Role for the Salmonella Flavohemoglobin in Protection from Nitric Oxide*

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Hemoglobin homologs are being identified in an expanding number of unicellular prokaryotic and eukaryotic organisms. Many of these hemoglobins are two-domain proteins that possess a flavin-containing reductase in their C terminus. Determination of a function for these flavohemoglobins has been elusive. A Salmonella typhimurium strain harboring a deletion in the flavohemoglobin gene shows no difference in growth under oxidative stress conditions but does display an increased sensitivity to acidified nitrite and S-nitrosothiols, both of which produce nitric oxide. The effect is seen aerobically or anaerobically, indicating that oxygen is not required for flavohemoglobin function. These results suggest a role for the bacterial flavohemoglobins that is independent of oxygen metabolism and provide evidence for a bacterial route of protection from nitric oxide that is distinct from oxidative stress responses.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF020388.

1 The abbreviations used are: hmp, flavohemoglobin; GSNO, S-nitrosothiols; SNAC, S-nitrosoylated N-acetylcysteine; PCR, polymerase chain reaction; bp, base pair(s); WT, wild type.
**FIG. 1.** *S. typhimurium* hmp locus and deletion of hmp. **A**, using a primer from the published upstream *S. typhimurium* glyA sequence (21) and a degenerate primer based on the C terminus of the *E. coli* hmp (22), the *S. typhimurium* hmp gene was amplified by PCR from ATCC *S. typhimurium* strain 14028s genomic DNA. The insertion of the Kan<sup>R</sup> cassette into the hmp gene and subsequent creation of the hmp<sup>-</sup> strain was performed as described.SalI sites (S) used in panel B are shown. **B**, for Southern analysis, 10 µg of SalI-digested genomic DNA was probed with an hmp<sup>R</sup>-labeled PCR product.

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**EXPERIMENTAL PROCEDURES**

Cloning and Deletion of the *S. typhimurium* hmp—All PCR reactions used KlenTaq/LA (W. Barnes, Barnes Biotech, St. Louis, MO). Using the RN1 primer (5'-GATCGAATTCATGTTGAGC-GCCAAACAT-3') from the published upstream *S. typhimurium* glyA sequence (21) and a degenerate primer RN4 (5'-GATCGGATCCCNAG-NAC/C/TTTAGC/GTNGGNC/GA/GA/A/G/C/A/C/T/TG/A/G/T/A/3') based on the C terminus of the *E. coli* hmp (22), the *S. typhimurium* hmp gene was amplified by PCR from ATCC *S. typhimurium* strain 14028s genomic DNA. The product was cloned into the TA cloning vector (Invitrogen) and found to contain an open reading frame homologous to other hmp genes. For creation of an hmp insertion/deletion construct, PCR products were generated using SKO1/SKO2 primers for hmp upstream/5' sequence and SKO3/SKO4 primers to hmp 3' sequence. The sequences of the knockout primers are as follows: SKO1, 5'-GATCGAATTCATGTTGAGC-GCCAAACAT-3'; SKO2, 5'-GATCGC-CGGGGTTAGAATGTGGCCGGTACAG-3'; SKO3, 5'-GATCGGATCCCTTACAAGACCATACCCGAAC-3'; SKO4, 5'-GATCGGATCCTTA-GTGAAAGAAAGGCATA-3'.

The two PCR primers internal to the gene (SKO2/SKO4) were created with *SmaI* sites to facilitate the ligation of the 1.4-kilobase pair kanamycin resistance cassette from pUC-KIXX (Amersham Pharmacia Biotech), creating the final knockout construct (pSKO). This construct was transduced by phP2 into the genome of 14028s (23), creating the hmp<sup>-</sup> strain. For Southern analysis, 10 µg of *SmaI*-digested genomic DNA was probed with an hmp<sup>α</sup>-labeled PCR product (24). The blot was stripped and reprobed with the Kan<sup>R</sup> cassette to confirm site-specific integration. To recover the DNA sequence downstream of hmp, the 4.3-kilobase pair SalI fragment from the hmp<sup>-</sup> strain was cloned using Kan<sup>R</sup> as selection and partially sequenced. A native hmp expression construct was created by PCR using SKO1 (213 bases upstream of the hmp start codon) and SH4: (5'-GTTAAAGAAAGGACATA-AAAGAAGCAGC-3'), an antisense oligo starting 48 bp downstream of a predicted 12-bp inverted repeat, which is centered 37 bp downstream of the hmp stop codon. The product was cloned into the Xhol/HindIII site of pUHE21–SalCP (25), making pShmp. The hmp nucleotide sequence was deduced from sequencing three separate clones of pShmp (26), all of which were identical and deposited in GenBank.

Materials—All chemicals were purchased from Sigma. S-Nitrosothiols were made as described previously (27). Briefly, the S-nitroso-derivatives of glutathione (GSNO) or N-acetylated cysteine (SNAC) were prepared by making 1 mM solutions of the thiol in 1 N HCl and adding an equal volume of 1 M sodium nitrite. M9 minimal salts medium (pH 7.0): 7 g of Na<sub>2</sub>HPO<sub>4</sub>, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of NH<sub>4</sub>Cl, 0.5 g of NaCl, 2 g of glucose, 120 mg of MgSO<sub>4</sub>, 15 mg of CaCl<sub>2</sub>, and 40 mg of thiamine/liter. For plates, agar was added to 1.5%. When needed, ampicillin (100 µg/ml) or kanamycin (50 µg/ml) was added to the medium.

Growth Conditions—For nitrite growth curves, 1 × 10<sup>6</sup> cells of overnight cultures grown in LB-ampicillin were diluted into 250-ml culture flasks with 100 ml of fresh LB-ampicillin medium containing various concentrations of sodium nitrite. Cells were shacked at 225 rpm at 37 °C, and growth curves were measured by a Klett colorimeter with a red filter. Minimal inhibitory concentrations for oxidative stress compounds and NO derivatives were obtained by taking overnight cultures grown in LB and diluting in phosphate-buffered saline. 1 × 10<sup>6</sup> bacteria were then added to a test tube with 1 ml of M9 medium containing the inhibitory compound, which varied in concentration from tube to tube by 2-fold. The tubes were incubated with shaking at 37 °C. The minimal inhibitory concentration is the concentration of the compound where no visible growth occurred after 24 h. For plate cultures, 10<sup>6</sup> cells of overnight cultures grown in LB-ampicillin medium were washed in phosphate-buffered saline and streaked onto M9-ampicillin plates containing 10 mM GSNO. For liquid growth curves, 10<sup>6</sup> cells of overnight cultures grown in LB-ampicillin medium were washed in phosphate-buffered saline and streaked onto M9-ampicillin plates containing 100 mM of LB-ampicillin medium. The cells were then sealed with a two-holed stopper and ultrapure argon (Cee-Kay, St. Louis, MO) was vigorously bubbled into the medium for 30 min. The flasks were then incubated without shaking at 37 °C. Anaerobic indicator strips were used (Becton Dickinson Microbiology Systems) to confirm a proper seal.

**RESULTS**

To determine if flavohemoglobins are involved in protection from oxidative and/or nitric oxide-mediated stress, we cloned and deleted the hmp gene of *S. typhimurium*. The coding region of the *S. typhimurium* hmp predicts a two-domain hemoglobin/reductase protein with 94% amino acid identity to the *E. coli* *hmp* (22). As in *E. coli*, the gene upstream of the *S. typhimurium* hmp is the divergently transcribed glyA, encoding...
serine hydroxymethyltransferase (21). Sequence similarity to the E. coli hmp locus, however, abruptly halts after the hmp stop codon. Instead of glnB (22), the cadC gene, a regulator of lysine decarboxylase in E. coli (28), is directly downstream of the S. typhimurium hmp (Fig. 1A). The hmp gene does not appear to be part of an operon with cadC. The 189-bp intergenic region contains a 12-bp inverted repeat downstream of the hmp thought to terminate the hmp transcript and standard basal promoter elements immediately upstream of cadC (not shown). For phenotypic characterization, we created a strain wherein most of the hmp coding region was replaced with a Kan^R cassette. This was confirmed by Southern blot analysis (Fig. 1B).

No detectable growth differences between the deletion (hmp^-) and wild-type strains were observed under standard in vitro growth conditions in 100% oxygen, air, hypoxic, or anaerobic conditions (not shown). With 3 mM nitrite at pH 7, no significant difference in growth between strains was detected (Fig. 2A). Increasing the nitrite concentration to 30 mM, however, resulted in a substantial increase in the doubling time of the hmp^- strain (Fig. 2A). Nitrite will protonate to form HNO2, which quickly dismutates to produce several species of nitrogen oxides, including NO (29, 30). When the pH of the solution was lowered to 6 to promote nitrite protonation, a delay in growth of the hmp^- strain was seen with only 3 mM nitrite (Fig. 2B).

These results suggest that it is not nitrite but nitrogen oxides created by the protonation and subsequent dismutation of nitrite, that produce the difference in growth between the wild-type and hmp^- strains.

Given the association of both hemoglobin and nitric oxide-

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TABLE I

| Strain | Paraoquat | H2O2 | GSNO | SNAC | SIN-1 |
|--------|-----------|------|------|------|-------|
|        | μM        | μM   | mM   | mM   | mM    |
| WT     | 15.1 ± 4.0 | 250  | 10 ± 4.9 | 5.5 ± 1.2 | 1.0   |
| hmp^-  | 15.1 ± 4.0 | 250  | 0.70 ± 0.3 | 0.62 ± 0.7 | 1.0   |

Minimal inhibitory concentrations of various stressors on 14028s (WT) and hmp^- S. typhimurium measured as the mean ± S.E.

Where there is no S.E., results were identical for every experiment. Experiments were performed at least six times. The minimal inhibitory concentration values were obtained as described (28). SIN-1, 3 morpholinosydnonime hydrochloride.
mediated stress responses to oxygen metabolites, we were interested in determining whether the flavohemoglobin protects against both O₂ and NO-mediated insult. Cultures were assayed for the minimal inhibitory concentrations of oxidative and nitric oxide-producing stressors. Both the superoxide generator paraquat and hydrogen peroxide inhibited growth of the wild-type and mutant strains equally (Table I). Profound disparities between wild-type and hmp strains were seen with the NO-donating S-nitrosothiols. These compounds possess a broad spectrum antimicrobial activity, presumably by donating nitrosonium (NO⁺) to critical intracellular thiol targets, disruption of iron-sulfur clusters, and by damage to DNA (31–33). The NO derivatives of both GSNO and SNAC inhibited the growth of the hmp strain by approximately an order of magnitude more than wild-type (Table I). We also wanted to ascertain whether the flavohemoglobin is involved in protection from peroxynitrite (ONOO⁻), which is formed by the reaction of O₂⁻ with NO and is a major effector in the NO-dependent bacteriocidal activity of macrophages (34). The minimal inhibitory concentrations of the peroxynitrite generator 3-morpholinosydnonimine hydrochloride (35) were identical between the two strains (Table I), indicating that hmp does not protect from peroxynitrite.

To further characterize the involvement of hmp in the NO-mediated stress response, both wild-type and hmp strains were cultured in medium containing GSNO. Whether on plates (Fig. 3A) or in liquid culture (Fig. 3B), the parental organism was able to grow, whereas the growth of the hmp strain was greatly slowed. Growth of the hmp strain could be restored by reintroduction of the hmp gene on a low copy number plasmid. A slight toxicity of the hmp rescue plasmid was seen in liquid culture, even in the wild-type strain (Fig. 3B). A similar restoration from the nitrite-mediated growth inhibition was observed using the hmp-expressing plasmid (not shown). To determine if the flavohemoglobin requires oxygen to exert its protective effect against NO donors, liquid cultures were subjected to anaerobiosis. The hmp cultures grown anaerobically continued to be hypersensitive to GSNO (Fig. 3C), suggesting that the flavohemoglobin is important in nitric oxide-mediated stress protection even in the absence of oxygen.

DISCUSSION

The flavohemoglobin gene is hypothesized to have been present in organisms before the divergence of prokaryotes and eukaryotes (36) and, like cytochrome oxidase, may predate the existence of significant levels of atmospheric oxygen (37). Interaction with NO may be a universal feature of hemoglobins throughout phylogeny. Even the vertebrate hemoglobins, well understood as facilitators of oxygen delivery, display physiologically important interactions with nitric oxide. Human hemoglobin binds NO tightly in the heme pocket and may assist in NO sequestration (38). S-Nitrosohemoglobin, formed by a NO adduct to the thiol group of Cys-93 in the β-chain, may be a major intermediate in transduction of the NO vasodilating signal in humans (39). Formation of a physiologically relevant S-nitrosoflavohemoglobin in Salmonella or another microorganism is unlikely. No cysteine residues are conserved in the heme binding domain of these molecules, and indeed there are no cysteines at all in the heme binding domain of the Salmonella hmp. Instead the NO moiety likely interacts with hmp inside the heme pocket. This type of interaction is supported by experimental evidence showing that E. coli hmp can form a nitrosylated complex with a reduced heme iron (41). NO production can occur endogenously in E. coli using nitrite as an electron acceptor. Ferrous iron, nitrate reductase, and low pH can compete with the preferred enzymatic reduction of nitrite to NH₃ and instead create nitric oxide (42–44). NO compounds also threaten bacteria from other sources, including the S-nitrosothiols produced in the vertebrate host (45, 46).

The flavohemoglobin may function by simply sequestering NO in its heme pocket, allowing other enzymes to detoxify the
moiety. The homocysteine molecule, which forms a more stable S-nitrosoylated form than GSNO or SNAC, may also serve as a NO sink in Salmonella. Mutants deficient in homocysteine biosynthesis are 2–3-fold more sensitive to S-nitrosothiols by zone of inhibition assays (47). Alternatively, hmp may directly detoxify NO by an oxido-reductase reaction, leading to a less toxic nitrogen oxide product. It should be noted, however, that no NO reductase activity could be demonstrated using the A. eutrophus flavohemoglobin (17). Another intriguing possibility is that hmp, upon binding NO in its heme domain, might be an NO-sensing protein-containing, two-component system that senses oxygen leading to the positive regulation of protective genes. A heme hmp, upon binding NO in its heme domain, might be flavohemoglobin (17). Another intriguing possibility is that hmp, upon binding NO in its heme domain, might be an NO-sensing protein-containing, two-component system that senses oxygen leading to the positive regulation of protective genes. A heme-binding domain (48). Whichever mechanism applies, it is apparent that this bacterial hemoglobin can function independently of oxygen to protect the organism from stress effects by nitric oxide.

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