The Flavohemoglobin of *Escherichia coli* Confers Resistance to a Nitrosating Agent, a “Nitric Oxide Releaser,” and Paraquat and Is Essential for Transcriptional Responses to Oxidative Stress*

(Received for publication, March 24, 1998, and in revised form, October 27, 1998)

Jorge Membrillo-Hernández‡‡, Malini D. Coopamah‡‡, Muna F. Anjum‡, Tania M. Stevanin‡, Andrew Kelly, Martin N. Hughes‡‡, and Robert K. Poole‡‡

From ‡The Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, United Kingdom, the ¶Life Sciences Division, King’s College London, Campden Hill Road, London W8 7AH, United Kingdom, and the **Chemistry Department, King’s College London, Strand, London WC2R 2LS, United Kingdom

*This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC) Grant P05184 and by a BBSRC Studentship (to T. M. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Present address: Dept. of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115.

‡‡ Recipient of Biochemical Society Krebs Memorial Scholarship and an Overseas Research Student Award.

‡‡ To whom correspondence should be addressed: The Krebs Institute for Biomolecular Research, Department of Molecular Biology & Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK. Tel.: 44-114-222-4447; Fax: 44-114-272-8697; E-mail: r.poole@sheffield.ac.uk

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**The Flavohemoglobin of *Escherichia coli* possesses a flavohemoglobin (Hmp), product of *hmp*, the first microbial globin gene to be sequenced and characterized at the molecular level. Although related proteins occur in numerous prokaryotes and eukaryotic microorganisms, the function(s) of these proteins have been elusive. Here we report construction of a defined *hmp* mutation and its use to probe Hmp function. As anticipated from up-regulation of *hmp* expression by nitric oxide (NO), S-nitrosothioligone (GSNO) or sodium nitroprusside (SNP), the *hmp* mutant is hypersensitive to these agents. The *hmp* promoter is more sensitive to SNP and S-nitroso-N-penicillamine (SNAP) than is the *soxS* promoter, consistent with the role of Hmp in protection from reactive nitrogen species. Additional functions for Hmp are indicated by (a) parallel sensitivity of the *hmp* mutant to the redox-cycling agent, paraquat, (b) inability of the mutant to up-regulate fully the *soxS* and *sodA* promoters in response to oxidative stress caused by paraquat, GSNO and SNP, and (c) failure of the mutant to accumulate reduced paraquat radical after anoxic growth. We conclude that Hmp plays a role in protection from nitrosating agents and NO-related species and oxidative stress. This protective role probably involves direct detoxification of those species and sensing of NO-related and oxidative stress.

The best known members of the ancient globin superfamily are the hemoglobins of vertebrate blood and intramuscular myoglobin (1), which are primarily responsible for oxygen delivery and storage in animals, although the circulating hemoglobin has also been implicated in transport of NO (2). It is now clear that homologous hemoglobins also occur in many bacteria and yeast as well as in invertebrates and higher plants (3). Microbial hemoglobins are divisible into two groups: dimeric hemoproteins comprising two polypeptides each having one heme, as in *Vitreoscilla* VGB (4), and monomeric, chimeric flavohemoproteins composed of a single polypeptide having both a single heme and FAD. The sequence of the *hmp* gene (5), encoding the prototype of the latter class, *Escherichia coli* Hmp, has revealed an N-terminal domain homologous to vertebrate, plant, and *Vitreoscilla* globins, whereas a C-terminal domain has FAD- and NAD(P)H-binding sites as in proteins in the ferredoxin-NADP reductase family (6). Closely related flavohemoglobins occur in the yeasts *Saccharomyces cerevisiae* (7) and *Candida norvegensis* (8) and in the bacteria *Alcaligenes eutrophus* (9), *Erwinia chrysanthemi* (10), and *Bacillus subtilis* (11). On the basis of polymerase chain reaction experiments (12) and genome sequencing projects, e.g. that on *Mycobacterium tuberculosis* (13), related hemoglobins are predicted to be also present in many other bacteria.

The functions of microbial globins have been elusive. Based on up-regulation of the *Vitreoscilla* globin at low oxygen tension (14) and the ability of this protein to restore aerobic respiration when expressed in oxidase-deficient *E. coli* mutants (15), VGB has been implicated in oxygen storage, delivery, or reduction (16). The *E. coli* Hmp protein also consumes oxygen (17) and reduces various acceptors, including cytochrome c (18), Fe(III) (6, 19, 20), and the *Azotobacter* regulatory flavoprotein NifL (21). This dual ability might allow Hmp to act as an oxygen sensor (17, 22).

The first evidence that Hmp might function in responses to NO came from the discovery that its expression is markedly up-regulated by NO, both aerobically and anaerobically (23). *Bacillus subtilis* *hmp* is also induced by nitrite (11). Furthermore, in *A. eutrophus*, mutation of the *hmp* homologue, *fhp*, results in failure to detect nitrous oxide as an intermediate during denitrification (9). Recently the *Salmonella typhimurium* flavohemoglobin has been shown to confer resistance to acidified nitrite (and thus presumably NO) and S-nitrosothiols (24), and *E. coli* Hmp has been shown to have NO dioxygenase activity (25). These findings implicate bacterial flavohemoglobins in detoxification or utilization of NO.

However, other evidence suggests that microbial flavohemoglobins are involved in responses to oxidative stress. Paraquat (1,1’-dimethyl-4,4’-bipyridinium dichloride; methyl viologen) is a strong inducer of the *hmp* gene, independently of the SoxRS regulatory system (26), and Hmp itself generates superoxide anion, detectable using a superoxide-sensitive F(sodA-lacZ) fusion or with the purified protein (20). The yeast flavohemo-
Oxidative Stress in an E. coli Flavohemoglobin Mutant

TABLE I

| Strains and plasmid used in this study | Relevant genotype | Source/Ref. |
|----------------------------------------|-------------------|-------------|
| C-6007                                  | pfr, kmβ         | 31          |
| JC7623                                  | recB21 recC22 sbcB15 sbcC201 | 32          |
| QC772                                   | Δlac rpsL Φ(odoA-lacZ) | 33          |
| RKP2178                                 | As VJS676 but Φ(hmp-lacZ):1 | 23          |
| RKP4232                                 | JC7623 hmp::Tn5  | This work   |
| RKP4323                                 | QC772 but hmp    | This work   |
| RKP4324                                 | TN530 but hmp    | This work   |
| RKP4545                                 | VJS676 but hmp::Tn5 | This work |
| VJS676                                  | ΔargF-lacZ:U169  | Valley Stewart |
| Plasmid                                 | glnA + hmp::Tn5 glnB in a 13-kb EcoRI insert | 34          |

moglobin encoded by the YHB1 gene is also induced by agents that promote oxidative stress and antimycin A (27), but a subsequent re-examination has produced conflicting views (28).

To resolve whether E. coli Hmp is important in responses to oxidative stress or NO, or both, we have constructed the first defined null allele of hmp and used this mutant to test responses to paraquat, sodium nitroprusside (SNP, a nitrosating agent) and S-nitroso glutathione (GSNO) and S-nitroso-N-penicillamine (SNAP), the last two being widely used as NO-releasing agents. In addition, since the SoxRS system has been shown to respond to both oxidative stress and NO (29, 30), we have compared the response of the hmp and soxS promoters to challenge with “NO-releasing” agents. These data suggest that Hmp is pre-eminently involved in responses to NO and related reactive nitrogen species.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Growth Conditions—**Strains and plasmid used are listed in Table I. Cells were grown in rich medium (LB) or MOPS-glucose defined medium, initial pH 7.0 (23). Kanamycin, chloramphenicol, tetracycline, spectinomycin, and ampicillin were used at final concentrations of 100, 40, 15, 100, and 200 μg/ml, respectively. Culture optical density was measured with a Pye-Unicam SP6–550 spectrophotometer at 600 nm, after dilution to bring A_600 to below 0.7 when measured in cells of 1-cm path length. All cultures were grown at 37 °C with shaking (200 rpm) in conical flasks containing 1/2 to 1/3 of their own volume of medium.

**Determination of Resistance to Paraquat, SNP, and GSNO—**GSNO was prepared by the method of Hart (35); SNP was from Sigma. Cultures (10 ml) of strains VJS676 (wild-type) and RKP4545 (hmp::Tn5) were grown aerobically in LB medium to an OD_600 of 0.3: the cultures were then divided, one-half being treated with paraquat, SNP, or GSNO at concentrations of 100, 40, 15, 100, and 200 μM, respectively. In addition, since the SoxRS system has been shown to respond to both oxidative stress and NO (29, 30), we have compared the response of the hmp and soxS promoters to challenge with “NO-releasing” agents. These data suggest that Hmp is pre-eminently involved in responses to NO and related reactive nitrogen species.

**RESULTS**

**Construction of an hmp Null Mutant—**To determine the consequences of Hmp deletion, we constructed a strain carrying a null mutation in its structural gene, hmp. Plasmid pGS16 (34, 40) comprises the pACYC184 vector and a 13-kb hmp fragment.

β-Galactosidase Assays—β-Galactosidase activity measurements (36, 38) were carried out at 21 °C on CHCl_3 and sodium dodecyl sulfate-permeabilized cells by monitoring the hydrolysis of o-nitrophenyl-β-D-galactopyranoside. Activities are expressed per A_600 of cell suspensions (36).

Visible Electronic Spectroscopy of Cells Grown with Paraquat—These spectra were recorded using an SDB4 dual-wavelength scanning spectrophotometer (University of Pennsylvania School of Medicine Biomedical Instrumentation Group and Current Designs Inc., Philadelphia PA 19104-2420) (39). For direct observation of the paraquat radical in anaerobically cultivated cells, the unopened culture bottle was mounted directly adjacent to the photomultiplier. Spectral data were analyzed and plotted using SoftSDB (Current Designs Inc.) and CA-Cricket Graph III.

**RESULTS**

**Construction of an hmp Null Mutant—**To determine the consequences of Hmp deletion, we constructed a strain carrying a null mutation in its structural gene, hmp. Plasmid pGS16 (34, 40) comprises the pACYC184 vector and a 13-kb EcoRI insert containing the hmp, glnA, and glnB genes, with a Tn5 insertion located in that portion of hmp encoding the heme domain (243 base pairs downstream of the translational start site). pGS16 was digested with EcoRI, and the gel-purified linear fragment containing the disrupted hmp::Tn5 was used to transform strain JC7623; this strain has mutations in the RecBCD enzyme (product of recBC) and exonuclease I (shb gene product), allowing linear DNA to have a longer half-life in vivo and recombine (32). Six colonies were selected on plates containing kanamycin, one of which (strain RKP4322) was selected for further study. The Km<sup>+</sup> phenotype of strain RKP4322 was transduced to strain VJS676 (∆lac) to give strain RKP4545. Confirmation that the genomic copy of hmp was disrupted was obtained by comparing genomic Southern blots of strains VJS676 and RKP4545. Digestion of genomic DNA from VJS676 (hmp<sup>+</sup>) with PvuII and EcoRI, which cut outside...
The 

hmp

gene (Fig. 1A), yielded a single band, equivalent to a fragment of about 2.5 kb, when probed with the 

hmp

gene (Fig. 1B). Digestion of genomic DNA from RKP4545 with 

Pvu

II and 

EcoRI

yielded two bands (3.3 and 2.1 kb; Fig. 1B), the expected result for interruption of 

hmp

. The larger is the 

Pvu

II-

EcoRI

fragment comprising 1.42 kb from 

Tn5

, 1.1 kb from the interrupted 

hmp

gene, and 0.82 kb from the region flanking the 3' end of the 

hmp

gene. The 2.1-kb band represents the 

Pvu

II-

Pvu

II fragment comprising 1.42 kb from the 

Tn5

, 0.2 kb from the interrupted 

hmp

gene, and 0.47 kb from the region flanking 

hmp

at the 5' end. Fragments generated from the 

Pvu

II sites internal to the 

Tn5

are not revealed with this probe.


growth under common laboratory conditions. Growth of the two strains was similar either in LB medium or MOPS minimal medium, with glucose or glycerol as carbon and energy sources, aerobically or anaerobically, and with or without nitrate as electron acceptor (not shown). Thus, 

Hmp

is not essential for aerobic or anaerobic growth under these conditions. However, in all cases, turbidity of the 

hmp

mutant cultures declined slightly (data not presented), but significantly and reproducibly, during the stationary phase (10–30 h after inoculation).

An 

hmp

Null Mutant Shows Increased Sensitivity to Paraquat, SNP, and GSNO—The demonstration that 

hmp

is induced by paraquat and nitric oxide (23, 26) suggested that 

Hmp

participates in bacterial defenses against oxidative stress, NO, or the deleterious effects of related, reactive nitrogen species. We therefore investigated the killing action of paraquat, SNP (a nitrosating agent), and GSNO (a compound widely used as a NO-releasing agent) on the 

hmp

mutant strain RKP4545 and its isogenic parental strain VJS676. We also included for comparison an 

fpr

mutant; the gene product, ferredoxin (flavodoxin) 

NADP+ oxidoreductase (41), belongs to the ferredoxin-in-

NADP+

reductase family as does the flavin domain of 

Hmp

. Mutation of 

fpr

results in increased sensitivity to paraquat (31). The wild-type strain VJS676 gave a biphasic response to paraquat; as the paraquat concentration was increased to 800 

µM

, viability in LB medium decreased to about 80% of the control value after 45-min incubation (Fig. 2A). At higher concentrations, the decline in viability was precipitous, reaching 25% of the control value at 1 mM paraquat. No viable cells could be cultured after 45-min treatment with 2 mM paraquat. The 

hmp

mutant was markedly more sensitive, particularly at lower paraquat concentrations. Most sensitive was the 

fpr

mutant, the viability of which was decreased to about 5%, even at 800 

µM

(Fig. 2A). The 

hmp

and 

fpr

mutants were also more sensitive to GSNO (Fig. 2B) and SNP (Fig. 2C). Exposure for 45 min to 2 mM GSNO (Fig. 2B) and 2 mM SNP (Fig. 2C) gave similar results. A 25% loss of viability was measured for the wild-type strain, whereas viabilities of the 

hmp

and 

fpr

mutants were reduced by about 70 and 80%, respectively.

Since expression of the yeast flavohemoglobin is enhanced by 

H2O2

(27), even though a 

YHB1

mutant is more resistant to this agent (28), we compared the growth of the 

hmp

mutant and its isogenic parent strain in a wide range of 

H2O2

concentrations. No significant differences were evident (data not shown).

The 

hmp

Mutation Prevents Full Response by the Sox System to Oxidative Stress—The sensitivity of the 

hmp

mutant to paraquat, SNP, and GSNO may result from 

Hmp

protein being directly involved in combating these agents (e.g. by reaction with superoxide or detoxifying NO and related species) and/or because 

Hmp

is needed to elicit the SoxRS response to oxidative stress (17). Therefore, we investigated the effects of an 

hmp

mutation on the Sox system, exploiting the Km3-marked mutant 

hmp

allele and transducing it to a strain carrying 

Φ(sosX-lacZ)

and monitoring 

β-galactosidase

activity. Strain TN530 ( 

hmp

) gave an activity of about 200 Miller units, which was increased 9-fold by 200 

µM

paraquat (Fig. 3A). In contrast, strain RKP4324, having 

Φ(sosX-lacZ)

but also the 

hmp

mutant allele, had a similar basal level of 140 Miller units, which was up-regulated only 5-fold. A more marked difference was observed when the 

hmp

mutation was transduced into a strain (QC772) carrying a 

Φ(sodA-lacZ)

fusion, which is up-regulated by the SoxS protein. This strain had a basal 

β-galactosidase

activity of 160 Miller units, which was increased 10-fold on challenge with paraquat (Fig. 3B). In the 

hmp

mutant (strain RKP4323), the same fusion had a similar basal level of 230 units, which was increased only 2.6-fold on adding paraquat (Fig. 3B). Therefore, the 

Hmp

protein is essential for full aerobic activation of the SoxS system and resistance to paraquat.

Similar results were found when SNP or GSNO was used (not shown). SNP (200 

µM

final concentration) increased 

Φ(sodA-lacZ)

and 

Φ(sosX-lacZ)

activities by 3.3- and 2.1-fold, respectively, but the presence of the 

hmp

mutation gave increases of only 1.7- and 1.3-fold, respectively. GSNO (500 

µM

final concentration) increased 

Φ(sodA-lacZ)

and 

Φ(sosX-lacZ)

activities by 3.5- and 1.5-fold, respectively, but the presence of the 

hmp

mutation gave increases of only 1.5- and 1.2-fold, respectively.

Transcription of 

hmp

in the Presence of SNP and NO—Since 

hmp

is important in the response to paraquat, NO, and NO-related species and is up-regulated by these agents (Fig. 2; Refs. 23 and 26), we sought direct evidence at the level of the 

hmp

transcript for 

hmp

up-regulation by growing strains VJS676 or RKP4545 ( 

hmp

) and challenging them with SNP in the exponential phase of growth. After 20-min incubation with SNP, total RNA was isolated and subjected to Northern blot analysis using the 

hmp

gene as probe (see Fig. 1A). RNA (10 

µg

) loaded to each well gave uniform loading (Fig. 4A). Northern hybridization (Fig. 4B) revealed that both strains exhibited low levels of 

hmp

expression during exponential phase in the absence of SNP, consistent with maximal expression of 

hmp

in the stationary phase of growth (42). SNP resulted in strong induction of the 

hmp

gene in strain VJS676 (mRNA of about 1.2
levels of SNP and NO than Is soxS Transcription—The above results show that Hmp is synthesized in response to SNP and that Hmp is needed for full expression of sodA in response to paraquat and for full expression of soxS, whose gene product is required for up-regulation of sodA and superoxide dismutase synthesis. A possible explanation is that Hmp is part of a regulatory cascade and that hmp transcription, triggered by low levels of oxidative stress, is required so that the Hmp may participate in the induction processes. We therefore compared, under identical conditions, the responsiveness of Φ(hmp-lacZ) and Φ(soxS-lacZ) to SNP and SNAP, an NO-releasing agent. Expression of Φ(hmp-lacZ) in strain RKP2178 was enhanced above the basal level by 18-fold at 50 μM SNP and by 37-fold at 200 μM SNP (Fig. 5). In contrast, Φ(soxS-lacZ) fusion activity in strain TN530 was enhanced only 2-fold at 200 μM SNP (Fig. 5). Φ(soxS-lacZ) fusion activity was significantly increased only at 1000 μM SNP (not shown). The NO releaser SNAP enhanced Φ(hmp-lacZ) activity in strain RKP2178 12-fold at concentrations as low as 50 μM (Fig. 5). Again, millimolar concentrations of SNAP were required for induction of Φ(soxS-lacZ) fusion activity (not shown).

Spectral and Respiratory Consequences of an hmp Mutation—Hmp is a soluble flavohemoglobin (5) with features in the visible spectral region attributable to heme B and FAD (17, 44). In yeast, deletion of the flavohemoglobin Yhb results in the loss of the characteristic spectral forms of the oxyhemoglobin at about 575 nm (28), and so we sought spectral changes in soluble extracts of ultrasonically disrupted cells. Reduced minus oxidized difference spectra of extracts from wild-type cells showed a Soret peak at 421 nm, an α-peak at about 553 nm, and a weak β-band (not shown). These signals probably arose from low levels of cytochrome c and are not characteristic of pure Hmp (Soret, β-, and α-bands at 435.5, 560, and about 590 nm, respectively, in reduced minus oxidized difference spectra; Ref. 43). These signals were quantitatively and qualitatively similar in the mutant (not shown), indicating that Hmp does not make a significant spectral contribution in such extracts. The region at wavelengths > 553 nm was featureless, indicating the absence of cytochrome bd, a marker for contamination by membrane fragments (22). Reduced minus oxidized spectra of intact cells also revealed no significant differences between wild-type and mutant cells.

To determine the contribution of the oxidase activity (17, 44) of Hmp to total cellular respiration, oxygen uptake rates in the wild-type strain VJS676 and the hmp mutant RKP4545 were compared. Respiration rates were unaffected whether measured using endogenous substrates present in cells harvested from overnight aerobic cultures in rich medium or after stim-

FIG. 4. Levels of hmp mRNA in the absence or presence of SNP in strains VJS676 (wild-type) and RKP4545 (hmp). A, ethidium bromide-stained gel as a control for RNA loading. Lane 1, RNA markers; lanes 2–4, RNA from strain VJS676; lanes 5–7, RNA from strain RKP4545. Numbers below lane labels (0, 50, 100) indicate the concentration of SNP (micromolar) used to treat cultures. B, autoradiography of the blot hybridized with the probe shown in Fig. 4A. Lane descriptions are the same as described in A. The arrow at the left indicates hmp mRNA.

kb; indicated by the arrow in Fig. 4B). When strain RKP4545 was similarly challenged, no mRNA at 1.2 kb was detected, confirming that strain RKP4545 was carrying an hmp null mutation.

Up-regulation of hmp Transcription Is Triggered by Lower Levels of SNP and NO than Is soxS Transcription—The above results show that Hmp is synthesized in response to SNP and that Hmp is needed for full expression of sodA in response to paraquat and for full expression of soxS, whose gene product is required for up-regulation of sodA and superoxide dismutase synthesis. A possible explanation is that Hmp is part of a regulatory cascade and that hmp transcription, triggered by low levels of oxidative stress, is required so that the Hmp may participate in the induction processes. We therefore compared, under identical conditions, the responsiveness of Φ(hmp-lacZ) and Φ(soxS-lacZ) to SNP and SNAP, an NO-releasing agent. Expression of Φ(hmp-lacZ) in strain RKP2178 was enhanced above the basal level by 18-fold at 50 μM SNP and by 37-fold at 200 μM SNP (Fig. 5). In contrast, Φ(soxS-lacZ) fusion activity in strain TN530 was enhanced only 2-fold at 200 μM SNP (Fig. 5). Φ(soxS-lacZ) fusion activity was significantly increased only at 1000 μM SNP (not shown). The NO releaser SNAP enhanced Φ(hmp-lacZ) activity in strain RKP2178 12-fold at concentrations as low as 50 μM (Fig. 5). Again, millimolar concentrations of SNAP were required for induction of Φ(soxS-lacZ) fusion activity (not shown).

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FIG. 3. Activities of Φ(soxS-lacZ) and Φ(sodA-lacZ) fusions in response to paraquat. Strain TN530 Φ(soxS-lacZ) and its hmp derivative (RKP 4324) (A) and strain QC772 Φ(sodA-lacZ) and its hmp derivative (RKP4323) (B) were grown in rich medium. In the exponential phase, cells were treated with or without 200 μM paraquat, and the incubation was continued for 45 min. Cells were pelleted, and β-galactosidase activity was determined and expressed as Miller units. The experiment was repeated at least three times with similar results.
ulation with exogenous succinate (10 mM). The oxygen uptake rates of "soluble" supernatant fractions from ultrasonically disrupted cells were low and were stimulated by NADH by 4-fold (to about 4 nmol of O₂/min/mg of protein) in soluble fractions from both wild-type and hmp mutant cells (not shown).

**Paraquat Reduction Is Affected in the hmp Mutant**—Hmp is an oxidoreductase with a broad specificity for electron acceptors. Since NADPH-dependent paraquat diaphorases, i.e. enzymes capable of reducing the paraquat cation (PQ₂⁻), have been reported in *E. coli* (45, 46), we tested the possibility that Hmp might be such a diaphorase. To detect directly the reduction of paraquat by the hmp mutant and its parent strain, cells were grown aerobically in rich medium for 6 h and then subcultured (4% inoculum) into fresh medium containing 1 mM paraquat. The tubes were sealed and growth allowed to continue in the anoxic atmosphere of an anaerobic jar. It was anticipated that paraquat would increase synthesis of paraquat reductases (diaphorases) identified in *E. coli* (45, 46), have been reported in *E. coli* and characterized the *hmp* gene function by derepression of a particulate activity (8). Following reaction of paraquat with oxygen to generate superoxide, the oxidized divalent paraquat cation formed can be reduced leading to redox cycling and sustained superoxide production. Paraquat reductases (diaphorases) identified in *E. coli* are ferredoxin:NAD⁺ oxidoreductase, thioredoxin reductase, and NADPH:sulfite reductase (31, 45, 52). Although hmpX is normally induced in stationary phase (42), the hmpX mutant is in the stationary phase of growth where it attained a slightly lower population density; it may be significant that hmpX is normally induced in stationary phase (42).

An important outcome of this work is that the hmp mutant is more sensitive than its isogenic parent to SNP and GSNO. Although the latter is widely used as an NO-releasing agent, both compounds may act similarly as nitrosating agents (50). Responses to SNP and GSNO are of special interest, since we have recently elucidated a novel mechanism for hmp up-regulation via nitrosation of homocysteine thus modulating binding of MetR to the *glsA-hmp* intergenic region (50). Other mechanisms of toxicity of SNP and GSNO are poorly understood, but such nitrosating agents are expected to be reactive with thiols and may interact with the Fe-S cluster of SoxR preventing full induction of *soxS*, *sodA*, and other stress-responsive genes.

The finding that Hmp is involved in surviving the oxidative stress caused by paraquat or "NO releasers" is consistent with the results of previous studies with the hemoglobins of *S. cerevisiae* and *E. chrysanthemi* (10, 27). Mutations in the *S. cerevisiae* *YHB* gene conferred increased sensitivity to oxidative stress from the thiol oxidants diamide and diethylmaleate, but paraquat had only a minor effect on the *YHB* mutant (27). In *E. chrysanthemi*, mutations in the gene *hmp* conferred loss of plant pathogenicity (10); it was speculated that this was due to increased sensitivity to oxygen radicals, but the recent discovery (51) that plants utilize NO to resist pathogenic bacteria suggests that *hmpX* might also be involved in NO responses in *Erwinia*.

Following reaction of paraquat with oxygen to generate superoxide, the oxidized divalent paraquat cation formed can be reduced leading to redox cycling and sustained superoxide generation. Paraquat reductases (diaphorases) identified in *E. coli* are ferredoxin:NAD⁺ oxidoreductase, thioredoxin reductase, and NADPH:sulfite reductase (31, 45, 52). Although hmpX is up-regulated by paraquat, purified Hmp is not itself an effective reductant of paraquat with NADH or NADPH as substrate,² consistent with the much higher midpoint potential of the flavin (around ~150 mV) for the 2-electron reduction from FAD to FADH₂ (53) than of paraquat (E₉₀ = -446 mV) (54).

² S. O. Kim and R. K. Poole, unpublished data.
It is therefore unlikely that the accumulation of reduced paraquat in anoxic cell suspensions (Fig. 6) is due to direct reduction by Hmp. More likely, the presence of Hmp is required for full induction of components of the oxidative stress response (Fig. 3). One candidate for the paraquat-reducing enzyme is NADPH:ferredoxin oxidoreductase (45), a member of the \textit{soxRS} regulon.

A paradoxical aspect of the induction of \textit{hmp} by paraquat is that Hmp itself generates free superoxide (20). This, and the present finding that an \textit{hmp} mutant fails to elicit full responses to the presence of paraquat (Fig. 3), may be reconciled by considering Hmp as an amplifier of oxidative stress. In this model, paraquat (possibly via superoxide anion) induces synthesis of Hmp, which generates further superoxide, resulting in the activation of SoxR and the cascade of regulatory processes that result in oxidative stress responses. Such a mechanism may explain the increased sensitivity of the \textit{hmp} mutant to paraquat (Fig. 2). The extreme paraquat sensitivity of the \textit{fpr} mutant confirms the findings of Bianchi et al. (31), and we report for the first time the additional sensitivity of the \textit{fpr} mutant to GSNO and SNP; the mechanism for neither effect is known.

Interestingly, \textit{hmp} up-regulation appears to respond to lower concentrations of SNP and SNAP than does transcription of \textit{soxS}. This lends support to the view that Hmp may be an early component of the regulatory cascade. Several possible mechanisms can be envisaged. First, superoxide generation by Hmp might directly facilitate conversion of SoxR to the active form, perhaps by reaction with the FeS cluster (55). NAD(P)H:flavin oxireductase activity (Fre) has also been demonstrated to induce \textit{soxRS}-regulated genes by superoxide generation (56). Second, Hmp is an NADH (43) and NADPH oxidase (57), and its activity will contribute to reducing the anabolic reduction charge ([NADPH]/[NADP] + [NADP⁺]) (58) that has been regarded as one of the possible signals for SoxR. Such regulatory influences of Hmp on genes involved in stress response would be reinforced by the ability of Hmp to directly convert NO to the relatively innocuous NO₃⁻ ion by reaction of NO with oxy-Hmp, recently demonstrated in Ref. 25, but such a mechanism cannot explain the anaerobic roles of Hmp in protecting cells from NO reported recently (24, 25). Such mechanisms are currently under investigation in this laboratory.

\textbf{Acknowledgments}—We thank Drs. Vera Bianchi, Bruce Demple, George Stauffer, and Daniele Touati for providing the strains and plasmids used in this work.

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\textbf{FIG. 6. Absorption spectra of cultures grown anaerobically in the presence of paraquat.} Strains RKP4545 (\textit{hmp}) and VJS676 (wild-type) were grown in LB medium anaerobically in the presence of paraquat for 16 h at 37 °C. The base line (unlabeled) is the spectrum of LB medium.
