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Bacterial Hemoglobins and Flavohemoglobins for Alleviation of Nitrosative Stress in *Escherichia coli*

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***Escherichia coli* MG1655 cells expressing novel bacterial hemoglobin and flavohemoglobin genes from a medium-copy-number plasmid were grown in shake flask cultures under nitrosative and oxidative stress. *E. coli* cells expressing these proteins display enhanced resistance against the $NO^{\cdot}$ releaser sodium nitroprusside (SNP) relative to that of the control strain bearing the parental plasmid. Expression of bacterial hemoglobins originating from *Campylobacter jejuni* (CHb) and *Vitreoscilla* sp. (VHb) conferred resistance on SNP-challenged cells. In addition, it has been shown that $NO^{\cdot}$ detoxification is also a common feature of flavohemoglobins originating from different taxonomic groups and can be transferred to a heterologous host. These observations have been confirmed in a specific in vitro $NO^{\cdot}$ consumption assay. Protein extracts isolated from *E. coli* strains overexpressing flavohemoglobins consumed authentic $NO^{\cdot}$ more readily than protein extracts from the wild-type strain. Oxidative challenge to the cells evoked nonuniform responses from the various cell cultures. Improved oxidative-stress-sustaining properties had also been observed when the flavohemoglobins from *E. coli*, *Klebsiella pneumoniae*, *Deinococcus radiodurans*, and *Pseudomonas aeruginosa* were expressed in *E. coli*.**

Oxidative and nitrosative stresses are encountered in all living organisms. Either reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed as undesired and noxious metabolic side products or organisms might also be challenged by such stresses generated exogenously. Oxidative stress is an unavoidable side effect of an aerobic lifestyle. ROS are formed in vivo during respiration when molecular oxygen is reduced to water (36). In particular, reduced flavoproteins have been implicated in this redox process in *Escherichia coli* (13, 19). RNS, such as the nitric oxide radical ($NO^{\cdot}$) (nitrogen monoxide is the name recommended by the International Union of Pure and Applied Chemistry [IUPAC]) and the more potent peroxynitrite ($ONOO^{-}$), directly or indirectly cause nitrosative stress (17).

In mammals, elevated levels of $NO^{\cdot}$ in combination with ROS serve as potent toxins in cellular responses against infections, foreign bodies, and neoplastic tissues. In plants, ROS appear to play key roles in early and late stages of responses to pathogens. ROS seem to act both as cellular signals and as killing molecules. The presence of $NO^{\cdot}$ in plants is also well documented, and recent results suggest clearly that $NO^{\cdot}$ appears to play important roles in the plant-pathogen interaction (3). Therefore, infectious bacteria may be challenged by ROS and RNS as part of host defense mechanisms when they invade mammals and plants.

In denitrifying bacteria, $NO^{\cdot}$ is produced during growth when nitrate or nitrite is used as a terminal electron acceptor (14). Additionally, in nondenitrifying bacteria such as *E. coli*, $NO^{\cdot}$ may be produced by nonenzymatic reactions (40) or by reduction of nitrite to $NO^{\cdot}$ by an oxidase (4).

Oxidative and nitrosative damage to proteins, nucleic acids, and cellular membranes can occur when the concentration of reactive species exceeds the capacity of the cell's mechanisms for elimination (7). *E. coli* has developed (constitutive and inducible) enzymatic mechanisms to detoxify ROS and RNS under the control of the SoxRS and OxyR regulatory networks (31).

Whereas the induction of *hmp* expression by $NO^{\cdot}$ has been known for some time (32), the implication of *E. coli* flavohemoprotein (HMP) in the inducible response against nitrosative stress was derived later (11, 16). HMP plays a significant role in the detoxification of RNS in *E. coli* under aerobic conditions (26). The reaction catalyzed by HMP converts $NO^{\cdot}$ into nitrate with consumption of O_2 and NADH. Although the overall reaction balance is widely accepted, two different mechanisms for the HMP-mediated reaction have been proposed. One is a route including a dioxygenase (11, 16), where the heme-bound oxygen reacts with $NO^{\cdot}$ to yield nitrate. Alternatively, a "denitrosylase" activity at physiologically more relevant $NO^{\cdot}$ and O_2 concentrations has been proposed (15), where heme-bound $NO^{\cdot}$ reacts with oxygen to form nitrate.

The impact of bacterial flavohemoglobins on oxidative stress is a subject of discussion. It has been shown that in *E. coli*, overexpression of native HMP leads to accumulation of superoxide and peroxide (1, 29). In contrast, an *E. coli hmp* null mutant has been found to be more sensitive to ROS (26).

Similar functions or putative roles in nitrosative or oxidative stress responses have also been proposed or demonstrated for the flavohemoglobins from diverse microorganisms such as *Saccharomyces cerevisiae*, *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Typhimurium, and *Ralstonia eutropha* (6, 10, 18, 39).

While most bacterial species express flavohemoglobins, some bacteria such as a *Vitreoscilla* sp. and *Campylobacter jejuni* express single-domain hemoglobins (2, 37). The most thoroughly investigated bacterial hemoglobin is that from a *Vitreoscilla* sp. (VHb) (24). VHb is a homodimeric hemoglobin. It has been postulated that expression of heterologous

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TABLE 1. Plasmids used in this study

Plasmid	Flavohemoglobin or hemoglobin[a]	Origin of protein	Reference
pKQV4	Control		22
pPPC1	VHb*	*Vitreoscilla* sp.	22
pAX1	FHPg*	*R. eutropha*	8
pAX4	VHb-Red[b]	Chimeric protein	8
pAX5	FHP	*R. eutropha*	8
pECS14	HMP	*E. coli*	2
pBSS15	HmpBs	*B. subtilis*	2
pPAS6	HmpPa	*P. aeruginosa*	2
pDRS3	HmpDr	*D. radiodurans*	2
pCJS8	CHb*	*C. jejuni*	2
pSTS28	HmpSt	*S. enterica* serovar Typhi	2
pKPS10	HmpKp	*K. pneumoniae*	2

[a] Asterisks indicate hemoglobins.
[b] Fusion protein of *Vitreoscilla* VHb and the C-terminal reductase domain of *R. eutropha* flavohemoglobin FHP.

VHb yields increased intracellular oxygen concentrations, and in turn oxygen-dependent cellular activities are stimulated in recombinant host cells (21). The VHb protein has been successfully expressed in various biotechnologically relevant organisms such as *E. coli*, *Bacillus subtilis*, *Streptomyces coelicolor*, *S. cerevisiae*, and Chinese hamster ovary (CHO) cells. Expression of VHb results in improved growth and enhanced productivity of recombinant proteins or improved synthesis of primary or secondary metabolites (20). Recently, similar growth-stimulating effects and improved carbon utilization have also been observed when novel flavohemoglobins, which have been isolated from several bacterial species, have been expressed in *E. coli* (2, 8).

In this study, we have expressed the flavohemoglobin genes from *Pseudomonas aeruginosa*, *S. enterica* serovar Typhi, *Klebsiella pneumoniae*, *Deinococcus radiodurans*, *E. coli*, and *R. eutropha* (previously *Alcaligenes eutrophus*) and the hemoglobin genes from a *Vitreoscilla* sp. and *C. jejuni* in *E. coli* MG1655. Our goal was to study the feasibility of using these hemoglobins to provide protection from nitrosative and oxidative stress to a heterologous host. In view of further biotechnological applications such as bioremediation (30), the NO consumption activities of these hemoglobins have also been tested in vitro.

MATERIALS AND METHODS

Bacterial strain, plasmids, and growth conditions. *E. coli* MG1655 K-12 (F^- λ^-; Cold Spring Harbor Laboratory) has been used as a host throughout this study. Plasmids used in this study are listed in Table 1. All cultivations were performed either in M9 minimal medium (34) supplemented with 4 g of glucose/liter, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, thiamine (5 mg/liter), trace elements, and a vitamin mix or in Luria-Bertani (LB) medium (22, 34). Ampicillin (100 mg/liter) was added to all cultures for plasmid maintenance. To obtain identical cultivation conditions for precultures and for oxidative- and nitrosative-stress experiments, shake flasks were filled with medium to a fixed ratio of 1/10 (medium to total shake flask capacity). All cultivations were performed at 37°C on a rotatory shaker at 300 rpm. Cell growth was monitored by measuring culture turbidity at 600 nm (A_{600}) with a Perkin-Elmer λ1 spectrophotometer.

Determination of resistance to SNP and paraquat. Precultures were inoculated with overnight-grown cultures of the various bacterial strains to an A_{600} of 0.1. At an A_{600} of 0.5, precultures were first induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to obtain a final concentration of 0.1 mM and then grown to an A_{600} of 1. To determine the resistance of hemoglobin- and flavohemoglobin-expressing strains and a control strain to toxic ROS and RNS species, IPTG-induced precultures were used to inoculate fresh minimal medium containing either 1 mM nitroprusside sodium dihydrate (SNP; Fluka), 1 mM $K_4[Fe(CN)_6]$, or 100 μM 1,1′-dimethyl-4,4′-bipyridinium dichloride (paraquat; Fluka) to an initial A_{600} of 0.2. As a reference, stressor-free cultures were also prepared from each preculture. Both stressor-containing and stressor-free cultures were grown further under identical conditions, and the A_{600} was routinely measured over a 3-h period.

Specific growth rates (μ) were calculated by applying linear regression analysis on the natural logarithms of A_{600} values plotted against the time course of the cultivation. The ratio of μ from a culture subjected to a stressor ($\mu_{stressor}$) to μ from the respective reference culture ($\mu_{no\ stress}$) was calculated ($\mu_{stressor}/\mu_{no\ stress}$) in order to quantify the effects of the stressors on growth of the *E. coli* cultures. The assay was performed in triplicate and repeated at least twice for each experiment. One-way analysis of variance (ANOVA) and Dunnett's multiple comparison test were applied for statistical analysis.

Growth of cells and preparation of soluble protein fraction for in vitro NO· assay. *E. coli* cells were grown in LB medium in the presence of ampicillin (100 mg/liter). Precultures were diluted into fresh LB medium to an A_{600} of 0.1 and grown to an A_{600} of 1 where expression of hemoglobins and flavohemoglobins was induced by addition of IPTG to a final concentration of 1 mM. Cultures were grown for 10 h at a low shaking rate of 170 rpm to ensure production of the heme cofactor.

Cells were harvested by centrifugation and resuspended in assay buffer (50 mM NaCl, 1 mM EDTA, 100 mM Tris-HCl [pH 7]). Soluble protein fractions were obtained as described previously (8).

Determination of protein and heme concentrations. Total soluble protein concentrations of the extracts were determined by the Bradford method (5) using reagents obtained from Bio-Rad and bovine serum albumin as a standard.

Heme content in crude extracts was determined using the pyridine hemochrome method (2). Briefly, an equal volume of a stock solution containing 200 mM NaOH, 40% pyridine, and 0.8 mM $K_3Fe(CN)_6$ was added to an aliquot of the soluble protein fraction and mixed. The absorbance of this oxidized sample was measured at 556 and 539 nm. A few grains of sodium dithionite were added to reduce the hemichromes to hemochromes, and the maximum and minimum absorbance values (A_{556} and A_{539}, respectively) were measured after 3 min of incubation at room temperature. The absorbance values of the oxidized form were subtracted from those recorded for the reduced sample, and the minimum absorbance was subtracted from the maximum absorbance. A pyridine hemochrome standard curve was generated using hemin (Sigma) as a standard. An extinction coefficient of 19 mM^{-1} cm^{-1} was obtained for hemin and was used to calculate the heme contents of the samples.

Measurements of nitric oxide consumption. Gaseous nitric oxide was obtained from Linde (Höllriegelskreuth, Germany) and passed through a degassed NaOH solution and a column packed with NaOH pellets to remove higher nitrogen oxides before use. NO· solutions were prepared by degassing assay buffer for at least 30 min with N_2 and then saturating it with NO·.

NO· decay was measured with an ANTEK Instruments nitric oxide analyzer with a chemiluminescence detector, which was adapted to liquid measurements by using a 2-ml stirred cuvette connected to a bundle of capillaries that allow gas diffusion. A 1 μM concentration of NO· corresponds to a signal of 42 mV.

An appropriate amount of crude extract as indicated in Results was added to the cuvette, which was then filled to 2 ml with assay buffer. NADH was added to a final concentration of 200 μM unless otherwise stated. The signal of this mixture was set as the baseline, and after 10 s, NO· was added from an NO·-saturated solution (2 mM) by using a gastight SampleLock Hamilton syringe. The decay of NO· was recorded until the baseline was reached. All measurements were performed at room temperature and in duplicate. NO· consumption rates in the presence of crude extracts were calculated by subtracting the rate of NO· consumption in buffer and were expressed relative to heme content.

Quantification of nitrate and nitrite. Nitrate and nitrite concentrations in samples were analyzed by using the Griess reagent. Nitrate was enzymatically converted to nitrite and quantified by use of a colorimetric assay kit (Roche Molecular Biochemicals). Nitrite was determined directly with the Griess reagent (Molecular Probes, Eugene, Oreg.). Nitrate and nitrite standards were used for calibration. Each sample was measured twice.

RESULTS

Determination of resistance of hemoglobin- and flavohemoglobin-expressing *E. coli* cells to SNP and paraquat. *E. coli* cells expressing either flavohemoglobins or hemoglobins

TABLE 2. $\mu_{stressor}/\mu_{no\ stress}$ ratios of cultures exposed to SNP

Strain[a]	$\mu_{SNP}/\mu_{no\ stress}$[b]
Control	0.68 ± 0.06
FHP	0.88 ± 0.02, a[c]
VHb-Red	0.88 ± 0.05, a
HmpKp	0.84 ± 0.03, a
HmpPa	0.86 ± 0.04, a
HmpSt	0.89 ± 0.05, a
HmpBs	0.92 ± 0.06, a
HMP	0.90 ± 0.08, a
HmpDr	0.93 ± 0.02, a
FHPg	0.92 ± 0.02, a
VHb	0.91 ± 0.06, a
CHb	0.87 ± 0.07, a
HMP[d]	0.99 ± 0.08, a
VHb[d]	0.96 ± 0.06, a

[a] Strains expressing various hemoglobins and flavohemoglobins in *E. coli* MG1655 cells. The control strain contained the parental plasmid pKQV4.

[b] Ratio of growth rate of a culture exposed to 1 mM SNP to that of a stressor-free control culture. Means and standard deviations are given. Values are calculated from the results of at least two independent experiments performed in triplicate.

[c] For statistical significance, data were analyzed by ANOVA ($F = 24.25$) and compared to data for the control culture by Dunnett's multiple comparison test (confidence interval, 99%). Different letters indicate significant differences ($P \leq 0.05$).

[d] Cultures of *E. coli* MG1655::pPPC1 and MG1655::pECS14 grown in the presence of 1 mM $K_4[Fe(CN)_6]$.

TABLE 3. $\mu_{stressor}/\mu_{no\ stress}$ ratios of cultures exposed to paraquat

Strain[a]	$\mu_{paraquat}/\mu_{no\ stress}$[b]
Control	0.32 ± 0.06
FHP	0.36 ± 0.04, a[c]
HmpKp	0.33 ± 0.08, a
HmpPa	0.43 ± 0.05, a
HmpSt	0.48 ± 0.05, b
HmpBs	0.24 ± 0.01, a
HMP	0.52 ± 0.07, b
HmpDr	0.52 ± 0.05, b
VHb	0.32 ± 0.06, a
CHb	0.28 ± 0.05, a

[a] Strains expressing various hemoglobins and flavohemoglobins in *E. coli* MG1655 cells. The control strain contained the parental plasmid pKQV4.

[b] Ratio of growth rate of a culture exposed to 100 μM paraquat to that of a stressor-free control culture. Means and standard deviations are given. Standard deviations were calculated from the results of at least two independent experiments performed in triplicate.

[c] For statistical significance, data were analyzed by ANOVA ($F = 18.47$) and compared to data for the control culture by Dunnett's multiple comparison test (confidence interval, 99%). Different letters indicate significant differences ($P \leq 0.05$).

achieved significantly higher $\mu_{SNP}/\mu_{no\ stress}$ ratios than the control strain (0.68) (Table 2). All recombinant *E. coli* strains investigated showed $\mu_{SNP}/\mu_{no\ stress}$ ratios of approximately 0.9. Thus, the growth rates of *E. coli* cultures expressing either flavohemoglobins or hemoglobins were only minimally affected by nitrosative stress, clearly indicating that expression of these hemoproteins helps the cells to resist the toxicity exerted by SNP. However, it was not possible to distinguish between the effects exerted by hemoglobins and flavohemoglobins. *E. coli* cells expressing either type of protein were equally capable of protection from nitrosative stress. To exclude the eventuality that cyanide liberated from SNP is responsible for growth inhibition, experiments using 1 mM $K_4[Fe(CN)_6]$ were performed. Growth rates of $K_4[Fe(CN)_6]$-containing cultures expressing either VHb or FHP were not affected by the presence of this complex relative to control cultures (Table 2).

The effect of paraquat treatment on the growth of the cultures was heterogeneous. Increased resistance to $O_2^{\cdot -}$ generated from paraquat was seen only in MG1655 cells expressing the flavohemoglobin HMP, HmpSt, HmpDr, or HmpPa. The specific growth rates of these cultures in the presence of paraquat were decreased by 50% at most from that of the stressor-free culture, resulting in $\mu_{paraquat}/\mu_{no\ stress}$ ratios of about 0.5. Cultures expressing FHP, HmpBs, HmpKp, CHb, or VHb were more severely affected by paraquat treatment, and $\mu_{paraquat}/\mu_{no\ stress}$ ratios were approximately 0.3, a value which does not significantly differ from that for the control strain (0.32) (Table 3).

In vitro NO· consumption assay. NO· decay was monitored with the nitric oxide analyzer. Soluble protein fractions were added to the reaction chamber, and the reaction was started by addition of an aliquot of an NO·-saturated solution. In the absence of a reducing agent, the consumption of NO· by flavohemoglobins and hemoglobins was only slightly accelerated compared to consumption in the protein-free buffer. Therefore, NADH was added to the reaction mixture. Addition of NADH dramatically increased the consumption of NO· by flavohemoglobins and hemoglobins relative to the decay observed in protein-free solutions. NADH addition was necessary for both flavohemoglobins, such as FHP, and classical hemoglobins, such as CHb (Fig. 1). The control cell extract, at the same heme concentration as CHb and FHP, showed no acceleration in NO· decay in the presence of additional NADH (data not shown).

The NO· consumption activities of various cell extracts are displayed in Fig. 2. The NO· turnover rates of the different flavohemoglobins and hemoglobins were calculated by subtracting the rate of NO· decay in buffer and standardizing to the heme content (Table 4). The NO· consumption rates for VHb, CHb, HmpDr, FHPg, and VHb-Red were not significantly different from that for the control. Protein extracts containing FHP or HmpPa had intermediate rates, leading to an acceleration of NO· turnover of approximately 10-fold relative to that of the control. Protein extracts containing the flavohemoglobins of *K. pneumoniae*, *S. enterica* serovar Typhi, *B. subtilis*, and *E. coli* showed the highest activities, up to 100-fold higher than the turnover numbers for the native hemoglobins (Table 4).

Due to the fast NO· removal, the NO· turnover rates of cell extracts of *S. enterica* serovar Typhi, *K. pneumoniae*, *B. subtilis*, and *E. coli* were also determined at an NO· concentration of 20 μM. The rates at a higher NO· concentration were not significantly different from the turnover determined at 10 μM except for the extract of *B. subtilis*, which reached a substantially higher rate at 20 μM than at 10 μM NO· (Table 4).

Previously, it has been shown that the aerobic detoxification of NO· by purified *E. coli* HMP stoichiometrically yields nitrate (11, 16). In contrast, the nonenzymatic reaction of NO· and O_2 in the aqueous phase should produce nitrite (38). To further prove the involvement of flavohemoglobins and hemoglobins in the observed NO· removal, nitrate and nitrite concentrations were determined. Mixtures identical to those used for deter-

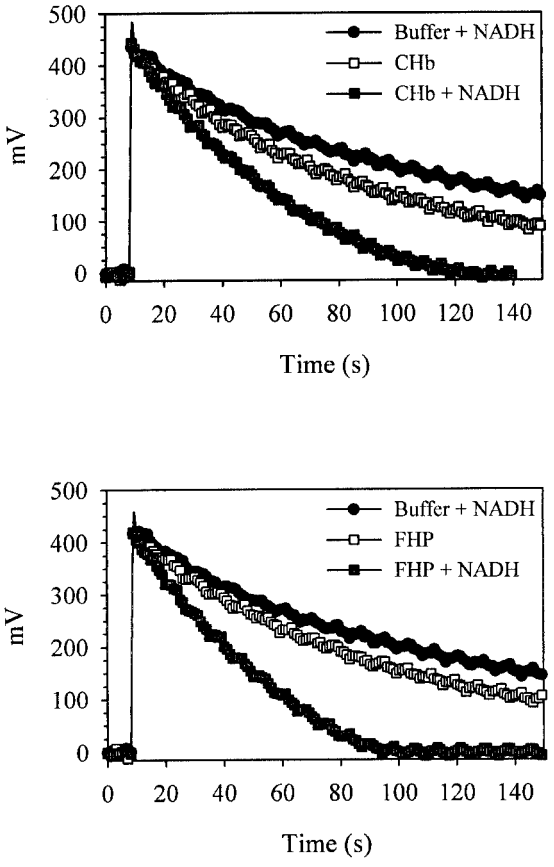

FIG. 1. NO· consumption curves of soluble protein fractions of cells expressing the *C. jejuni* hemoglobin CHb (upper panel) and *R. eutropha* FHP (lower panel) in the presence and absence of NADH. NO· consumption was measured with a nitric oxide analyzer using a chemiluminescence detector. The assay was performed with or without the addition of 200 μM NADH. A signal of 42 mV corresponds to 1 μM NO·.

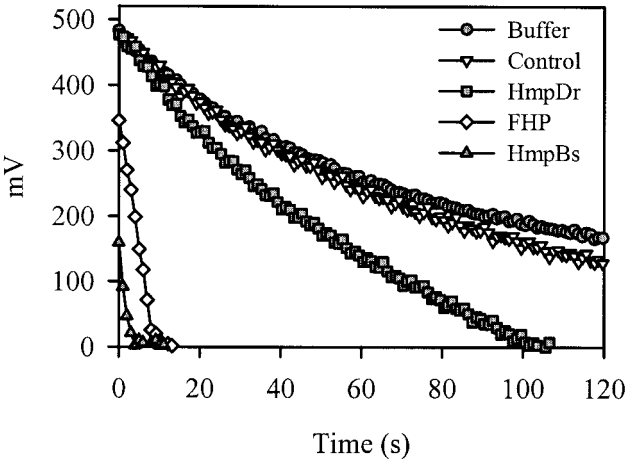

FIG. 2. NO· consumption curves of soluble protein fractions containing various bacterial hemoglobin and flavohemoglobin proteins. Protein extracts were added to a final concentration of 0.05 mg/ml in an air-equilibrated buffer containing 200 μM NADH. The reaction was initiated by addition of an aliquot of an NO·-saturated solution to a final concentration of 10 μM. NO· consumption was monitored with a nitric oxide analyzer using a chemiluminescence detector. A signal of 42 mV corresponds to 1 μM NO·.

mination of NO· consumption, including air-equilibrated buffer and 200 μM NADH, were prepared in sealed glass vials, and an NO·-saturated solution was injected to give a final concentration of 20 μM. After 30 min of incubation, the samples were analyzed for nitrite and nitrate. Almost all NO· was recovered as nitrate; nitrite was almost undetectable in all samples (0 to 1 μM) (Table 5). Thus, these data confirm that the acceleration of NO· decay by hemoglobin- and flavohemoglobin-containing protein extracts is due to the presence of these proteins and not to any nonspecific reaction.

DISCUSSION

E. coli cells expressing the different flavohemoglobins and hemoglobins displayed nonuniform responses to oxidative stress. Despite numerous reports, no clear indication for a common role of these proteins isolated from various organisms is obvious under oxidative stress. For *S. enterica* serovar Typhimurium no significant differences in the MICs of paraquat and H_2O_2 were observed between an *hmp* null mutant and a wild-type strain (6). Overexpression of *E. coli* HMP causes oxidative stress, but deletion leads to increased sensitivity to oxidative stress (1, 27). Therefore, it has been concluded that HMP might have a regulatory role (26). All these observations point to an important role for the dose of flavohemoglobin in the response to oxidative stress. This may be a possible reason for the differences observed between the different flavohemoglobins and hemoglobins tested in this study.

Although all hemoproteins were equally capable of sustaining the growth of *E. coli* under nitrosative-stress conditions, clear differences in their abilities to detoxify NO· were observed in the in vitro assay. Cell extracts containing the flavohemoglobins of *B. subtilis* or of the pathogenic bacteria *E.*

TABLE 4. NO· consumption of soluble protein fractions

Protein extract[a]	NO· turnover rates (s^{-1})[b] at the following NO· concn:	
	10 μM	20 μM
Wild type	1.9 ± 0.1	ND
Control	2.0 ± 0.1	ND
CHb	0.4 ± 0.1	ND
FHPg	3.4 ± 1.3	ND
VHb	0.9 ± 0.1	ND
VHb-Red	4.3 ± 1.0	ND
HmpDr	3.1 ± 1.5	ND
FHP	15 ± 1	ND
HmpPa	26 ± 4	ND
HmpKp	95 ± 1	86 ± 9
HmpSt	76 ± 4	78 ± 6
HmpBs	68 ± 11	128 ± 6
HMP	91 ± 4	90 ± 4

[a] Soluble protein fractions were obtained from *E. coli* MG1655 cells overexpressing either hemoglobins or flavohemoglobins. The control strain contained the parental plasmid pKQV4.

[b] NO· turnover rates were calculated with a correction for background rates of NO· decomposition and were expressed relative to heme content. Means of duplicate measurements ± standard deviations are given. ND, not determined. See the text for details.

TABLE 5. Nitrate production of cell extracts during NO· turnover

Protein extract[a]	Nitrate (μM)[b]
Wild type	17 ± 4
Control	18 ± 5
FHPg	19 ± 5
VHb-Red	15 ± 5
FHP	19 ± 6
VHb	17 ± 4
CHb	16 ± 3
HMP	22 ± 1
HmpSt	23 ± 3
HmpKp	18 ± 4
HmpBs	24 ± 4
HmpPa	24 ± 7
HmpDr	26 ± 3

[a] Soluble protein fractions were obtained from *E. coli* MG1655 cells overexpressing either hemoglobins or flavohemoglobins. The control strain contained the parental plasmid pKQV4.

[b] Nitrate recovery after conversion of 20 μM NO· by cell extracts. Means from two independent assays and standard deviations are given. Each measurement has been done twice.

coli, *K. pneumoniae*, *S. enterica* serovar Typhi, or *P. aeruginosa* proved to be the fastest. This is not surprising, since pathogenic bacteria are potentially exposed to large amounts of RNS when infecting their host organisms. The measured rates for NO· degradation by HMP and FHP are in good agreement with previously reported data (10).

The absence of a significant NO·-degrading activity of soluble protein fractions containing either VHb or CHb is in contrast to the results of the in vivo experiments. This could be due to the fact that a membrane-bound protein is involved in the enzymatic reduction of these hemoglobins, as in the reduction of myoglobin (25). It is noteworthy that in *Vitreoscilla* a nonlinked NADH-cytochrome *o* reductase exists, which copurifies with VHb (12). By analogy, an endogenous reductase system of *E. coli* might be able to reduce the heme iron of hemoglobins to the biochemically active ferrous form, leading to the acceleration of NO· degradation by the addition of NADH (Fig. 1). Another feasible explanation is that O_2 and NO· concentrations in the two assays differ.

NO· detoxification by HMP converts equistoichiometric amounts of NO· and O_2 to nitrate with the consumption of NADH (11, 16). The NO· removal mediated by our hemoproteins yielded mainly nitrate, indicating that the observed acceleration of NO· removal by protein extracts is indeed due to the hemoprotein-mediated conversion of NO· and is not caused by any nonspecific reaction.

To study the effect of the reductase domain on protection against nitrosative stress, a strain expressing the hemoglobin domain of FHP (FHPg) and a strain producing a chimeric protein consisting of VHb fused to the reductase domain of FHP (VHb-Red) were included (8). The results suggest that the presence or absence of the C-terminal reductase domain does not significantly change the protective effect in vivo. However, in vitro NO· consumption activity is clearly improved in the presence of the reductase domain, accelerating the activity of FHPg and VHb by a factor of 4 to 5. The lack of an appropriate external reductase system necessary for the full activity of hemoglobins could also explain the superior performance of flavohemoglobins relative to hemoglobins under those conditions, since the necessary rereduction of the heme iron for the NO· turnover in vitro is probably more effective if the reductase domain is bound directly to the globin domain. Kaur et al. (23) have also analyzed the effect of addition of a reductase domain to VHb in SNP-stressed cells, by using a construct very similar to that of VHb-Red, previously described by Frey et al. (8). Faster NO· consumption activity in the extracts containing the chimeric protein termed VHb-R relative to the VHb protein was also observed (23). Unfortunately, no NO· turnover value was reported. In agreement with our results, growth experiments under similar nitrosative-stress conditions do not result in significant differences between the growth performances of VHb- and VHb-R-expressing *E. coli* cells.

VHb and, more recently, FHP and related proteins have been routinely used to improve growth and protein production in various bacterial and eukaryotic species (20). VHb has been proposed to increase intracellular oxygen levels, concomitantly leading to more-efficient energy generation. (20, 21). FHP- or FHPg-expressing *E. coli* strains had higher oxygen uptake rates than strains producing VHb or VHb-Red under microaerobic conditions (9). These results correlate well with the observed higher NO· turnover rate of FHP and FHPg relative to VHb and VHb-Red. Therefore, it is tempting to assume that the role of VHb and FHP is to protect the respiratory chain from endogenously produced NO· rather than to increase the intracellular oxygen concentration. This hypothesis is further supported by recent reports that HMP induced protection of cytochromes against NO· and that the oxygen uptake of a hemoglobin-expressing strain was less affected in the presence of NO· (23, 35). Protection of cytochromes from NO· or an oxygen-providing role would require a close interaction of VHb with the cytochromes. Indeed, VHb has been shown to localize preferentially in the close vicinity of the respiratory chain in *Vitreoscilla* and also in *E. coli* (33).

In this study novel hemoglobins and flavohemoglobins originating from various bacterial species have been expressed in wild-type *E. coli* MG1655, which harbors a chromosomal copy of the endogenous *hmp* gene. Therefore, a background HMP level is likely to be present in our stress experiments, since *hmp* is known to be induced by paraquat and SNP (28, 32). Nevertheless, our results show clearly that overexpression of different flavohemoglobins and hemoglobins increases the natural resistance of *E. coli* cells under nitrosative stress.

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