# The flavohaemoglobin (HMP) of *Escherichia coli* generates superoxide in vitro and causes oxidative stress in vivo

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**Abstract** **Purified flavohaemoglobin (HMP) of *Escherichia coli* reduces Fe(III) in a superoxide dismutase (SOD)-sensitive reaction, demonstrating superoxide anion generation during aerobic NADH oxidation. In vivo, *sodA-lacZ* fusion activity was increased 3-fold by introducing plasmid pPL341, containing the *hmp* gene, or by growth with paraquat. The effects were additive and SOXS-dependent. Thus HMP activity causes oxidative stress in vivo. Activities of *sodA-lacZ* and *hmp-lacZ* fusions were stimulated in a *himA* mutant, demonstrating repression of both promoters by integration host factor (IHF), but the effects of pPL341 on *sodA-lacZ* activity were not due to titration of IHF by the *hmp* promoter.**

*Key words:* Hemoglobin; Superoxide; Superoxide dismutase; Flavohaemoglobin; Integration host factor; Transcription; *Escherichia coli*

## 1. Introduction

HMP, the product of the gene *hmp* of *Escherichia coli* [1], is a soluble protein with two domains. The N-terminal domain has striking sequence similarity to globins, including *Vitreoscilla* haemoglobin (VGB) [2]. The C-terminal domain of HMP is related to proteins in the ferredoxin-$NADP^+$ reductase (FNR) family [3]. Purified HMP [4] contains haem B and FAD and binds and reduces oxygen with electrons derived from NADH [5]. The behaviour of the flavin during turnover suggests that superoxide might be a product of oxygen consumption [6] and the midpoint potentials of the chromophores [7] are consistent with single-electron donation to oxygen with FAD mediating electron transfer from NADH.

Superoxide anion radical and other reactive by-products of oxygen metabolism are produced in all aerobic cells and, if not scavenged, can damage critical biomolecules during oxidative stress. Recent work has identified several sources of intracellular superoxide and peroxide generation in *E. coli*, particularly the aerobic respiratory chains and fumarate reductase [8,9]. An NAD(P)H:flavin oxidoreductase could also be a major source of intracellular superoxide [10]. No haem-containing oxidase has yet been reported in *E. coli* that reduces oxygen to superoxide. *E. coli* possesses several superoxide dismutases (SOD); Mn-SOD, encoded by *sodA*, is induced in response to oxidative stress via the SOXR/SOXS sensor/activator system and its synthesis is subject to highly complex regulation by six global regulators [11].

Here we demonstrate superoxide generation by purified HMP and use a *sodA-lacZ* fusion to demonstrate in vivo that overexpression of the *hmp* gene causes oxidative stress and enhances *sodA-lacZ* activity via the SOXR/SOXS system.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

The *E. coli* K12 strains and plasmids used in this study are described in Table 1. Genetic crosses used to introduce *soxS* and *himA* mutations were performed using bacteriophage P1-mediated transduction [17]. Transformations were carried out using the $CaCl_2$-procedure [18].

The chromosomal (*sodA-lacZ*)49 fusion in strain QC772 [11] is a protein fusion which faithfully mimics transcriptional induction of *sodA*. The *hmp-lacZ* operon fusion (RKP2178) was constructed on a plasmid and then transferred to λ phage by recombination in vivo, using the method of Simons et al. [16]. A 635 bp DNA fragment was excised from pPL341 using *Bam*HI and *Sma*I and ligated into the site created by digestion of pRS528 with *Sma*I, then *Bam*HI. The required recombinant plasmid was isolated by transformation of strain RK4353 (Δ*lac*). The fusion was recombined onto λ RS45 to make λ RKP1. Several single-copy fusions to the chromosome of VJS676 (Δ*lac*) were isolated and verified using β-galactosidase assays [17] and Ter tests as described before [19]; one such fusion strain (RKP2178) was used.

### 2.2. Media, growth conditions and ß-galactosidase assays

Cells were grown at 37°C in LB medium [17], pH 7.0, with ampicillin (Ap, 200 μg/ml) or chloramphenicol (Cm, 50 μg/ml) as appropriate. Cultures were grown in 500 ml Erlenmeyer flask containing 100 ml medium. Cells in mid-exponential phase ($OD_{600nm}$ 0.4, measured in a Pye-Unicam SP6-550 spectrophotometer) were treated with 200 μM paraquat (PQ; methyl viologen, Sigma) for 45 min, harvested, and assayed for ß-galactosidase [17]. Assay results given are means of three determinations from one experiment; similar results were obtained in three separate experiments.

### 2.3. Iron(III) reductase activity

This was assayed by measuring reduction of Fe(III) citrate; Fe(II) was trapped and determined at 562 nm as the magenta ferrozine complex [20]. The reaction mixture (1.33 ml) contained 0.1 M MOPS buffer (pH 7.0), 0.2 mM NADH, catalase (300 Sigma units), 1 mM ferrozine and equal volumes of 10 mM $FeCl_3$ and 10 mM citrate solutions to give final concentrations up to about 0.2 mM Fe(III) citrate; the reaction was started by the addition of 0.8 nmol HMP, purified as described by Ioannidis et al. [4]. In anaerobic assays of Fe(III) reduction, 7.5 mM glucose, 0.5 mg glucose oxidase (Sigma) and catalase (see above) were added to deplete residual oxygen levels in buffers and other reagents, before initiating the reaction, 5–7 min later, by adding HMP. Where indicated, SOD (50 Sigma units) was added to remove superoxide, the result of one-electron reduction of oxygen. Values of $K_m$ and $V_{max}$ shown were obtained by fitting the Michaelis-Menten rate equation to the data. Similar values were obtained by using the Hanes or Eadie-Hofstee plots (not shown).

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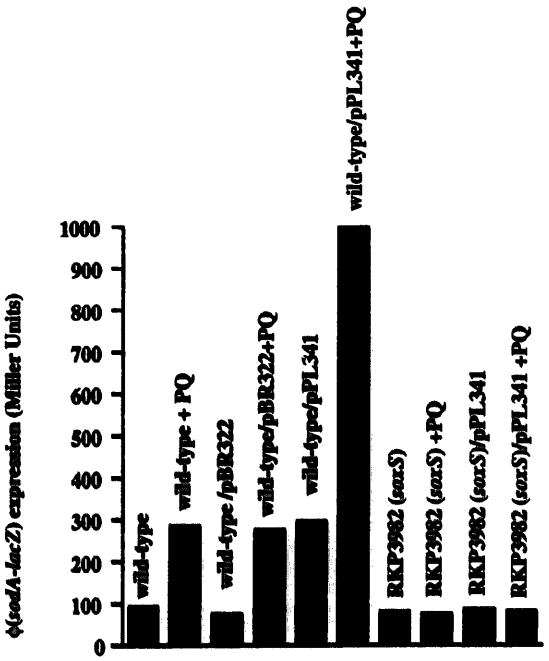


Fig. 1. Effect of PQ (200 μM) and HMP on the expression of *sodA-lacZ*.

## 3. Results and discussion

### 3.1. Hmp reduces Fe(III) and produces superoxide

HMP reduces Fe(III) [3,21] but the reaction is not well characterised and the complications of simultaneous oxygen reduction have not been addressed. Use of ferrozine to trap Fe(II) in a simple aerobic assay gave a $V_{max}$ of 0.09 $s^{-1}$ and a $K_m$ of 69.2 μM (Table 2). To determine whether HMP generates superoxide that contributes to the rate of Fe(III) reduction, the assay was repeated with SOD. The $K_m$ for Fe(III) was unchanged but $V_{max}$ decreased >3-fold (Table 2). This indicates that most of the Fe(III) reductase activity is due to superoxide anion; the SOD-insensitive portion of the activity may be due to reduction by electron transfer from FAD. When oxygen was removed from the assay mixture using glucose, glucose oxidase and catalase (which removed oxygen to sub-micromolar levels), the rate of Fe(III) reduction was not significantly different from the aerobic assay with SOD. However, the affinity for Fe(III) was increased about 10-fold, indicating that oxygen is a competitive inhibitor of Fe(III) reduction.

### 3.2. Paraquat and over-expression of hmp each increase transcription of a sodA-lacZ fusion

The *sodA* gene is regulated by a complex inter-connected regulatory system involving at least six global regulators (SOXR/S, FNR, SOXQ, FUR, ARCA/B and IHF) [11]. A consequence of such regulation is transcriptional activation of *sodA* in response to superoxide stress. To study the effect of over-producing HMP on *sodA* expression, we introduced plasmid pPL341 into strain QC772 (*sodA-lacZ*). Plasmid pPL341 induced *sodA* expression to a similar extent (about 3 times the basal level) as that achieved by growth in the presence of 200 μM paraquat, a redox cycling compound that increases the intracellular flux of superoxide radical in the presence of oxygen [22]. The levels of induction achieved by addition of paraquat were similar in the presence or absence of the vector pBR322 (Fig. 1). However, the effects of paraquat and pPL341 (*hmp*$^+$) were additive, giving together *sodA-lacZ* activity that was about 10 times the basal level (Fig. 1).

To determine if *sodA* induction by pPL341 was via the *soxRS* system, we introduced a *soxS* mutation into strain QC772. Induction by the presence of plasmid pPL341 or by adding paraquat was abolished, demonstrating that the induction caused by plasmid pPL341 is mediated by SOXS.

Table 1
Strains and plasmids used

| Strain, plasmid, phage | Relevant genotype/properties | Reference or source |
|---|---|---|
| ***E. coli* strains** | | |
| BW831 | *soxS*::Tn*10* | [12] |
| GS019 | *him*A::cat | [13] |
| QC772 | Δ*lacU*, *rpsL*, φ(*sodA-lacZ*) | [10] |
| RK4353 | *araD139* Δ(*ara-gpt-lac*)*U169* *gyrA219* *non-9* *rpsL150* | [14] |
| RKP2178 | φ(*hmp-lacZ*) | this work |
| RKP3981 | as QC772 but *himA*::cat QC772 × P1 (GS019) | this work |
| RKP3982 | as QC772 but *soxS*::Tn*10* QC772 × P1 (BW831) | this work |
| RKP3990 | as RKP2178 but *himA*::cat RKP2178 × P1 (GS019) | this work |
| VJS676 | Δ*lac* | Valley Stewart |
| **Plasmids** | | |
| pBR322 | Ap$^r$ | [15] |
| pRS528 | Ap$^r$ *lacZ*$^+$ *lacY*$^+$ *lacA*$^+$ | [16] |
| pPL341 | *hmp*$^+$ in 1.9 kb *Eco*RI-*Bam*HI fragment in pBR322, Ap$^r$ | [1] |
| **Phage** | | |
| λRKP1 | φ(*hmp-lacZ*)*1* (gene fusion) | this work |
| λRS45 | *bla'-lacZ*$_{SC}$ *att*$^+$ *int*$^+$ *imm*$^{21}$ | [16] |

### 3.3. Induction of sodA by HMP is IHF-independent

The increased expression of the *sodA-lacZ* fusion as a result of over-expressing HMP (Fig. 1) might be due to titration by the *hmp* promoter of repressors of *sodA*. Under the aerobic growth conditions employed here, FNR is unlikely to be active [23] and will not contribute to *sodA* regulation. We could detect no 'iron box' (GATAATGAT A ATCATTATC) for binding FUR [24] in the *hmp* promoter region. A third repressor of *sodA* is Integration Host Factor (IHF), product of the *himA* gene [25]. This was confirmed by transducing the *himA* mutation into QC772, giving strain RKP3981 (Fig. 2; compare with Fig. 1). The 1.8-fold increase in *sodA-lacZ* activity is in good agreement with data of Compan and Touati [11] and Pressutti and Hassan [26] who reported increases in *sodA-lacZ* activity of 1.4-and 1.6-fold, respectively, under aerobic conditions. We then tested the ability of pPL341 to induce *sodA-lacZ* expression in RKP3981 (*himA*) (Fig. 2). Plasmid pPL341, but not the vector pBR322, caused a >5-fold increase in activity, demonstrating that the effect of pPL341 on *sodA* does not require IHF. The activity of *sodA-lacZ* in the wild type (Fig. 1) and *himA* strains (Fig. 2) was induced to equal extents by paraquat (about 3-fold), confirming that induction of *sodA* by SOXS is independent of IHF [26]. Addition of PQ to cultures of RKP3981 (*himA*)/pPL341 did not increase *sodA-lacZ* expression further.

Introduction of the *himA* mutation into the *hmp-lacZ* fusion strain (RKP2178), to give RKP3990, increased transcriptional activity 3-fold (Fig. 2), showing that IHF also represses *hmp* gene expression.

### 3.4. Conclusions

We show that the flavohaemoglobin HMP produces superoxide in vitro and causes oxidative stress in vivo, as reflected in SOXS-dependent *sodA-lacZ* expression. The presence of a multicopy plasmid containing the *hmp* gene results in a 3-fold increase of *sodA-lacZ* expression; addition of 200 μM PQ gave a further 3-fold increase in *sodA* activity. The increased *sodA* expression caused by the presence of the plasmid pPL341 was not due to titration of the repressor IHF, since a *himA* mutant still responded to the introduction of pPL341. Since the effects on *sodA-lacZ* of paraquat and plasmid pPL341 are both mediated via the SOXR/S system, and therefore presumably can be explained by increased superoxide generation, their additive effects were unexpected. One intriguing possibility is that the expression of *hmp* from pPL341 is itself induced by paraquat or superoxide. Experiments are currently in progress to study the expression of *hmp*

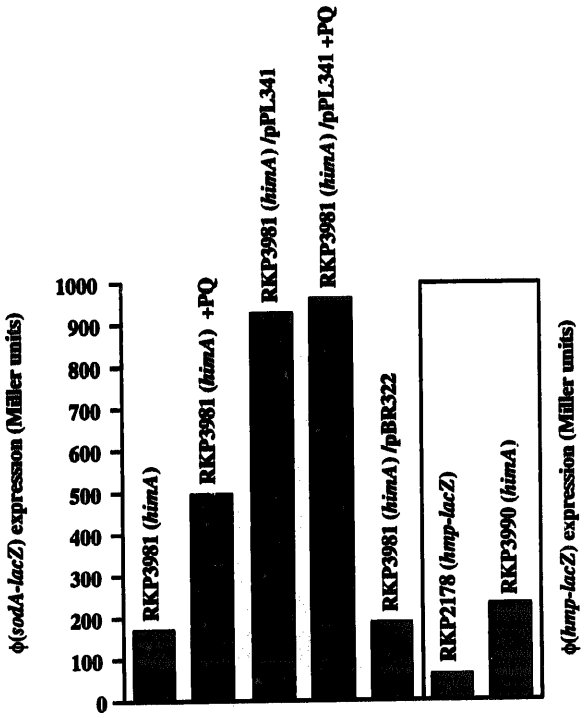


Fig. 2. Effect of *himA* mutation on the expression of *sodA-lacZ* and *hmp-lacZ* fusions.

Table 2
Fe(III) reductase activities of purified HMP

| Assay conditions | $K_m$ (μM) | $V_{max}$ ($s^{-1}$) |
|---|---|---|
| Aerobic | 69.2 ± 9.8 | 0.090 ± 0.004 |
| Aerobic + SOD | 65.9 ± 36.8 | 0.027 ± 0.004 |
| Anaerobic + SOD | 6.2 ± 0.8 | 0.020 ± 0.004 |

$V_{max}$ values are expressed as turnover numbers (activity per mol of enzyme). Values shown are means ± S.D.

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