Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide

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Abstract The flavohaemoglobin Hmp of *Escherichia coli* is inducible by nitric oxide (NO) and provides protection both aerobically and anaerobically from inhibition of growth by NO and agents that cause nitrosative stress. Here we report rapid kinetic studies of NO binding to Fe(III) Hmp with a second order rate constant of $7.5 \times 10^{6}$ M\textsuperscript{-1} s\textsuperscript{-1} to generate a nitrosyl adduct that was stable anoxically but decayed in the presence of air to reform the Fe(III) protein. NO displaced CO bound to dithionite-reduced Hmp but, remarkably, CO recombined after only 2 s at room temperature indicative of NO reduction and dissociation from the haem. Addition of NO to anoxic NADH-reduced Hmp also generated a nitrosyl species which persisted while NADH was oxidised. These results are consistent with direct demonstration by membrane-inlet mass spectrometry of NO consumption and nitrous oxide production during anoxic incubation of NADH-reduced Hmp. The results demonstrate a new mechanism by which Hmp may eliminate NO under anoxic growth conditions.

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1. Introduction

Nitric oxide (nitrogen monoxide, NO\textsubscript{2}, written here as NO) is now recognised to be of major importance in biology and medicine, particularly as a molecular messenger with a broad spectrum of activities in the central nervous, cardiovascular, and immune systems [1]. Microbiologically, NO is important as an intermediate in denitrification, being produced by nitrite reductase activity, and in turn serving as substrate for nitric oxide reductases which convert it to N\textsubscript{2}O [2]. NO is also a component of the arsenal of reactive oxygen and nitrogen intermediates produced by macrophages which exert oxidative and nitrosative stress on engulfed microorganisms [3,4].

Recent research has revealed a special role in NO metabolism or detoxification for a distinctive class of haemoproteins – the flavohaemoglobins of bacteria and yeast [5]. The *Escherichia coli* flavohaemoglobin Hmp is the best characterised such protein and the structural gene (*hmp*) was the first bacterial globin gene to be cloned and sequenced [6]. The C-terminal domain resembles ferredoxin-NADP\textsuperscript{+} reductase domains [7] and transfers electrons from NAD(P)H via FAD to the haem [8]. Oxygen, even at submicromolar concentrations, binds to the haem but is reduced to superoxide anion and released [9–12].

The first indication that Hmp might be involved in NO biochemistry was the demonstration of the marked up-regulation of *hmp* transcription in cultures treated with solutions of NO gas or nitrite [13] and, later, the ‘NO releaser’ S-nitrosothiol (GSNO) and sodium nitroprusside (SNP) [14]. Intriguingly, this induction is largely independent of the SoxRS system (which is responsive to superoxide stress as well as to NO [3,4]) but is instead explained by nitrosation of homocysteine and consequent attenuation of MetR binding to the *hmp* promoter [14]. Other evidence for flavohaemoglobin interactions with NO and related reactive species comes from: (1) the inability of an *Alcaligenes eutrophus* mutant defective in Fhp (an Hmp homologue) to accumulate nitrous oxide during denitrification [15]; (2) the sensitivity of a specifically engineered *hmp* mutant of *E. coli* [16] and of *Salmonella typhimurium* [17] to NO, redox-related species, and nitrosating agents; and (3) the demonstration that Hmp has NO oxygenase activity forming nitrate [18,19].

Aerobic conversion of nitric oxide to nitrate has long been known for other globins. However, *hmp* mutants are sensitive to NO and related species both aerobically and anaerobically [18] suggesting oxygen-independent modes of Hmp/NO interactions. In this paper, we report the reaction of NO with purified Fe(III) Hmp from *E. coli* and demonstrate its reversibility. Significantly, we report transient NO binding in the presence of CO and direct mass spectroscopic evidence for reduction of NO to N\textsubscript{2}O under anoxic reducing conditions.

2. Materials and methods

2.1. Purification of Hmp

*E. coli* strain RSC521 (RSC49 harbouring the multicopy plasmid pPL341, having the *hmp* gene under the control of its native promoter) [6] was grown aerobically, disrupted in a French pressure cell, and used to purify Hmp by two chromatographic steps [20]. Enzyme concentration was measured from spectra of the native (Fe(III)) enzyme using the absorption coefficients described by Ioannidis et al. [20].

2.2. Spectrophotometric studies of Hmp-NO interactions

Studies of reversible NO binding to the Fe(III) protein and NADH oxidation were performed in a Beckman DU-650 single-beam spectrophotometer. Hmp was diluted in 100 mM MOPS buffer (pH 7.0) and added as described in Section 3 to 4 ml cuvettes of 1 cm pathlength having a ground glass hole fitted with a rubber seal, to which NADH and NO (prepared as described in [13]) were added as anoxic.
solutions. The rapid reactions of NO and reduced Hmp complexed with CO were investigated at 25°C using the stopped flow rapid scan instrument described before [8]. The globin solution, in 50 mM MOPS/NaOH buffer (pH 7.0) containing 50 mM NaCl, was placed in one of the reservoirs of the 1:1 mixing device, bubbled with CO gas for 10 min and reduced with a small amount of Na dithionite. The reaction medium without enzyme was placed in the other reservoir and bubbled for 10 min with 20% NO/80% N₂. Gas mixtures were prepared using a mass flow controller system (Type EL-6640, Estec Co. Ltd, Kyoto).

2.3. Membrane-inlet mass spectrometry
Non-invasive, continuous measurements of changes in both NO and N₂O dissolved in the liquid assay system were made using membrane-inlet mass spectrometry essentially as described previously [21,22]. A probe with an external diameter of 0.8 mm was constructed and an orifice (0.08 mm diameter) was made just above the tip. The wall thickness of the stretched membrane was 100 µm. The inlet was fitted with a silicone rubber sleeve (1.0 mm external diameter, 0.5 mm internal diameter; Vygon GMBH, Aachen, Germany). The magnetically stirred reaction vessel had a working volume of 2.3 ml and was filled with buffer and sparged with argon for 15 min. To further minimise oxygen contamination, the entire vessel was contained at the bottom of an open chamber continuously flushed with pure argon. NADH was added (final concentration 0.5 mM) and carbonic anhydrase was included to minimise interference by CO₂ which has an identical m/z ratio (44) to that of N₂O. Calibration was achieved by injection of volumes of NO-saturated anoxic buffer.

3. Results

3.1. Reactions of NO with Fe(III) Hmp
Unlike dioxygen or carbon monoxide, which bind haemoproteins only in the Fe(II) form, NO binds to either Fe(II) or Fe(III) haemproteins. This reaction has long been of utility in studies of haemoglobin ligand-binding kinetics [23]. To date, only the spectra of the nitrosyl complex of Hmp prepared by adding nitrite in the presence of the reducing agent dithiothreitol [20] or by adding NO to NADH-reduced Hmp [19] have been reported. Therefore, to assess the ability of Hmp to sequester NO as a possible mechanism of NO detoxification, the kinetics of the reaction of NO with Hmp were investigated in the stopped-flow apparatus by mixing anoxic solutions of Hmp and NO, giving final concentrations of 2 µM and 0.2 mM, respectively. As prepared, Hmp is in the Fe(III) form [20] having a Soret band at 404 nm and a prominent, broad flavin absorbance centred near 450 nm. After the solution flow stopped, a species formed with maxima in the difference spectrum (with the 1 ms spectrum as reference) at 422.5, 534 and 568 nm, characteristic of the nitrosyl species [20]. An apparent isosbestic point at 413.5 nm and an absorbance minimum at 404.5 nm, due to reaction of the Fe(III) unligated form, were also observed (Fig. 1a). The 422.5 nm absorbance increased monophasically (Fig. 1b) giving an apparent rate constant of 150 s⁻¹ and, assuming an undiminished initial NO concentration of 0.2 mM, a second order rate constant of 7.5 × 10⁷ M⁻¹ s⁻¹ at 25°C. However, it should be noted that most of the reaction was completed in the dead time of
the apparatus and that this value was estimated from the residual reaction.

To determine the stability of the nitrosyl species, air was admitted to a cuvette in which the nitrosyl species had been formed by addition of a solution of NO to an anoxic sample of Fe(III) Hmp (Fig. 2). In absolute spectra (cf. difference spectra in Fig. 1a), the peak of the Ar-sparged Fe(III) Hmp was at 404 nm, which, on adding an NO solution, shifted to 420 nm, accompanied by substantial bleaching of the flavin absorbance and α/β bands at 534 and 568 nm (Fig. 2A). The spectrum was unchanged after 60 min further incubation (not shown) and injection of further NO produced no further spectral changes other than those attributable to dilution effects. Subsequent exposure of the sample to air (Fig. 2B) resulted in progressive loss of the 420 nm form. Although the Fe(III) form reappeared at 404 nm with time, as predicted in proposed schemes for NO oxygenase activity [18,19], no reductant was present, deoxy Hmp was not observed, and the reactions must therefore be attributed to reversible NO reaction with Fe(III) Hmp. In the absence of further NO addition, the Fe(III) form was again stable for at least 160 min. Further addition of NO (an additional 0.3 mM final concentration) produced again an immediate red shift of the Soret band (Fig. 2C), which was transient as judged from the reversion to a form at 404 nm after 10 min. These results confirm that NO binds to Fe(III) Hmp giving a nitrosyl complex which is stable under anoxic conditions but autoxidisable regenerating the Fe(III) form that may again bind added NO.

3.2. Reaction of NO with dithionite-reduced and CO-ligated Hmp

The reaction of NO with Fe(II) Hmp was studied by mixing in the stopped flow apparatus a solution of Hmp, reduced with Na$_2$S$_2$O$_4$, with an NO solution giving a final NO concentration of 0.2 mM. However, the reduced Hmp-NO species was formed almost completely during the dead time of the apparatus; the direct reaction of NO with deoxy Hmp was thus too fast to be followed by this method and will require a photolysis approach.

The very fast reaction of NO with a Fe(II) haemoprotein like cytochrome $c$ oxidase provides a means of obtaining the ‘off’ rate for CO, the reaction with NO being limited only by the rate of CO dissociation [24]. Fig. 3 shows a stopped-flow experiment in which a dithionite-reduced sample of Hmp (final concentration 2 µM) prepared under CO was mixed with NO to give a final NO concentration of 0.2 mM. During the first 2 s after the mix, the spectrum of the CO-ligated form decreased in amplitude and the peak shifted from 422 to 419.5 nm (Fig. 3a). As expected, these spectra resemble, respectively, those of the carbonmonoxy and nitrosyl forms [20]. However, during the course of such experiments with Hmp, we noted a remarkable feature of Hmp activity: an apparent displacement of CO by NO that lasted about 2 s was followed by reversal of the spectral changes, both the original signal intensity and peak position being reversed over 600 s (Fig. 3b). Difference spectra recorded during the phase of CO displacement (Fig. 3c) were mirrored by the changes occurring thereafter (Fig. 3d). In the presence of a slight excess of dithionite as initial reductant of Hmp and as electron donor to the nitrosyl complex, the possibility of NO loss by either oxygenation with contaminating O$_2$ or direct reduction by dithionite is eliminated.

![Fig. 3. Reaction of dithionite-reduced Hmp-CO with NO.](image-url)
3.3. Electron transfer to ligated NO
Since stopped-flow experiments revealed a release of CO from, and rebinding to, Hmp in the presence of NO and dithionite, we considered the possibility that NO was reduced at the haem and subsequently dissociated. We demonstrated previously that the FAD of Hmp is competent to transfer electrons to haem resulting in single electron reduction of bound oxygen with release of superoxide anion [9-12]. In the presence of O₂, both NADH and NADPH are oxidised by Hmp, but the affinity for the former (Kₘ ≈ 2 μM) is higher [25]. In the experiment of Fig. 4, therefore, Hmp was added to an anoxic solution of NADH and the cuvette flushed continuously with Ar. The peak at 430 nm (Fig. 4A) is that of Fe(II) Hmp and the 340 nm band is NADH. In the first spectrum (Fig. 4A), the small shoulder near 420 nm is probably due to a small amount of oxygenated Hmp reflecting the ability to react with submicromolar O₂ [9]. This signal was transient and thereafter the spectrum of the Fe(II) form was stable for over 1 h with no diminution of the NADH signal, indicating anoxia (Fig. 4A). NO was then added to a final concentration of 0.3 mM whereupon the haem signal immediately shifted to 416.5 nm (Fig. 4A). Spectra taken over the following 45 min (Fig. 4B) revealed progressive loss of the 340 nm absorbance indicating NADH oxidation but persistence of the 416.5 nm signal without detectable change in intensity or position. Since NO is the only potential electron acceptor in this experiment, we conclude that anoxically Hmp catalyses the reduction of NO by NADH, with an estimated turnover rate of 0.013 s⁻¹.

3.4. Hmp possesses NO reductase activity
To seek direct evidence for the consumption of NO by Hmp in the absence of oxygen, i.e. by mechanisms distinct from oxygenase activity [18,19], membrane-inlet mass spectrometry was used. After equilibration of the strictly anoxic chamber contents (see Section 2) at room temperature, Hmp was added to 10 μM, the vessel was sealed with a rubber stopper to displace the gas of the head space and the reaction started by injection of an anoxic solution of NO (final concentration 0.31 mM). The reaction was monitored over 330 s during which time a signal at m/z = 30 (NO) continuously decayed while a signal at m/z = 44 (N₂O) increased in amplitude. The true amount of NO analysed was calculated after correction for overlap of the cracking patterns of NO and N₂O produced by electron impact ionisation (NO 100% of maximum peak at m/z 30; N₂O 100% at 44 and 31% at 30). A turnover number for NO reductase activity of 0.24 s⁻¹ (mean of three determinations ± S.D. 0.06) was obtained.

4. Discussion
The flavohaemoglobin of Hmp is dramatically up-regulated aerobically by NO [13], GSNO [14] SNP [14,16] and S-nitroso-N-penicillamine (SNAP) [16]. The earliest of these studies led us to propose [13] that Hmp might function to sequester or reduce NO and that its induction by NO is an adaptive response. Taken with our previous demonstrations that Hmp generates free superoxide from the oxygenated protein [9-12], this work has provided physiological rationalisation and some mechanistic information to support the recent demonstrations...
[18,19] that NO and oxygen interact at the haem to generate nitrate. This enzymatic reaction provides one mechanism for removal of NO in E. coli and prevention of the cytotoxicity that ensues when bacteria are challenged with NO and redox-related reactive nitrogen species during, for example, phagocytosis. Indeed, a strain of E. coli in which the hmp gene is specifically mutated is hypersensitive to GSNO, SNAP and paraquat [16]. The pivotal role that Hmp plays in responses to NO and related species is further demonstrated by the finding [16] that up-regulation of hmp is more responsive to the presence of SNAP and GSNO than is the soxS promoter, previously the only NO-responsive regulatory gene identified in E. coli [3,4].

Despite the probable importance of the NO oxygenase reaction in resisting aerobic NO-related stresses, it is clear that Hmp must serve other functions. Evidence in support of this assertion includes the following. (1) Transcription of hmp is not shut down under anaerobic growth conditions [13]. The global regulator Fnr is a repressor of hmp transcription aerobically as revealed by hmp up-regulation in an fnr mutant [13], yet hmp transcription is slightly, but consistently, higher anaerobically than aerobically ([13], S.O. Kim, M.D. Coopamah, R.K. Poole, unpublished), suggesting involvement of an unidentified further mechanism of regulation. (2) In E. coli [18,26] and S. typhimurium [17], Hmp confers resistance to nitric oxide anaerobically, implying that the oxygenase reaction is not the only mechanism for combating nitrosative stress. (3) Finally, an hmp mutant fails to reduce paraquat anaerobically [16]; this may be an indirect effect on another action in resisting aerobic NO-related stresses, it is clear that Hmp may be involved in sequestering haem ligands [5]. In this sense, Hmp may act like insect nitrophorins [23] or perhaps bacterial cytochromes of the c' type [34]. Recent work [35] shows that purified cytochrome c' is able to react with NO formed as a freely diffusible intermediate of denitriﬁcation by Paracoccus denitriﬁcans and that denitriﬁing cells are able to remove NO from the nitrosyl cytochrome c' complex. Here we demonstrate that Hmp binds NO rapidly to form a nitrosyl species that is extremely stable in the absence of air. The binding is reversible in the presence of oxygen, but the Fe(III) Hmp formed cannot arise from the oxygenase reaction, since it is observed in the absence of a reducing substrate for oxy-Hmp (Fe(II)O₂) formation. If Hmp is shown to have a physiologically relevant role in sequestration of NO, it will resemble in some respects the green cytochrome b of Bacillus halodenitriﬁcans, which spectrally resembles cytochrome c'. This protein [36] binds NO and reacts with SNP only in the Fe(II) state. Like the reaction of Fe(III) Hmp with NO, the binding of the green protein with NO is reversible by air, with reformation of the Fe(III) form [36]. Mitochondrial ferricytochrome c may also act as a reversible sink for excess NO in these organelles generating NO⁻, thereby providing a means for producing peroxynitrite in the absence of superoxide [37].

The reactions of Hmp with oxygen and NO that appear to date to be relevant to resisting NO and nitrosative stress, both anaerobically and aerobically, are summarised in Fig. 5. The mechanism of N₂O formation is not understood but presumably occurs at the haem, as suggested by the ability of CO to recombine with the haem (Fig. 3). In view of the diversity of these reactions, the unexplained responses of hmp to oxidative stress [32] and the broad-speciﬁcity reductase capabilities of Hmp [7,10,12,14,25], it seems that the aerobic and anaerobic functions of Hmp are multifaceted.

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