Flavohemoglobin requires microaerophilic conditions for nitrosative protection of *Staphylococcus aureus*

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Abstract Flavohemoglobinns and flavodiiron proteins are two families of enzymes involved in nitrosative detoxification. However, the physiological oxygen-related conditions under which they work and their relative role are still a matter of debate. To address this question we analyzed the function of the putative flavohemoprotein of *Staphylococcus aureus*, an organism that lacks a flavodiiron-like gene. In this report we show that the recombinant protein contains all features typical of canonic flavohemoglobins and that the transcription of flavohemoglobin gene was upregulated by nitrosative stress in an oxygen-dependent manner. However, and in contrast to other bacterial flavohemoglobins, the *S. aureus* protein has no apparent role in aerobic nitrosative protection, being only beneficial when cells of *S. aureus* are submitted to nitrosative stress in a microaerophilic environment. The in vivo data corroborates the proposal that Hmp acts physiologically as a denitrosylase.

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1. Introduction

The prokaryotic defence systems against nitrosative stress involve at least two families of enzymes that directly detoxify nitric oxide (NO) or S-nitrosothiols: the flavodiiron NO reductases and the flavohemoglobins (Hmp) [1,2]. Flavohemoglobinns, which have a two-domain structure formed by a hemoglobin-like domain (containing a single B-type heme) and a NADP⁺:ferredoxin oxidoreductase-like domain (harbouring a FAD moiety and a NAD(P)H binding motif) are able to function as NO scavengers. In the presence of oxygen, Hmp oxidizes NO to nitrate, with a range of activities that varies from 7.4 to 128 s⁻¹, at 20 °C [3–5]. Anaerobically, Hmp is also able to reduce NO to N₂O, but with a much lower activity of ca 0.14–0.5 s⁻¹ [2,6]. Both the oxidation of NO to nitrate as well as the reduction of NO to N₂O are proposed to occur through a common NO-(nitroxyl anion-bound heme) intermediate. Since the former reaction can operate very efficiently under microaerobic conditions [3,4], Hmp may be adapted mainly to protect under microaerobic conditions, which are typical in vivo.

The beneficial role of Hmp in protection from nitrosative stress under aerobic and anaerobic conditions has been demonstrated for several microorganisms [2–9]. In *E. coli*, both under microaerobic and anaerobic growth conditions, flavohemoglobin was found to confer a degree of NO protection similar to that of the flavodiiron NO reductase [7,8,10]. It was also observed that, when compared with flavohemoglobin, the higher levels of Hmp expression are reached at longer times after the addition of the nitrosative stress, and that the lack of flavohemoglobin production was not compensated by an increase in the expression of flavohemoglobin and vice-versa [10]. These results strongly suggest that in *E. coli* the two enzymes play different roles in nitrosative cell protection. Flavodiiron NO reductases and flavohemoglobin-like proteins are widely distributed among bacteria, fungi and protozoa and, as in *E. coli*, in a large number of genomes the two genes are present in the same organism. On the contrary, in the *Staphylococcus aureus* genome sequence only the gene encoding for a putative flavohemoprotein is present, making *S. aureus* a good system to study the role of Hmp per se. In addition, *S. aureus* is a major human pathogen capable of causing from mild to life-threatening systemic diseases, which results from the ability to colonize different environmental niches, i.e., to survive a diverse range of stresses. Furthermore, the spread of antibiotic-resistance of *S. aureus* strains constitutes a major worldwide concern [11,12]. In spite of its clinical importance the *S. aureus* cellular components involved in the response to reactive nitrogen species, that constitutes a major mammalian defence mechanism against pathogens, remains almost unknown.

In the present work we have cloned and produced the recombinant *S. aureus* Hmp and performed its biochemical characterization. To elucidate its physiological function the *S. aureus* hmp gene was disrupted and the resistance to the nitrosative stress of the mutant was analysed.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

Strains *E. coli* XL2-Blue, *E. coli* BL21Gold(DE3), *S. aureus* NCTC8325 and its derivative *S. aureus* RN4220, and plasmids pET-28a (Novagen) and pSP64E [13,14] were used in this work. *S. aureus* strains and pSP64E were a kind gift of Prof. H. de Lencastre. *S. aureus* cells were grown in TSB or LB, at pH 7, aerobically in flasks filled with 1/5 of its volume, microaerophilically in closed flasks completely filled or anaerobically in rubber seal capped flasks that, once filled with media and closed, were extensively bubbled with nitrogen. Cells were cultivated at 37 °C and only the aerobically grown cultures were shaken at 180 rpm.
2.2. Cloning, expression and purification of S. aureus recombinant Hmp

To clone the hmp gene, primers based on flanking sequences that generated NcoI and EcoRI restriction sites were used to amplify a 1.2-kb fragment from the S. aureus NCTC8325 genomic DNA [15], isolated as described in [16]. The gene was ligated into pET28a (Novagen), yielding pETHmp, and introduced into E. coli XL2-Blue. Positive recombinant plasmids were selected from kanamycin-resistant colonies and DNA sequenced. Overexpression of the recombinant protein was achieved in pETHmp containing E. coli BI21Gold(DE3) cells, grown aerobically in LB supplemented with 3 μM FeCl3, 100 μM riboflavin and 30 μg/mL kanamycin. When cells reached OD600 of 0.4, 500 μM isopropyl-β-D-galactopyranoside and 50 μM aminolevulinic acid were added and growth continued for another 6 h. Cells were disrupted and the soluble extract loaded into a Q-Sepharose High Performance column, previously equilibrated with buffer A (Tris–HCl 10 mM, pH 7.6, and glycercol 20%). Hmp was eluted at ~240 mM NaCl, and applied to a Superdex S-75 gel filtration column equilibrated with buffer A + 150 mM NaCl. The protein was then reloaded on the Q-Sepharose column and the protein eluted at ~170 mM NaCl was found to be pure, as judged by SDS–PAGE. Protein concentration was assayed by the bicinchoninic acid method with BSA as the standard [17], flavin content was quantified after acid extraction with trichloroacetic acid [18], and heme content assayed using the hemochromopidine method [19].

2.3. EPR, UV–Vis spectroscopy and redox titration

EPR spectra were obtained on a Bruker ESP 380 spectrometer, equipped with an Oxford Instruments continuous flow helium cryostat. UV–Vis absorption spectra of S. aureus Hmp were acquired using a Shimadzu UV-1603 spectrophotometer, at room temperature. S. aureus Hmp (~11 μM) was titrated anaerobically as described in [20].

2.4. Construction of S. aureus hmp deletion strain and complementation analysis

To disrupt the hmp gene, an internal fragment (800 bp) of the S. aureus NCTC 8325 hmp was PCR amplified, using oligonucleotides (SAHECO: 5′-GAAAGGCGACAGAATTCACTC-3′ and SAHBAM: 5′-GTGTTGATGATGCCGATC-3′), and ligated into pSP64E. The resulting pSPHmp, was electroproporated into S. aureus RN4220 [21,22] and transformants were selected on TSA plates containing erythromycin (10 μg/mL). Chromosomal DNA isolated from single colonies and PCR analysis was used to confirm the correct integration of pSPHmp into the chromosome of RN4220. One of such colonies was designated LMS800 and used in subsequent studies.

For the complementation analysis, the plasmids pETHmp and pET28a were individually transformed into LMS2710 strain (E. coli norV mutant) [10]. Single colonies were grown overnight and used to inoculate minimal salt medium [19] containing chloramphenicol and kanamycin. The growth was performed under anaerobic conditions and monitored at 600 nm.

2.5. RNA extraction and RT-PCR analysis

Total RNA was isolated from cells grown under the indicated conditions using the hot-phenol method, and treated with DNaseI. The forward and reverse primers used to create the disruption of the hmp gene were also utilized in the RT-PCR assays, performed with USB Reverse Transcriptase Kit. After confirming the absence of any residual DNA, RT-PCR reactions were performed with 150 ng of RNA. The 16S rRNA gene was used to guarantee that equal amounts of RNA were compared.

2.6. NADH oxidase, NO denitrosylase and NO reductase activities of S. aureus Hmp

The kinetic experiments were recorded in a Shimadzu UV-1603 spectrophotometer and performed at 25 °C. The NADH oxidase activity of S. aureus Hmp (30 nM) was measured by monitoring the anaerobic NADH consumption (200 μM), using potassium ferricyanide (K3Fe(CN)6) (500 μM) as artificial electron acceptor, and following the absorbance decrease at 420 nm (ε420nm = 1020 M–1 cm–1). The NO denitrosylase activity of Hmp (36 nM) was measured aerobically by following the NADH oxidation (200 μM) (ε340nm = 6200 M–1 cm–1) upon addition of aliquots of a saturated NO solution [10]. The NADH:NO oxidoreductase activity was determined by anaerobically incubating NADH (200 μM) with Hmp (365 nM), and monitoring the NADH oxidation by NO. Activities are reported in terms of NO consumption, using the proposed stoichiometry of 2 NO molecules per NADH molecule, for both the denitrosylase and reductase reactions [23]. All activities were calculated taking into account the stoichiometric heme and flavin content of the as purified protein.

3. Results and discussion

3.1. S. aureus flavohemoprotein is a canonical flavohemoglobin

In all known genome sequences of S. aureus strains it is present a gene predicted to code a flavohemoprotein that shares ~30% sequence similarity with the E. coli Hmp. To assess the function of the S. aureus Hmp, the recombinant protein was produced and characterized. Upon purification, S. aureus Hmp exhibited a molecular mass of 44 kDa that corresponds to a monomeric protein, and contained 0.7 mol flavin and 1 mol heme. The UV–Vis spectrum of S. aureus Hmp displayed the features typical of the canonical Hmps in the oxidised, reduced and CO bound states (Fig. 1A). Also, the EPR spectrum is characteristic of high-spin ferric iron (data not shown). A reduction potential of −170 mV was measured for the heme centre and two identical reduction potentials of −190 mV were measured for the FAD centre (Fig. 1B).

Fig. 1. Characterization of the as-purified S. aureus Hmp. (A) UV–Vis spectra of Hmp oxidized (−−), reduced with sodium dithionite (●●●), and reduced and CO ligated (- - - ). (B) Redox titration of the heme centre (●) and of the FAD centre (□) of S. aureus Hmp. Full lines were calculated with the Nernst equation for a monoelectronic (reduction potential of −170 mV) and two monoelectronic consecutive processes (identical reduction potentials of −190 mV), for the heme and FAD centres, respectively.
The NADH oxidase activity of *S. aureus* Hmp was determined to be 34 s⁻¹, using potassium ferricyanide as the electron acceptor. Hmp exhibited an NO denitrosylase activity of 66 s⁻¹, measured upon addition of 20 μM NO, and a NADH:NO oxidoreductase activity of 0.7 s⁻¹. These values are within the range usually reported for homologous enzymes [23].

### 3.2. *S. aureus hmp transcription is mainly regulated by oxygen limitation*

The mRNA level of *hmp* under nitrosative stress conditions in aerobic and oxygen-limiting conditions was evaluated in RT-PCR experiments (Fig. 2). The transcription of *hmp* was found to be low under aerobic conditions and to increase considerably on switching to oxygen-limited growth conditions. *S*-nitrosoglutathione (GSNO) caused an increase in the *hmp* transcription level under aerobic conditions, and no major variations were observed in microaerophilic or anaerobic grown conditions. Hence, the results indicate that the major trigger for *hmp* induction is oxygen limitation, thus suggesting a physiological function under oxygen limited conditions. In general, *hmps* exhibit oxygen-dependent gene expression via the oxygen sensor-regulator Fnr [2], but analysis of the upstream sequence of the *S. aureus hmp* coding region did not allow identification of any obvious Fnr binding motif.

![Fig. 2. *S. aureus hmp* expression increases upon oxygen limitation.](image)

RNAs isolated from *S. aureus* grown aerobically (A), microaerophilic (B) or anaerobically (C) in LB for 4 h in the absence (−) or in the presence (+) of 200 μM (A,B) or 50 μM (C) GSNO. The equal loading of total RNA was confirmed by the same intensity of the 16S rRNA band (lower panel). Data are representative of reactions performed with two independent RNA samples.

![Fig. 3. Microaerophilic GSNO-protection conferred by flavohemoglobin to *S. aureus*.](image)

*S. aureus* wild type strain RN4220 (wt) and mutant strain LMS800 (Δ*hmp*), were grown aerobically (A,B), microaerophilically (C,D) or anaerobically (E) in LB medium and left untreated (●) or treated with 50 μM GSNO (▲), 100 μM GSNO (●) and 200 μM GSNO (●). Each growth curve represents the average of at least three independent cultures. Panel (F) Analysis of complementation of *E. coli* LMS2710 (Δ*norV*) with *S. aureus* Hmp. Growth curves acquired under anaerobic conditions in minimal medium for the *E. coli* flavohemoglobin mutant strain LMS2710 harbouring either vector alone (pET) (○) and with pETHmp expressing *S. aureus* Hmp (●) without addition of GSNO or in the presence of with 50 μM GSNO: LMS2710 (pET) (▲) and LMS2710 (pETHmp) (●).
3.3. Protection of S. aureus by flavohemoglobin in response to GSNO depends on the oxygen-related conditions

To infer the role for Hmp in nitrosative protection, the physiological effects of various concentrations of GSNO and oxygen on S. aureus hmp mutant and wild type strains were analysed (Fig. 3). Under aerobic conditions, GSNO concentrations up to 100 μM did not cause growth inhibition of wild type S. aureus, while 200 μM GSNO induced a severe growth arrestment (Fig. 3A). Under microaerophilic conditions, all tested concentrations of GSNO ranging from 50 to 200 μM only caused a negligible effect on growth of wild type S. aureus (Fig. 3C). However, when wild type S. aureus was grown under anoxic conditions the effect of similar concentrations of GSNO was more pronounced, as judged by the significant degree of growth impairment observed with 50 μM GSNO (Fig. 3E). Therefore, no additional work was done with GSNO and the hmp mutant under anaerobic conditions.

In the absence of GSNO, the S. aureus hmp mutant displayed an oxygen-dependent growth behaviour similar to that of wild type RN4220 strain (Fig. 3B,D). Under aerobic conditions, and for all the concentrations of GSNO tested, there was no discernible difference in the rates and extents of growth, as measured by the OD₆₀₀ between the parent and mutant strain (Fig. 3B). However, under microaerophilic conditions exposure of the wild-type S. aureus to 200 μM GSNO caused a decrease of the growth rate of ~7%, whereas the Δhmp strain exhibited a lag in growth of ~50% (Fig. 3D). A similar behaviour was also observed for the S. aureus hmp mutant in the presence of 50 μM of NO gas (data not shown). Hence, Hmp seems to be able to protect S. aureus submitted to a strong nitrosative stress only under microaerophilic conditions.

3.4. S. aureus Hmp attenuates the anaerobic NO damage in E. coli flavohemoglobin mutant

Our previous work showed that deletion of flavohemoglobin gene (norV) in E. coli, strain LMS2710, resulted in a mutant with increased sensitivity to anaerobically added NO [10]. Complementation studies were now conducted to test whether S. aureus Hmp could perform the role of flavohemoglobin in E. coli. The results showed that expression of S. aureus hmp in the E. coli norV mutant, LMS2710, lead to a significant increase in the anaerobic GSNO resistance of the mutant strain (Fig. 3F), indicating that S. aureus Hmp has the ability to perform anaerobic nitrosative detoxification. In fact, this ability could not be analysed in S. aureus since the wild type strain is itself highly sensitive to GSNO under anaerobic conditions. Nevertheless, the possibility that the low NO reductase activity of S. aureus Hmp was compensated by the presence of a large amount of protein generated by overexpression can not be excluded. Since only overexpression of S. aureus hmp could improve nitrosative protection, the level of anoxic expression S. aureus hmp and/or lower NO reductase activity seems to be insufficient to protect S. aureus anaerobically. Furthermore, it is already well documented in other organisms that, besides flavohemoglobin and Hmp, there are many other factors involved in the response to nitrosative stress, e.g. [10], which are so far unknown in S. aureus and may be also responsible for the high sensitivity of S. aureus to anaerobic nitrosative stress.

4. Conclusion

S. aureus infection is related to the versatility of the pathogen to grow in different and often hostile environmental niches. In this study we observed that wild type S. aureus grows, although differently, under aerobic and anaerobic conditions and also in the presence of GSNO. However, GSNO resistance is fully dependent on the degree of oxygenation. While, in anaerobic conditions S. aureus is very sensitive to GSNO since slow growth is observed in any of the tested concentrations, in microaerophilically S. aureus sustains growth up to 200 μM GSNO. Interestingly, Hmp confers S. aureus protection against nitrosative challenge only under microaerophilic conditions. This suggests that in vivo Hmp acts as a nitroxylase, i.e., under low oxygen concentration and high NO concentration the nitrosylase mechanism is operative, as previously proposed by Hausladen and coworkers [4]. Last but not least, it is quite interesting that recent studies on S. aureus indicated that the pathogenesis of this microbe, and in particular the production of virulence factors, is also dependent on the oxygen concentration [24,25].

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