Flavohemoglobin Hmp, but Not Its Individual Domains, Confers Protection from Respiratory Inhibition by Nitric Oxide in Escherichia coli*

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Escherichia coli possesses a two-domain flavohemoglobin, Hmp, implicated in nitric oxide (NO) detoxification. To determine the contribution of each domain of Hmp toward NO detoxification, we genetically engineered the Hmp protein and separately expressed the heme (HD) and the flavin (FD) domains in a defined hmp mutant. Expression of each domain was confirmed by Western blot analysis. CO-difference spectra showed that the HD of Hmp can bind CO, but the CO adduct showed a slightly blue-shifted peak. Overexpression of the HD resulted in an improvement of growth to a similar extent to that observed with the Vitreoscilla heme-only globin Vgb, whereas the FD alone did not improve growth. Viability of the hmp mutant in the presence of lethal concentrations of sodium nitroprusside was increased (to 30% survival after 2 h in 5 mM sodium nitroprusside) by overexpressing Vgb or the HD. However, maximal protection was provided only by holo-Hmp (75% survival under the same conditions). Cellular respiration of the hmp mutant was instantaneously inhibited in the presence of 13.5 μM NO but remained insensitive to NO inhibition when these cells overexpressed Hmp. When HD or FD was expressed separately, no significant protection was observed. By contrast, overexpression of Vgb provided partial protection from NO respiratory inhibition. Our results suggest that, despite the homology between the HD from Hmp and Vgb (45% identity), their roles seem to be quite distinct.

Flavohemoglobins have been identified in a number of bacteria and yeast (1–4). These proteins consist of an N-terminal heme binding domain integrated with a flavin binding reduction domain. Despite the high degree of conservation among these proteins in a variety of organisms and the biochemical characterization of yeast (5) and bacterial (6) examples, their function remained elusive for many years. A proposal for the involvement of flavohemoglobins in protection from NO stress was first prompted by the observation of a significant increase in hmp transcription in response to exogenous NO in Escherichia coli (7). This was later supported by (a) the increased sensitivity to NO-releasing agents of defined hmp mutant strains of E. coli (8) and Salmonella enterica serovar typhimurium (9–10), (b) biochemical evidence showing that the purified Hmp protein has NO dioxygenase or denitrosylase activity, forming nitrates under aerobic conditions (11–13), and (c) the additional ability of Hmp to sequester NO and reduce it to N2O anaerobically (12, 14). Together these observations strongly suggested that one of the main functions of flavohemoglobins is to provide protection from the toxicity of NO. Indeed, S. enterica serovar typhimurium flavohemoglobin-deficient mutants are impaired in pathogenicity due to their increased sensitivity to the NO-related killing process exerted by macrophages (10). Recently, it was reported that heterologous expression in E. coli of flavohemoglobins from Pseudomonas aeruginosa, S. enterica serovar typhi, Klebsiella pneumoniae, Deinococcus radiodurans, or Ralstonia eutropha also confers resistance to nitrosative and oxidative stresses (15).

The electron transport chain of aerobically grown E. coli contains two different quinol oxidases, cytochromes bo’ and bd, which reduce oxygen to water (16). Cytochrome bo’ is the predominant terminal oxidase during fully aerobic conditions, and its oxygen affinity is relatively low (Km < 1 μM (17)). Conversely, cytochrome bd is synthesized under low oxygen conditions and has an extremely high oxygen affinity (Km 3 to 8 μM (18)). Respiration catalyzed by either cytochrome bo’ or bd is sensitive to NO (19) so that, at oxygen tensions above the Km for O2 of these oxidases, neither oxidase provides NO-insensitive respiration, and the degree of NO inhibition increases at low oxygen tensions (19). Significantly, we demonstrated that the flavohemoglobin Hmp from E. coli provides effective protection in vivo from NO respiratory inhibition (19).

Some bacteria, such as Vitreoscilla sp. and Campylobacter jejuni, do not synthesize flavohemoglobins but possess single-domain hemoglobins lacking the FAD-containing domain (3, 4, 20–21). Vitreoscilla hemoglobin Vgb accumulates to high levels...
under microaerophilic conditions (22). Significant enhancement of growth and production of recombinant proteins and antibiotics is observed when vgb is expressed in E. coli, Streptomyces coelicolor, and Streptomyces lividans (23–25), and numerous other beneficial effects on culture growth and metabolism have been described (for review, see Ref. 4). The proposal that the function of Vgb is facilitation of $O_2$ delivery for respiration (25) has received considerable recent support from the finding that Vgb interacts specifically with the $O_2$-reducing subunit of the cytochrome $bo'$ terminal oxidase (26). However, roles in nitrosative and oxidative stress responses have also been suggested for one-domain hemoglobins (27). Whatever the role of Vgb, it has been assumed that the globin interacts with a cognate, separately encoded, reductase that is functionally analogous to the C-terminal domain of flavohemoglobins. Indeed, such a reductase has been purified from Vitreoscilla and characterized (28). Presumably, another reductase(s) assumes a similar role when Vgb is expressed in heterologous hosts.

In this communication we describe genetic engineering of the Hmp flavohemoprotein from E. coli to synthesize separately each domain and the effect of expressing them on growth, oxygen metabolism, and protection from inhibition of respiration by NO. Our results with the heme domain of the E. coli Hmp (flavohemoglobin) differed from those obtained using the Vitreoscilla sp. Vgb single-domain hemoglobin.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Growth Conditions**—The strains and plasmids used are listed in Table I. Transformations were done after CaCl$_2$ treatment (32). Cells were grown in rich medium (LB) (33) supplemented as appropriate with kanamycin (50 $\mu$g/ml) or ampicillin (200 $\mu$g/ml). Culture optical density at 600 nm (apparent $A_{600}$) 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 conical flasks containing about $\frac{1}{5}$ of their own volume of medium and inoculated with 1% of the culture volume using an overnight culture.

**Construction of Plasmids** pPL341 and pPL341AHD—Plasmid pMA2000U (34) was used for cloning a 1309-bp EcoRI-BamHI fragment containing the entire hmp structural gene plus its own promoter region from plasmid pPL304 (30), to yield plasmid pMAHmp. Single strand phagemid pMAHmp was obtained by infection with helper phage M13 RP408. Insertion of a stop codon (TAA) and an NdeI restriction site (see Fig. 1) was carried out by using a site-directed mutagenesis kit (Amer sham Biosciences) using the 5'-end phosphorylated primer RKPhmp1 (5'-GCCGAGCAAGCCCTAACATATGCGTGTTGGGAA-3'), where italics indicate the introduced stop codon (TAA) and the underlined bold indicates the NdeI site (Fig. 1B); the annealing step was performed following the manufacturer’s instructions. Extension and religation of the complementary strand was carried out by using the Klencow fragment of DNA polymerase and T4 DNA ligase (Amer sham Biosciences). The resulting mutated plasmid was called pMAHmp (see Fig. 2). NdeI digestion of this plasmid precisely excised the heme domain (HD) of the hmp gene. This plasmid was religated, and then the BamHI-EcoRI fragment was cloned in the cloning vector pBR322 digested with the same enzymes to yield plasmid pPL341AHD (see Fig. 2). This plasmid contains only the flavin (FD) domain of Hmp starting at codon 148 (see Fig. 1). On the other hand, partial NdeI-XmnI1 digestions of the pMAHmp plasmid allowed the excision of the flavin domain (Fig. 2), yielding the plasmid pPL341AFD. It is important to note that both constructs are under the control of the native hmp promoter and that both conserve an 11-bp repeat at the 3'-end that has been suggested to be the terminator sequence of hmp (30). Both constructs were verified by sequencing using Sequenase version 2.0 (U. S. Biochemical Corp.; data not shown).

**Treatment with NO, Sodium Nitroprusside (SNP), and Methyl Viologen (PV)—NO was prepared as in Poole et al. (7). SNP was from Sigma. Solutions were added to a culture 1.5 h after inoculation ($A_{600} = 0.5$), samples were taken at the stated times, and serial dilutions were performed to determine viability. Results were expressed as the percentage of viable cells present in the control culture without any treatment.

**Preparation and Use of Anti-Hmp Polyclonal Antibodies—Antibody polyclonal antibodies were obtained as described in Stevanin et al. (19). Western blot detection was done using the ECL chemiluminescence system (Amer sham Biosciences).**

**Determination of Cell Respiration Rates and the Effects of NO—Cells were grown for 6 h as described above until $A_{600}$ reached ~1.4. Cells were harvested by centrifugation, washed in sterile 0.9% saline, and resuspended in about 5 ml of buffer containing MOPS (50 mM, pH 7.4) and 50 mM NaCl. A Clark-type polarographic oxygen electrode system (Rank Bros, Bottisham, 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 $\mu$l of cell suspension was diluted in the chamber

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1 The abbreviations used are: HD, heme domain; FD, flavin domain; SNP, sodium nitroprusside; PV, paraquat or methyl viologen; MOPS, 4-morpholinopropanesulfonic acid.
with MOPS-NaCl 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 mM final concentration), and respiration rates measured in the
closed system.

Preparation of Cell-free Extracts and Assay of NO Denitrosylase
Activity—Cells were harvested by centrifugation at 6000 \times g for 20 min.
The cell pellet was washed and resuspended in 0.1 M phosphate buffer,
pH 7.0. The cell suspension was sonicated three times in an ice bath
using an MSE Soniprep 150-watt sonicator. Each sonication was for 1
min, with a 30-s interval between successive sonications. Cell debris
and unbroken cells were removed by centrifugation at 77,000
\times g for 20 min.

Visible Electronic Spectroscopic Analysis—Spectra for characteriza-
tion and quantification of hemoproteins in cells and cell-free extracts
were obtained using an SDB-4 dual-wavelength scanning spectropho-
tometer (University of Pennsylvania Biomedical Instrumentation
Group and Current Designs Inc., Philadelphia, PA) (35). Cells were
centrifuged from stationary phase cultures, suspended in 0.1 M phosphate buffer,
pH 7.0. The cell suspension was sonicated three times in an ice bath
with MOPS-NaCl buffer to give a working volume of 2 ml and a

RESULTS

Construction of Plasmids Carrying the Heme or the Flavin
Domain of Hmp Separately—The E. coli flavohemoglobin consists
of two domains, an NH\textsubscript{2} heme domain (HD) that is homol-
logous (45% identity; 67% similarity; Fig. 1A) to the single
domain Vgb Vitreoscilla hemoglobin, and a COOH flavin do-
main (FD) that belongs to the ferredoxin-NAD\textsuperscript{+} reductase
(FNR) family of reductases (30, 37). Several reports implicate
Vgb in the improvement of growth when the globin is heterolo-
gously expressed in other organisms (4, 24–25), and more im-
portantly, many of its functions overlap with those of flavohe-
moglobins (23). To examine whether the heme domain from the
Hmp protein of E. coli could play a similar role to that of the
Vgb protein, we genetically engineered the
hmp gene so that
each domain of the protein could be expressed separately. First,
we determined the length of the heme domain by comparing
the structural features of the Vgb and the Hmp proteins, taking
advantage of the available structures of Vgb (38) and of the
flavohemoglobin from E. coli (39) and R. eutropha (40). We
determined that the heme domain extends up to residue 146
(Fig. 1). Plasmid pMAHmp carrying the whole hmp gene but
with the addition of 1) an Ndel restriction site, 2) a TAA codon
that stops translation at residue 146, and 3) an ATG starting
codon at residue 147 was constructed according to the proce-
dures described under “Experimental Procedures” (Figs. 1B
and 2). The new Ndel site allowed the excision of the HD or the
FD domain to yield plasmids pPL341\textsubscript{HD} and pPL341\textsubscript{FD},
respectively (Fig. 2).

Expression of the Separate Domains of Hmp Encoded by
Plasmids pPL341\textsubscript{HD} and pPL341\textsubscript{FD}—Polyclonal Hmp an-
tibodies were used to detect Hmp expression in high speed
cell-free extracts prepared from cells of strain RKP4545 (hmp:
Th5, hereafter referred to as hmp) carrying different plas-
mids. Western blot analysis of extracts from RKP4545 cells,
which harbored plasmid pPL341 (hmp\textsuperscript{+}), showed a band of 44
kDa, corresponding to the Hmp polypeptide, and a smaller
weak band that might be due to a degradation product (Fig. 3, lane 1). When extracts from RKP4703 cells were used, which harbored plasmid pPL341ΔHD, a 28-kDa band (corresponding to the expected size of the FD domain of Hmp) was evident (Fig. 3, lane 2). Similarly, extracts from RKP4702 cells, which harbored plasmid pPL341/H9004FD, displayed only one band of 16 kDa, the expected size of the heme domain (Fig. 3, lane 6). As expected, extracts from strain RKP4545 (hmp/H11002, no plasmid) did not react with Hmp antibodies (Fig. 3, lane 3).

To test whether the hmp constructs present in plasmids pPL341ΔHD and pPL341ΔFD were regulated in a similar fashion to the wild-type gene, we grew cell cultures of strain RKP2206 (wild type) and RKP4702 (hmp/H11002, harboring plasmid pPL341/H9004FD) in the absence or in the presence of 50 μM SNP, a potent inducer of hmp (41). Cell extracts were obtained and subjected to Western blot analysis. Extracts from wild-type strain RKP2206 displayed a 44-kDa band that increased markedly in intensity in the presence of SNP (Fig. 3, compare lanes 4 and 5). Likewise, when extracts from cultures of strain RKP4702 (hmp/H9004FD, harboring plasmid pPL341) treated with SNP were used, an increase in the intensity of the 16-kDa reactive band was observed (Fig. 3, compare lanes 6 and 7). Taken together, these results demonstrate that plasmids pPL341ΔHD and pPL341ΔFD encode truncated versions of the Hmp protein and that the regulation of the genetic constructs remains unaltered.

CO-difference Spectroscopy of Cell-free Extracts and Whole Cells from Cultures of Strains Carrying Plasmids pPL341ΔHD and pPL341ΔFD—To confirm the identity of the proteins expressed from the mutated hmp gene and determine whether heme was still incorporated into the ΔFD construct, cultures of strains RKP4701 (hmp−, harboring plasmid pPL341ΔHD), RKP4702 (hmp−, harboring plasmid pPL341ΔFD), RKP4703 (hmp−, harboring plasmid pPL341ΔHD), RKP4545 (hmp−), and RKP2206 (wild type, no plasmid) were grown in LB media, and difference spectra (CO + reduced – reduced) were run on the whole cells in a dual wavelength spectrophotometer (Fig. 4A). Cell-free extracts were also prepared and centrifuged at 77,000 × g to remove membrane-associated terminal oxidases (cytochromes bo and bd) that would confound the signals of
Hmp. Glucose was used to reduce the whole cells (Fig. 4A), and sodium dithionite was used to reduce cell-free extract samples (Fig. 4B).

CO-difference spectra of whole cells from all strains showed a signal with a peak at ~421 nm and a trough at ~437 nm. The similarity of spectra 1 to 5 in Fig. 4A suggests that the signals are due not only to Hmp (42) but also to the presence of other CO-binding proteins in whole cells (Fig. 4A). By contrast, CO-difference spectra of the cell-free extract of strain RKP4701 (Fig. 4B, trace 1) showed strong signals at 421 nm (peak) and 439 nm (trough), very similar to the band positions observed with purified Hmp (42). Strain RKP4702, which expressed only the heme domain, also gave an intense signal similar to that seen with Hmp, but with a slightly blue-shifted peak at 419 and a broad trough with a \( \lambda_{\text{min}} \) of 430.5 nm (Fig. 4B, trace 2). Strain RKP4703, which expressed only the flavin domain, and mutant strain RKP4545 (Fig. 4B, traces 3 and 4, respectively) revealed no detectable CO-binding hemoproteins. These results suggest that neither strain RKP4545 nor RKP4703 expresses the heme domain, which is the site of oxygen and CO binding, and that the remaining heme domain produced by plasmid pPL341FD could still bind CO, albeit with altered spectral characteristics.

**Effect of the Expression of Plasmids pPL341\(\Delta HD\) and pPL341\(\Delta FD\) on the Growth Physiology of E. coli**—The benefits of expressing the single-domain hemoglobin Vgb for the growth physiology of different microorganisms and several eukaryotes have been widely reported (4, 24–25). Vgb is 45% identical and 67% similar to the heme domain of Hmp (Fig. 1). To determine whether strains carrying plasmids pPL341\(\Delta HD\) and pPL341\(\Delta FD\) display alterations in growth physiology, such as those reported for cells overexpressing Vgb, we grew cultures of the hmp\(^-\) (RKP4545) strain transformed with different plasmids in LB media at 37 °C under aerobic conditions. As shown in Fig. 5A, differences in growth behavior became apparent after ~10 h of cultivation, probably suggesting that, beyond that point, oxygen concentration or utilization became growth-limiting (Fig. 5A). \( A_{600} \) values at this time point gave a clear indication that the strains expressing the holo-Hmp protein, the heme-only Hmp, or the Vgb hemoglobin grew significantly better relative to the control strain RKP5107 or RKP5108 carrying the pBR322 or pUC18 control plasmids. However, for strains carrying plasmids pPL341 (hmp\(^-\)) and pPL341\(\Delta FD\), the growth improvement was less pronounced than for the strain expressing Vgb. No growth improvement was observed for cells harboring the pPL341\(\Delta HD\) plasmid (Fig. 5A). These results demonstrate a slight growth improvement for all strains expressing hemoglobin from a plasmid-borne gene.

**Sensitivity to PQ and SNP of Cells Carrying Plasmids pPL341\(\Delta HD\) and pPL341\(\Delta FD\)**—Hmp and many other bacterial types of hemoglobin (including single-domain hemoglobins) have been implicated in detoxification of NO and related species (for review, see Refs. 2–4). To test whether the truncated domains of Hmp still confer resistance to nitrosative stress, cultures of strain RKP4545 (hmp\(^-\)) carrying different plasmids were grown to mid exponential phase (\( A_{600} = 0.5 \)) and treated with 1 mM SNP, and changes in the growth curves were recorded. As shown in Fig. 5B, RKP4545 cells transformed with either pUC18 or pBR322 suffered an abrupt cessation of growth after the addition of SNP. Cells carrying pPL341\(\Delta HD\) were also severely affected. However, strain RKP4545 (hmp\(^-\)) bearing plasmids pPL341 (holo-Hmp), pPL341\(\Delta FD\) (heme only), or pUC8:16 (vgb\(^+\)) showed an increased resistance to the SNP stress relative to the vector controls. Importantly, Vgb and holo-Hmp consistently conferred more resistance to SNP than did plasmid pPL341\(\Delta FD\) (heme only) over the first 30 h of culture, whereas cells bearing plasmid pPL341\(\Delta FD\) restarted growth only after 20–30 h of SNP treatment (Fig. 5B). This delayed tolerance is not understood, but the protein structural differences revealed by CO-difference spectra (Fig. 4B) may impede the protective response of the heme-only Hmp protein. Clearly, however, the heme-only form of Hmp is not as effective in conferring SNP tolerance as Vgb or the holo-Hmp.

It is well established that a defined hmp mutant strain is impaired in its response to NO and to the superoxide-generating agent PQ (8). To determine whether the truncated versions of Hmp produced by the plasmids pPL341\(\Delta HD\) and pPL341\(\Delta FD\) could increase viability in an hmp\(^-\) background on challenge by SNP, we treated exponentially growing cultures of strains RKP4701 (hmp\(^-\) but harboring pPL341 hmp\(^-\)), RKP4703 (hmp\(^-\) but harboring pPL341\(\Delta HD\)), RKP4702 (hmp\(^-\) but harboring pPL341\(\Delta FD\)), RKP5108 (pBR322 control), RKP5107 (pUC18 control), and RKP5104 (harboring pUC8:16 vgb\(^+\)) with a lethal concentration of SNP (5 mM). As shown in Fig. 5C, maximal protection was provided by expression of plasmid pPL341 (hmp\(^-\); 70% survival after 120 min). The plasmids encoding the heme domain of Hmp (pPL341\(\Delta FD\)) or the Vgb protein maintained viability to a similar extent (30% after 120 min). Surprisingly, the FAD domain of Hmp was more effective because survival was 40% after 120 min. By contrast, control experiments with cells carrying cloning vectors pUC18 or pBR322 displayed no viable counts after 120 min of exposure to SNP. This suggests that the heme domain of Hmp and the
Vgb protein prevents killing by SNP by similar mechanisms. Interestingly, when PQ sensitivity was tested, cells expressing Vgb displayed an increased sensitivity when compared with cells carrying each of the single domains or the holo-Hmp (data not shown), suggesting that PQ resistance can be conferred by Hmp but not by Vgb (results not shown).

**Effects of NO on Respiration and the NO Denitrosylase (Oxygenase) Reaction of Strains Carrying Plasmids (pPL3413HD and pPL3413FD)—Hmp protects cellular respiration catalyzed by cytochrome bo or cytochrome bd from the toxic effects of NO (19). To determine the extent of the contribution of each domain of Hmp, whole cell suspensions were prepared of strains RKP4701 (pPL341 hmp−), RKP4702 (pPL3413FD), RKP4703 (pPL3413HD), and RKP4545 (hmp−). Known amounts of cells were added to a closed oxygen electrode chamber as described under “Experimental Procedures.” Because the toxicity of NO is dependent on dissolved O2 concentration (19), additions of 36 μM NO were made at two separate oxygen tensions (at 160–180 and 70–90 μM oxygen). Fig. 6A shows the oxygen consumption traces. In the case of strain RKP4701 (hmp−, but harboring pPL341 hmp+), the addition of NO gave no inhibition of oxygen uptake (trace a). This indicates that Hmp is able to detoxify NO before it is able to inhibit respiration of whole cells, as we reported previously. On addition of NO to whole cells of strains RKP4702 (ΔFD; trace b), RKP4703 (ΔHD; trace c), and RKP4545 (hmp−, no plasmid; trace d) there followed a period of inhibition of respiration. Similar experiments were carried out using strains RKP5109 (hmp−, but harboring pUC8:16 vgb+) and RKP5107 (hmp− and with vector control; Fig. 7). Increasing concentrations of SNP progressively inhibited cell respiration. The presence of plasmid-encoded vgb+ did not markedly affect the tolerance of wild-type E. coli to SNP inhibition. An hmp mutant containing only the plasmid vector (without vgb+) was hypersensitive to SNP, but expression of vgb+ restored resistance. These results show that either Hmp or Vgb can protect cell respiration from SNP. Interestingly, cells overexpressing Vgb appeared to be slightly more sensitive to respiratory inhibition by SNP than the wild-type strain (Fig. 7); this may be due to the production of superoxide that has been previously reported in cells overexpressing Hmp (43). By contrast, neither the Hmp heme nor FAD domains separately were able to provide protection to the respiratory chain. This indicates that both domains are necessary for respiratory protection by Hmp.

To test whether the separate domains of Hmp conserve the NO denitrosylase activity, we determined the oxygen consumption in cell-free extracts using NADH as the electron donor. NADH was added after establishing no oxygen uptake before the addition (Fig. 6B). NO (13.5 μM) was added at each of two separate oxygen tensions (160–180 and 70–90 μM), and the resulting mean O2/NO ratio at each addition was calculated. RKP4701 (expressing holo-Hmp) showed an O2/NO ratio of 1.1 (mean of 4 determinations, S.D. 0.17), indicative of NO denitrosylase activity, i.e. the stoichiometric consumption of O2 and NO to yield nitrate (11–13). In further quadruplicate experiments, strains RKP4702 (ΔFD; ratio 0.4, S.D. 0.05), RKP4703 (ΔHD; ratio 0.6, S.D. 0.13), and RKP4545 (hmp−; ratio 0.5, S.D. 0.21) all showed much lower O2/NO ratios, indicating that Hmp with the presence of both domains is necessary for full NO denitrosylase activity. The low ratio seen with RKP4545 (hmp mutant strain) was expected, and the ratio was similar to the O2/NO ratio seen on the addition of NO to MOPS buffer only (44). Taken together, these results demonstrate that both the heme and flavin domains are necessary for efficient detoxification of NO by Hmp.

**DISCUSSION**

Reactive oxygen and nitrogen species are effective molecules for bactericidal action against invading pathogens (45). The best understood microbial response to nitrosative stress involves flavohemoglobins (2), but these proteins have been also implicated in oxidative stress responses in bacteria (8) and yeast (46). Interestingly, single domain hemoglobins such as Mycobacterium tuberculosis HbN (47) and Vgb from Vitreoscilla have also been implicated in NO detoxification processes (15, 27). It has been suggested that, because Vgb interacts with a reductase from Vitreoscilla (48), this globin may function in a manner similar to flavohemoglobin. The NADH-cytochrome c reductase has been shown to constitute an electron-transferring path for the oxidation of NADH and to increase the oxygen uptake severalfold in Vitreoscilla (21, 28). When Vgb is functionally expressed in heterologous hosts (for review, see Ref. 4), it seems probable that it interacts in such cells with an unidentified reductase(s) appropriate for trans-
ferring electrons to the globin heme, allowing turnover and ligand chemistry. The wide variety of beneficial consequences that overexpression of Vgb displays has motivated many researchers to genetically modify its structure. A reductase domain from the Fhp \((R. eutropha)\) flavohemoglobin was genetically linked to Vgb with an improvement in the beneficial effects on microaerobic bacterial growth (15, 23, 27). Expression of a double Vgb-Vgb hemoglobin also enhanced growth and alters ribosome and tRNA levels in \(E. coli\) (49).

The marked shift in the spectral properties of the heme in the \(\Delta H9004\) FD construct (Fig. 4B, trace 2) points to substantial changes in the environment of the heme affecting ligation and/or spin state. Whereas the CO spectrum of the native Hmp protein (Fig. 4B, trace 1) resembles that of the pure protein (42) and myoglobin (50), i.e. a peak near 420 nm and a trough near 440 nm, both bands in the \(\Delta H9004\) FD protein are blue-shifted. Indeed, the peak (419 nm) and trough (430.5 nm) render the spectrum much more similar to that of oxidases of the cytochrome \(o\)-type (peak at 415 nm, trough at 432 nm) (50, 51) than to Vgb (peak at 419 nm, trough at 437 nm) (50). The closeness of the trough position to 427 nm and the blue-shifted band of the CO adduct suggest some low spin character (48), but the \(\Delta H9251\)/\(\Delta H9252\) regions are insufficiently resolved in spectra of crude extracts for further discussion on this point. Nevertheless, the growth data in Fig. 5A demonstrate that, despite the modified ligand binding of the \(\Delta F D\) construct (Fig. 4B, trace 2) points to substantial changes in the environment of the heme affecting ligation and/or spin state. Whereas the CO spectrum of the native Hmp protein (Fig. 4B, trace 1) resembles that of the pure protein (42) and myoglobin (50), i.e. a peak near 420 nm and a trough near 440 nm, both bands in the \(\Delta F D\) protein are blue-shifted. Indeed, the peak (419 nm) and trough (430.5 nm) render the spectrum much more similar to that of oxidases of the cytochrome \(o\)-type (peak at 415 nm, trough at 432 nm) (50, 51) than to Vgb (peak at 419 nm, trough at 437 nm) (50). The closeness of the trough position to 427 nm and the blue-shifted band of the CO adduct suggest some low spin character (48), but the \(\alpha/\beta\) regions are insufficiently resolved in spectra of crude extracts for further discussion on this point. Nevertheless, the growth data in Fig. 5A demonstrate that, despite the modified ligand binding of the \(\Delta F D\) construct (Fig. 4B), this protein has properties that enhance growth, perhaps by involvement in oxygen metabolism. Furthermore, the \(\Delta F D\) construct conferred resistance to inhibition and killing by SNP (Figs. 5, B and C). Therefore, despite the altered spectral properties, the \(\Delta F D\) construct is functional in these assays yet does not protect cell respiration from NO or exhibit NO consumption (Fig. 6).

The goal of our experiments was to express separately the heme and FAD domains of the flavohemoglobin Hmp of \(E. coli\) and study the effects of these constructs on growth, oxygen metabolism, and the protection of respiration from NO and nitrosative stress. Surprisingly, expression of the heme domain of \(E. coli\) Hmp had effects quite different in some respects to
Heme and FAD Domains of Flavohemoglobin Hmp

those observed with Vgb. Thus, in contrast to holo-Hmp, the heme-only truncated Hmp failed to provide protection from NO to the respiratory chain in E. coli (Fig. 6A). However, Vgb, holo-Hmp and the heme-only truncated Hmp all conferred resistance to growing cells from SNP (Fig. 5, B and C). Frey et al. (15) also report that several diverse hemoglobins were equally holo-Hmp and the heme-only truncated Hmp all conferred re-

is shown not to be absolutely required for all globin functions. Furthermore, although the Hmp holoenzyme provided protection from PQ-killing, neither Vgb nor the single heme or FAD domains of Hmp did so (results not shown). It has been suggested that Vgb arose during the course of evolution by excision of the FAD-containing reductase domain from a flavohemoglobin (52). The present growth data (Fig. 5) and the alleviation of nitrosative stress provided by the heme domain from the R. eutropha flavohemoglobin, Vgb, and a single-domain globin from C. jejuni (15) lend experimental support to this proposal, since an “on-board” reductase domain is shown not to be absolutely required for all globin functions.

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