unclear. Second, NO, nitrite, the nitrosating compound sodium nitroprusside (SNP) and S-nitrosothiathanine (GSNO) are potent inducers of hmp gene expression in E. coli (22, 23) and Mycobacterium tuberculosis (24). The E. coli gene product HMP is a flavohemoglobin first proposed by Poole et al. (22) to detoxify NO. Recent studies support this proposal: mutants carrying a poorly defined deletion in the glnB-hmp region of the genome are more sensitive to growth inhibition by NO than are control strains (25). Such mutants are also unable to catalyze NAD(P)H-dependent NO consumption (25, 26) and...
are compromised in inducible resistance to nitrosative stress exerted by S-nitrosocysteine (SNO-Cys) (26). Unequivocal evidence that the hmp gene itself is responsible for aerobic resistance to NO and nitrosative stress has come from construction of genetically defined hmp mutants. An E. coli K12 hmp mutant (RKP4545) was shown to be hypersensitive to killing by GSNO and SNP (27) and a Salmonella hmp mutant showed increased sensitivity to acidified nitrite and S-nitrosothiols (28).

The roles of Hmp in protecting growth and aconitate activity from NO (27, 29) have been largely attributed to the NO oxygenase activity of the protein. NO is proposed to attack oxygenated Hmp (Fe(III)-O2) to form the relatively innocuous nitrate ion (25, 26). The additional ability of Hmp to sequester NO and reduce it to N2O (30) may explain the protective effects of Hmp observed during anaerobic or microaerobic growth (25) and may be of greater importance in natural environments of E. coli, including the gut (31). Thus, Hmp, a member of the ancient globin family, plays key roles in resisting NO in several bacteria and probably other microorganisms (32).

The aim of this work was to determine the sensitivity to NO of respiration catalyzed by each of the major respiratory oxidases of E. coli, cytochromes bo and bd. We show that NO inhibits both oxidases but that expression and activity of Hmp provides effective protection in vivo. Since the natural environment of E. coli in the gut is microaerobic (31), where NO inhibition of respiration is maximal (Ref. 20, this work), it is likely that Hmp plays a critical role in enterobacterial growth and survival.

**Experimental Procedures**

**Strains, Media, and Growth Conditions**—Strains and the plasmid used are listed in Table I. Transformations were done after CaCl2 treatment (35). Cells were grown in rich medium (TY) (36) supplemented as appropriate with kanamycin (50 μg/ml) or ampicillin (100 μg/ml). Culture optical density at 600 nm (A600) was measured with a Jenway 6100 spectrophotometer in cells of 10-mm path-length after appropriate dilution. Cultures were grown at 37 °C with shaking (200 rpm) in baffled conical flasks containing about 1/5 of their own volume of medium, and inoculated with 1% of their volume using an overnight culture.

**Treatment with NO, SNP, GSNO, and Paraquat—**NO was prepared as in Ref. 22 and GSNO synthesized as described in Ref. 23. SNP was from Sigma. Non-sterilized solutions of these species were added to a culture 1.5 h after inoculation; after 3 h further growth, the cultures were harvested and used to prepare cell extracts.

**Purification of Hmp—**E. coli strain RSC521 (with multicopy plasmid pPL341, having the wild-type hmp gene under the control of its native promoter) (34) was grown aerobically, disrupted in a French pressure cell, and used to purify Hmp by two chromatographic steps (37). An improved purification protocol was used for the Hmp employed in the experiment shown in Fig. 7.2 Enzyme concentration was measured from spectra of the native (ferric) enzyme using the absorption coefficients described by Ioannidis et al. (37).

**Preparation and Use of Anti-Hmp Polyclonal Antibodies—**Rabbits were administered subcutaneous injections with a homogeneous mixture (250 μl) of 2.7 mg of purified Hmp in Freund’s complete adjuvant. Subsequently, two booster injections of similar composition were administered at 3 and 7 weeks, respectively, after the first injection. At the end of the procedure, rabbits were sacrificed, and bled out for serum. E. coli cells were disrupted by ultrasonication using an MSE/Sanyo Soniprep 150 sonicator delivering three 30-s periods at full power, with 30-s cooling intervals. Extracts were clarified by centrifugation at 132,000 × g for 1.5 h at 4 °C and used in Western blots (39); detection was done using the ECL chemiluminescence system (Amerham Pharmacia Biotech).

**Determination of Respiration Rates and the Effects of NO—**Cells were grown for 6 h as above until A600 reached approximately 1.4. Cells were harvested by centrifugation, washed in 0.9% sterile saline, and resuspended in 5 ml of buffer containing HEPES (50 mM, pH 7.4), 100 mM NaCl, 5 mM KCl, and 1 mM each of MgCl2, NaH2PO4, N-glucose, and CaCl2 (20). A Clark-type polarographic oxygen electrode system (Rank Bros., Betcham, Cambridge, UK) was used comprising a water-jacketed (37 °C) Perspex chamber stirred magnetically; the membrane-covered electrode was situated at the bottom of the chamber below the stirrer. About 25–50 μl of cell suspension was diluted in the chamber with buffer to give a working volume of 2 ml and a close-fitting lid, with a fine hole for injections using a Hamilton syringe, was inserted. The suspension was further supplemented with glucose (10 μM final concentration) and respiration rates measured in the closed system. Additions of NOx to NO-saturated solutions were made in the same way. The electrode was calibrated with air-equilibrated water (taken to contain 220 μM O2) and on adding sodium dithionite to achieve anoxia.

**Measurement of NO Oxygenase Activity—**The above oxygen electrode system was modified to permit simultaneous measurements of O2 and NO consumption.2 Briefly, the chamber top was sealed with a Perspex cap having a concave bottom surface and drilled with a central vertical hole (6 mm in diameter to a depth of approximately 33 mm, and 2.5 mm diameter for the remaining 11-mm depth) to accept an ISO-NOP2 stainless steel shielded NO electrode of 2-mm diameter (World Precision Instruments). The electrode shaft was fitted with a plastic sleeve to hold the membrane at the required depth in the chamber. A second vertical hole (approximately 1.5-mm diameter, 44-mm depth) near the cap permitted addition of solutions of NADH, enzymes, and NO. Current from the O2 electrode was amplified and displayed on a two-channel recorder, along with the amplified current of the NO electrode, (polarizing voltage of 0.865 V) processed using a World Precision Instruments ISO-NO Mark II Isolated Nitric Oxide Meter. Experiments with purified Hmp were performed in a buffer containing 50 mM MOPS and 50 mM NaCl, pH 7.0.

**Visible Electronic Spectroscopy of Cells—**Spectra for determining the cytochrome composition of mutant strains were obtained using an SDB-4 dual-wavelength scanning spectrophotometer (University of Pennsylvania Biomedical Instrumentation Group, and Current Designs Inc., Philadelphia, PA) (40). Cells were centrifuged from stationary phase cultures, suspended in 0.1M potassium phosphate buffer, pH 7.4, and used to record dithionite-reduced minus persulfate-oxidized difference spectra (CO or dithionite minus dithionite difference spectra (41)). Spectral data were analyzed and plotted using Soft/SDB (Current Designs Inc.) and CA-Cricket Graph III software. To assess the presence or absence of cytochrome bo’ photodissociation spectra (photolysed, i.e. reduced minus unphotolysed, i.e. CO-ligated) were obtained at -100 °C in the above apparatus (42).

**Hmp Reaction with Cyanide—**Purified Hmp is largely in the ferric state (37). Protein (final concentration typically 3–4 μM) was incubated in 1 ml of 50 mM MOPS/NaOH buffer, pH 7.0, containing 50 μM potassium ferricyanide to ensure complete conversion to the ferric form. Cyanide was added as a solution of NaCN, adjusted to pH 7.0, to give the final concentrations shown under “Results” and the samples (1 ml final volume) incubated at 4 °C overnight. Transition of the high-spin form to the low-spin cyanide complex was measured as ΔΔ at 423–387 nm.

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RESULTS

NO Sensitivity of Respiration Catalyzed by Cytochromes bo' and bd in E. coli—Among the many possible targets for the cellular toxic effects of NO and related reactive species, respiratory heme enzymes are prime candidates (8, 12, 19). Yu et al. (20) have shown that respiration of E. coli is also sensitive to NO in an O₂-dependent fashion, with inhibition increasing as O₂ tension decreases. Since whole cell respiration in the range of O₂ tensions used in that study has contributions from the activity of both cytochromes bo' and bd, each having a kᵣ₉ for O₂ in the submicromolar range (43, 44), we tested separately the NO sensitivity of cytochrome bo' and cytochrome bd-mediated respiration. A set of isogenic strains was used in which either cytochrome bd (strain AN2343, cydD) or bo' (strain RKP4544, cyo) was absent. The phenotype of the strains was confirmed by room temperature absorption difference spectroscopy (cydD) or low temperature photodissociation spectroscopy (cyo); in the latter case absence of the characteristic 415/430 nm Soret bands of cytochrome o' was confirmed (not shown). Strain AN2342 is a wild-type strain having both oxidases. Injection of NO solutions to respiring cell suspensions immediately inhibited oxygen uptake but the degree of inhibition was strongly dependent on the oxygen tension in the reaction chamber at the time of NO addition (Fig. 1A). Thus, when 9 μM (final concentration) NO was added to a respiring cell suspension in which the O₂ concentration was 33 μM (bottom trace), inhibition of respiration was virtually instantaneous and the residual respiration rate was only about 4% of the control value. After about 3 min, respiration resumed at a rate that slightly exceeded the initial rate and proceeded linearly until the oxygen in the chamber was depleted, reflecting the high oxygen affinities of the oxidases. However, when NO was added at successively higher dissolved O₂ tensions, the extent of inhibition markedly decreased (e.g. to 25% of the control rate at 120 μM O₂) and the period of inhibition was decreased. Qualitatively similar results were obtained with strain RKP4544 (cyo, not shown) and strain AN2343 (cydD, Fig. 1B), although in both of these strains the period of respiratory inhibition at low O₂ tensions (25–33 μM) was always significantly greater than in the wild-type strain (Fig. 2A). Thus total respiratory capacity, rather than the properties of individual oxidases, may be the main determinant of NO inhibition of respiration at low oxygen tensions. At a fixed O₂ concentration, the period of inhibition by NO was dose-dependent (shown for the wild-type strain only in Fig. 2B). In view of the marked effects of O₂ tension on the NO sensitivity of cell respiration, in subsequent experiments NO was added at predetermined O₂ tensions, which are indicated on Figs. 3, 4, and 5.

Protective Role of Hmp in Vivo—Since Hmp is spectrally undetectable in non-induced E. coli and the levels of Hmp requiring β-galactosidase activity are low (22, 23), it might be assumed that normal levels of Hmp are not functionally significant in protection against NO. To test this, we measured the NO sensitivity of respiration in strain RKP4545, carrying a defined hmp null allele (27). Fig. 3A shows that the response of this mutant to added NO was, as in wild-type (hmp+) strains (Fig. 1, A and B), dependent on oxygen concentration at the time of NO addition. However, in marked contrast to hmp+ strains, the period of inhibition elicited by 9 μM NO was much greater (Figs. 2A and 3A). For example, at 100 μM O₂, the period of inhibition elicited by 9 μM NO in all three hmp+ strains was about 1 min, but was about 12 min in the hmp mutant (Fig. 2A). Similarly, when increasing concentrations of NO were added at a fixed O₂ concentration (60 μM) to respiring hmp mutant cells (Fig. 2B) the period of NO inhibition was considerably greater in the case of the hmp mutant. These results show that even basal levels of Hmp provide temporary NO resistance and that the NO inhibition data shown in Fig. 1 and previously (20) include a contribution from the protective effect of Hmp. However, in the absence of NO, the respiratory rates of wild-type and hmp mutant strains were not significantly different (<5%) in numerous experiments, demonstrating that the superoxide-generating O₂ consumption by Hmp in non-induced cells (45, 46) does not contribute significantly to cellular O₂ uptake. The hmp knockout mutant (Fig. 3B) allowed us to assess the true inhibition of oxidase activity by NO in vivo, if it is assumed that Hmp catalyzes the major protective activity. Fig. 3C shows that half-maximal inhibition of respiration in this strain was achieved at extracellularly added NO concentrations around 0.8 μM.

Protective Effects of Hmp in Adapted Cells and with High hmp+ Copy Number—To test directly the hypothesis that the Hmp protein confers protection from NO inhibition, we used plasmid pPL341 which results in overexpression of Hmp leading to cells colored brown-pink by the presence of excess globin (37). Compared with wild-type cells (Fig. 4A, trace 1), cells containing this plasmid were insensitive to addition of 9 μM NO (Fig. 4A, trace 2). Much higher NO concentrations (60 μM) added at higher O₂ tensions, to allow for the extensive subsequent O₂ uptake (Fig. 4A, inset), caused an immediate increase.

FIG. 1. Sensitivity of respiratory oxygen consumption of E. coli cells to NO. Measurements of respiration of washed cell suspensions (approximately 7 mg of cell protein/ml) of E. coli wild-type strain AN2342 (A) and the Cyd− strain AN2343 (B) were made in an oxygen electrode apparatus. A solution of NO was added at the arrows to give a final concentration in each case of 9 μM NO. The O₂ concentration (μM) at the point of each addition is shown alongside the arrow that marks the point of NO addition. Other figures on the traces are respiration rates expressed as nanomole of O₂ consumed per min/mg of cell protein. The experiment was repeated at least 4 times with similar results.

A

B

![Graphs showing respiration rates expressed as nanomole of O₂ consumed per min/mg of cell protein for E. coli wild-type strain AN2342 (A) and the Cyd− strain AN2343 (B).](image-url)
in O2 consumption, attributable to NO oxygenase activity of Hmp, in both wild-type cells (trace 5) and cells containing pPL341 (trace 6). However, whereas in the former case this phase was followed by a period of respiratory inhibition (trace 5), Hmp-overexpressing cells exhibited respiration that was completely insensitive to 60 μM NO (trace 6).

When wild-type cells were treated during growth with SNP, which markedly up-regulates Φ(hmp-lacZ) expression (27), sensitivity of respiration to NO was largely, but not entirely, abolished (compare traces 1 and 2 in Fig. 4A). pPL341-containing cells grown with SNP exhibited respiration that was also insensitive to 9 μM NO (Fig. 4A; trace 4). The extreme NO sensitivity of hmp mutant cells was overcome by introducing plasmid pPL341 (Fig. 4B; compare traces 7 and 8). However, growth of the hmp mutant with SNP did not overcome the prolonged inhibition of respiration by NO (trace 9); the slight (<20%) reduction in the period of inhibition compared with trace 8 may be the result of a marginally higher O2 concentration, slight differences in cell concentration, and/or up-regulation by SNP of an unknown gene that confers modest NO tolerance.

To date, the effects of NO, GSNO, and SNP on flavohemoglobin expression and function have been restricted to studies on Φ(hmp-lacZ) expression (22, 23) and hmp mRNA accumulation (27). To demonstrate that the resistance of cells treated with SNP (Fig. 4A, trace 2) is due to increased Hmp protein synthesis, we assayed Hmp levels with polyclonal antibodies raised against the purified protein. In Fig. 4C, track 1 was loaded with purified Hmp as control. Significant overexpression of the same protein was achieved in wild-type cells transformed with pPL341 (track 2) compared with wild-type cells (track 3). Growth with SNP (track 4) or GSNO (track 5) also raised Hmp to clearly detectable levels. The higher protein expression levels observed in cells containing pPL341, compared with cells treated with SNP, is consistent with the somewhat greater NO resistance of the former cells (see Fig. 4A). No Hmp was detected in extracts from an hmp mutant (track 6).

Cyanide Is a More Potent Inhibitor of Hmp Detoxifying Activity Than of Oxidase-mediated Respiration—The O2-reactive hemes of Hmp and terminal oxidases bind CN− (14, 37). Protection from NO inhibition of respiration was much more sensitive to cyanide than was respiration itself. Fig. 5 shows that even 100 μM cyanide was without measurable effect on whole cell respiration (trace 2), yet markedly increased the susceptibility of cells to a subsequent addition of 9 μM NO (trace 3), compared with cells to which cyanide had not been added (trace 1). Cyanide at 200 μM (trace 4) partially inhibited respiration after a delay, and further increased susceptibility to 9 μM (final

**Fig. 2.** Dependence on O2 and NO concentrations of NO sensitivity of respiration of E. coli mutants. Measurements of respiration of washed cell suspensions of E. coli cells were made as described in the legend to Fig. 1. The period of inhibition is calculated as the period between addition of the NO solution and the point obtained by extrapolation of the residual respiration rate (after inhibition was relieved) and the inhibited rate (see Fig. 3A for an example). In A, the NO concentration was 9 μM throughout and in B the O2 concentration was 62 μM throughout. Shown are results for the wild-type (○), the cyo mutant (●), the cydD mutant (□), and the hmp mutant (△). Similar results were obtained in four experiments.

**Fig. 3.** Sensitivity of respiratory oxygen consumption in an hmp mutant. Measurements of respiration of washed cell suspensions of E. coli strain RKP4545 (hmp) were made in an oxygen electrode apparatus. In A, a solution of NO was added at the arrows to give a final concentration in each case of 9 μM NO. The O2 concentration (μM) at the point of each addition is shown alongside each addition. The uninhibited respiration rate in each trace was 58 nmoles O2 consumed per min/mg of cell protein. The dashed lines in the top trace in A shows the method of calculation of the period of inhibition. In B, lower concentrations of NO (2.5, 1.0, and 0.5 μM, top to bottom) were titrated to the cell suspension, and the initial inhibition is shown. The O2 concentration (μM) at each addition was 60 μM; traces are offset for clarity. C shows the inhibition of respiration calculated from the steady state respiration rate immediately after addition of NO relative to the rate immediately before addition of the NO solution, as a function of NO concentration. The experiments were repeated at least three times with similar results.
concentration) NO (trace 5). The period of respiratory inhibition by NO increased almost linearly with cyanide concentration in the range 20 (3 min) to 200 μM (9 min) (not shown). Cyanide Reaction with Purified Hmp—To test directly the assertion (29) that Hmp is highly cyanide sensitive, we performed titrations of purified Hmp with cyanide solutions. Transition of the high-spin form to the low-spin cyanide complex was measured as ΔA at 423–387 nm. The transition was also accompanied by formation of an asymmetrical band at 553 nm and loss of the charge transfer band in the visible spectrum (not shown); measurements made in the visible region gave essentially the same conclusions as illustrated in Fig. 6 for the Soret band. In Fig. 6A, the fractional saturation of cyanide-binding sites was calculated by dividing the absorbance of the heme-CN species at each concentration of added cyanide by the absorbance observed at very high cyanide concentrations (about 6 mM). Free cyanide was determined using the measured absorbance coefficient (37) to calculate bound cyanide and subtracting this from the total cyanide added. The fitted curve gave a Kd of 2.96 μM and a Hill coefficient of 1.43. The Hill plot (see inset) suggests an n value of 1.3. The Scatchard plot (Fig. 6B) indi-
cates positive cooperativity of cyanide binding. Similar values were obtained from measurements in the α-region (not shown).

**NO Oxygenase Activity and Its Sensitivity to Cyanide—**Direct evidence for inhibition by cyanide of NO oxygenase activity was sought. Hmp consumes O₂ with a kₘ of about 2 μM as measured by deoxygenation of oxymyoglobin (46) and reduces O₂ to superoxide. This has been detected by superoxide dismutase-sensitive reduction by Hmp of cytochrome c (47), dismutation to peroxide,² and by the effects of Hmp on Φ[ΔαΔlacZ] up-regulation in vivo (48). Hausladen et al. (26) showed that addition of NO to purified Hmp in aerated buffer results in enhanced O₂ uptake but the concentrations of NO used (100 μM) were approximately 10-fold higher than those necessary to inhibit whole cell respiration (Fig. 1). Addition of a solution of NO (final concentration 36 μM) to purified Hmp oxidizing NADH in aerated buffer (Fig. 7A) immediately and markedly stimulated O₂ consumption from a basal activity of 150–450 nmol of O₂ min⁻¹ (mg Hmp protein)⁻¹. Further additions of NO at successively lower O₂ concentrations in the chamber each resulted in accelerated O₂ uptake followed by a decline in rate to pre-NO levels within 20 s. The increases in respiration were too rapid to follow at these concentrations of protein but greatly exceeded the 2-fold increase in rate described in Ref. 26. At O₂ concentrations near or above 100 μM, the NO electrode registered small transient responses significantly lower than anticipated from calibration of the apparatus with anoxic NO solutions in the absence of Hmp (not shown). At successively lower O₂ concentrations, however, the size of the NO signal increased (Fig. 7A). The lifetime of NO (measured as the time required for the NO electrode signal to decline to half its maximal intensity) also dramatically increased below 100 μM O₂ (Fig. 7A, inset). The ratio of O₂ uptake to NO added was 1–1.2 over the range 50–210 μM O₂ (Fig. 7A, inset).

In the absence of Hmp, additions of NO elicited slower uptake of O₂ due to the non-enzymatic reaction of NO with O₂ in solution to give nitrite (23), with a ratio of O₂ to NO added of about 0.4 (not shown). Likewise, addition of cyanide prior to NO (Fig. 7B) reduced the extent of NO-stimulated O₂ uptake (O₂/NO ratio approximately 0.4) and markedly increased the half-life of the added NO at all O₂ concentrations (Fig. 7B, inset).

**DISCUSSION**

*E. coli* is not a denitrifying bacterium and is generally considered not to produce NO from reduction of nitrate or nitrite (13). Nitrite reduction yields ammonia (13) although some NO accumulation has been reported during nitrate respiration (49). We have added NO solutions extracellularly and assume that NO penetration through cell membranes to O₂-reducing sites is facile (50). Respiration catalyzed by either cytochrome bd or bd is sensitive to NO. At O₂ tensions above the kₘ for O₂ of these oxidases, neither oxidase provides NO-insensitive respiration, despite the absence of a redox-active copper at the active site of cytochrome bd. The degree of inhibition increased at low oxygen concentrations (Fig. 1), as noted previously for *E. coli* wild-type cells (20), and plant mitochondria (19). Thus, aerobic respiration of *E. coli* in the microaerobic contents of the gut (31) will be highly sensitive to NO. Under such conditions, oxidative phosphorylation could be supported by anaerobic respiration, these pathways being up-regulated anaerobically by the global transcriptional regulator and [4Fe-4S] cluster-containing protein Fnr (51). Interestingly, hmp expression in *E. coli* is repressed by Fnr, as revealed by measuring Φ[hmp-lacZ] activity in an fnr mutant, and confirmed by inspection of the hmp promoter to reveal Fnr-binding sites (22). We have recently demonstrated that an Fnr homologue, CydR in *Azobacter vinelandii*, is sensitive to NO, site-specific DNA binding being prevented by low concentrations of NO (52). It follows

![Fig. 7. NO oxygenase activity of purified Hmp and its sensitivity to cyanide. A, to a solution containing 0.3 μM Hmp and 0.5 mM NADH, NO (final concentration 36 μM) was added at each vertical arrow and the electrode responses to O₂ (top trace) and NO (bottom trace) recorded. The traces are not corrected for the 4-s delay in the NO recorder channel. The inset shows the measured O₂/NO ratio (□) and the half-life of the NO signal (■). In B, a similar experiment is shown in which 100 μM cyanide was added 10 min before the first NO addition.](image-url)
that, if *E. coli* Fnr is also inactivated by NO, as is likely, the effect of NO in a microaerobic or anaerobic environment would be to inactivate Fnr, thus achieving maximal up-regulation of Hmp synthesis and enhancing NO tolerance.

Prolonged exposure to NO of mitochondria (53) causes a gradual and persistent inhibition of complex I that results from S-nitrosylation of critical thiols. Nevertheless, the reversibility of inhibition of respiration by NO shown in this work has also been reported in *E. coli* (20), and plant (19) and animal (53, 54) mitochondria. The differential sensitivity of Hmp and respiration to cyanide allowed us to explore the nature of the oxygen uptake that was invariably observed after transient inhibition of respiration by NO (see Figs. 1, 3, and 4). Despite the presence of 100 or 200 μM cyanide, recovery of respiration after NO inhibition was complete (e.g. Fig. 5, trace 3). Thus, the respiration that occurs after transient inhibition by NO cannot be attributed to the highly cyanide-sensitive O2 consuming activity of Hmp, and is presumably due largely to terminal oxidase activity. It is possible, however, that rates of O2 uptake observed after transient inhibition that are higher than those before adding NO (e.g. in Figs. 1 and 4) are due to the combined activities of (a) oxidase-catalyzed respiration, relieved of significant inhibition by Hmp-catalyzed detoxification of NO, and (b) substantial O2 consumption by Hmp stimulated by residual, subtoxic concentrations of NO. Supporting this is the fact that cells lacking Hmp (Fig. 3) exhibit post-inhibition rates of O2 consumption that never exceed those before NO addition. NO inhibits total cellular O2 consumption by >95% confirming the minor contribution made by Hmp to cellular O2 consumption in the absence of NO.

Inhibition of respiration, measured as either the degree or duration of inhibition, was proportional to NO concentration in both Cyd− and Cyd− mutants. Temporary cessation of respiration with 9 μM NO was observable in strains having wild-type (non-induced) levels of Hmp, irrespective of the oxidase component (Figs. 1 and 2). In the absence of Hmp (Fig. 2 and 3), inhibition was markedly increased and half-maximal inhibition was achieved at NO concentrations around 0.8 μM NO (Fig. 3C). It is difficult to assess the extent to which bacterial respiration will be inhibited by NO in natural environments, given the difficulties of measuring NO in such situations, the sustained production of NO and its balance with NO consumption rates, and since the effects of NO are also strongly dependent on O2 concentrations. NO synthase activities in murine macrophages of 1.4–2.2 μmol/h/10^6 cells have been reported (55), whereas bulk NO concentrations in soils are reported to be around 0.1 μM (56). Gastric NO concentration has been reported near 60 μM (57). Thus, under microaerobic conditions, the enhanced toxicity of NO observed here is to be expected. In the microaerophilic bacterium *Helicobacter pylori*, substantial generation of superoxide seems sufficient to react with added NO to form peroxynitrite, which is an irreversible inhibitor of respiration (38). In *E. coli*, protection from NO is afforded by the NO reductase activity of Hmp (30) and perhaps, in natural microaerobic environments, other unrecognized mechanisms.

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