Escherichia coli flavohaemoglobin (Hmp) with equistoichiometric FAD and haem contents has a low affinity for dioxygen in the absence or presence of nitric oxide

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A purification procedure for flavohaemoglobin Hmp (NO oxygenase) is described that gives high yields of protein with equistoichiometric haem and FAD contents. H_2O_2 accumulated on NADH oxidation by the purified protein and in cell extracts with elevated Hmp contents. H_2O_2 probably arose by dismutation from superoxide, which was also detectable during oxygen reduction; water was not a product. In the absence of agents that scavenge superoxide and peroxide, the mean K_m for oxygen was 80 μM; the addition of 15 μM FAD decreased the K_m for oxygen to 15 μM without a change in V_{max} but catalysed cyanide-insensitive oxygen consumption, attributed to electron transfer from flavins to O_2. Purified Hmp consumed NO in the absence of added FAD (approx. 1 O_2 per NO), which is consistent with NO oxygenation. However, half-maximal rates of NO-stimulated O_2 consumption required approx. 47 μM O_2; NO removal was ineffective at physiologically relevant O_2 concentrations (below approx. 30 μM O_2). On exhaustion of O_2, NO was removed by a cyanide-sensitive process attributed to NO reduction, with a turnover number approx. 1% of that for oxygenase activity. These results suggest that the ability of Hmp to detoxify NO might be compromised in hypoxic environments.

Key words: bacterial NO detoxification, haemoglobin, NO oxygenase.

INTRODUCTION

Flavohaemoglobins occur in a variety of bacteria and yeast [1]. The *Escherichia coli* protein Hmp is the best characterized of these and the structural gene (*hmp*) was the first bacterial globin gene to be cloned and sequenced [2]. Hmp is a 44 kDa protein having haem B in an N-terminal domain homologous with other globins [2,3]. The C-terminal domain resembles ferredoxin:$NADP^+$ reductase [4]. Both NADH (K_m 1.8 μM) and NADPH (K_m 19.6 μM) are oxidized at this domain [5]; electrons are transferred via FAD to the haem [6], where oxygen is bound and reduced [7]. In the absence of NO, reduced oxygen species are released [7,8] in an oxidase-like manner.

For a long time the function of microbial flavohaemoglobins was obscure. The first indication that Hmp might be involved in NO biochemistry was the marked up-regulation of *hmp* transcription in *E. coli* cultures treated with solutions of NO gas or nitrite [1,9], *S*-nitrosoglutathione and sodium nitroprusside [10,11]. Other evidence for interactions of flavohaemoglobin with NO and related reactive species comes from the following: (1) the inability of an *Alcaligenes eutrophus* mutant defective in Fhp (an Hmp counterpart) to accumulate nitrous oxide during denitrification, perhaps suggesting a role for Hmp in NO reduction [12]; (2) the sensitivity of growth and respiration of defined *hmp* mutants of *E. coli* [11,13] and of *Salmonella typhimurium* [14] to NO, redox-related species and nitrosating agents; and (3) the demonstration that Hmp has NO oxygenase activity forming nitrate [15–17].

However, certain evidence suggests functions in addition to NO oxygenase. First, *hmp* transcription is consistently higher anaerobically than aerobically [9] and *hmp* mutants are sensitive to NO and related species both aerobically and anaerobically [15], suggesting oxygen-independent modes of Hmp–NO interactions. Secondly, anoxically, Hmp reduces NO to nitrous oxide [16–18], albeit with lower activity, providing an alternative mechanism for combating nitrosative stress. Thirdly, *hmp* transcription is also up-regulated by methyl viologen (paraquat) [19] and *hmp* mutants are hypersensitive to this agent of oxidative stress [11].

Here we describe an improved method for Hmp purification that yields protein with an equistoichiometric complement of redox centres. We report that, when superoxide-scavenging and peroxide-scavenging agents are omitted from assays *in vitro*, Hmp reduces O_2 with an affinity for oxygen several orders of magnitude lower than terminal oxidase-catalysed respiration. FAD increases the O_2 affinity ($K_m \approx 15$ μM) but provokes haem-independent O_2 consumption. In both the absence and the presence of exogenous FAD, NO-stimulated O_2 uptake and NO removal from solution require high O_2 concentrations that are unlikely to be encountered by *E. coli* in many natural environmental niches.

EXPERIMENTAL

Bacterial strains and media

E. coli strain RSC2057 (AN1459 harbouring the plasmid pCL775 [20]) was used for the purification of Hmp and for the assay of peroxide production in Hmp-rich cell extracts. Plasmid pCL775

Abbreviation used: SOD, superoxide dismutase.

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contains the *hmp* gene cloned in pPL452, a stable, high-copy-number λ promoter vector conferring ampicillin resistance [20]. The *hmp*-null mutant RKP4600 has a kanamycin cartridge insertion and is the same as RKP4545 described previously [11]. The wild-type strain was VJS676 [11]. All strains were grown in Luria–Bertani medium [21], supplemented where appropriate with kanamycin or ampicillin (each at 50 μg/ml).

Purification of Hmp

E. coli strain RSC2057 was grown aerobically in Luria–Bertani medium supplemented with $FeCl_3$, δ-aminolaevulinic acid and riboflavin at final concentrations of 3, 50 and 100 μM respectively. Thermal induction of gene expression was achieved by shifting cultures from 30 to 42 °C for 4 h. Cells were disrupted by sonication and the crude extract obtained after centrifugation was used to purify Hmp by anion-exchange chromatography on DEAE-Sepharose Fast Flow at pH 8.0, followed by chromatography on DEAE-Toyopearl 650S at pH 6.5 and gel filtration on Superdex-200. Coloured fractions at each step were combined and A_{410}/A_{280} was determined. Purified Hmp (typically approx. 6 mg from 0.4 litre of culture) was a monomer in solution, had an A_{410}/A_{280} of 0.9, migrated on SDS/PAGE with an apparent molecular mass of 44 kDa and was estimated to be 95 % pure.

Polarographic studies of Hmp interactions with NO and O_2

Oxygen consumption was measured with a digital Clark oxygen electrode system (Model 10; Rank Bros, Bottisham, Cambridge, U.K.) modified to permit simultaneous measurements of O_2 and NO consumption, as well as sample addition. The electrode was situated at the base of the water-jacketed Perspex chamber maintained at 37 °C, beneath a magnetically driven stirrer bar. The top of the chamber (working volume 2 ml) was sealed with a tightly fitting adjustable Perspex cap with a concave bottom surface. The cap was drilled with a central vertical hole (6 mm in diameter to a depth of approx. 33 mm, and 2.5 mm in diameter for the remaining 11 mm depth) to accept an ISO-NOP2 stainless steel shielded NO electrode 2 mm in diameter (World Precision Instruments). The electrode shaft was fitted with a plastic sleeve to hold the membrane at the required depth in the chamber. A second vertical hole (approx. 1.5 mm diameter, 44 mm depth) near the cap perimeter permitted the addition of solutions of NADH, enzymes and NO (prepared as described in [9]) with Hamilton microlitre syringes, without significant introduction of O_2. Current from the O_2 electrode operating at a polarizing voltage of 0.60 V was amplified and displayed on a two-channel recorder, along with the amplified current of the NO electrode (polarizing voltage 0.865 V) processed with a World Precision Instruments ISO-NO Mark II Isolated Nitric Oxide Meter. The O_2 electrode was calibrated with air-saturated water and sodium dithionite crystals. The NO electrode was calibrated with anoxic solutions of freshly prepared NO solution. Experiments with purified Hmp were performed in a buffer containing 50 mM Mops and 50 mM NaCl, pH 7.0.

Assay of peroxide generation

Cell-free extracts were prepared by ultrasonication of cells grown to a D_{600} of 0.8 and centrifuged at 24000 ***g*** for 25 min. H_2O_2 production was measured with the scopoletin fluorescence assay as described previously [22].

Other analytical methods

Electronic absorption spectroscopy of purified Hmp was performed with a dual-wavelength scanning spectrophotometer. Hmp was quantified from the Soret region of CO difference spectra (438–421 nm), taking ϵ as 186 $mM^{-1} \cdot cm^{-1}$ [23]. The flavin content of Hmp was determined by fluorimetry (excitation at 460 nm; emission at 530 nm) after flavin had been extracted by boiling a sample at 100 °C for 3 min followed by centrifugation at 13000 ***g*** for 5–10 min [23]. Haem was quantified by the pyridine haemochrome method [24]. Protein assays were performed with the Markwell method with BSA as standard [25].

RESULTS

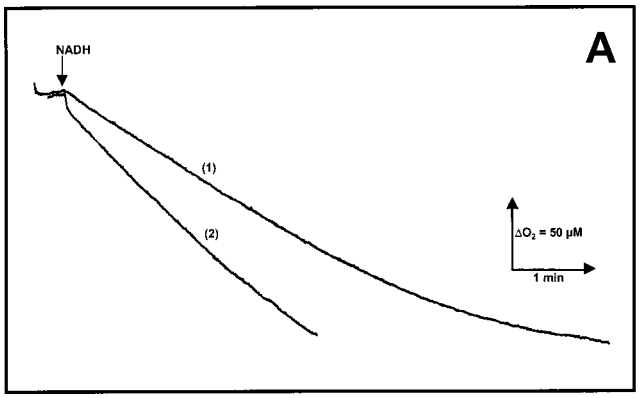
Purification and properties of Hmp

The yield of Hmp from aerobic cultures was approx. 3-fold higher than recently reported with anaerobic expression under nitrate-inducing conditions from the natural promoter of *hmp* cloned in pAlter [17]. Furthermore, typical preparations of Hmp had equimolar contents of haem and FAD as predicted by sequence and structural information on Hmp and its counterparts [1,2,4]. Concentrations of redox centres, given as nmol/mg of protein (means $\pm$ S.D.) and numbers of preparations, were as follows: FAD (13.3 ± 1.9, 4), haem assayed as the pyridine haemochrome (13.2 ± 1.2, 3), and haem assayed as the CO complex (13.0 ± 2.3, 5).

Oxygen reduction by Hmp and the effect of exogenous FAD

Previous studies of Hmp-catalysed O_2 consumption used ascorbate to remove superoxide anion [7], a product of O_2 reduction by Hmp [8]. In the present work, no such additions were made, in view of the possibility that superoxide, peroxide or scavenging agents might contribute to NO removal. Addition of saturating concentrations [5] of NADH (500 μM) to purified Hmp in buffer markedly stimulated O_2 uptake (Figure 1A) and the initial rate of O_2 consumption was higher in the presence of 15 μM FAD (trace 2) than in its absence (trace 1). Incubation in the sealed oxygen electrode chamber eventually depleted O_2 to levels below the sensitivity of the polarographic probe in both cases, but the rates of O_2 uptake were non-linear in both the absence and the presence of added FAD. Calculation of oxidase activities at successively lower O_2 concentrations (Figure 1B) allowed an estimation of the apparent affinity for oxygen under these conditions. In both the absence and the presence of added FAD, the data were well fitted by Michaelis–Menten kinetics in four experiments, one of which is shown in Figure 1(C). In the presence of FAD, the V_{max} was 330 nmol of O_2 consumed/min per mg of protein (mean $\pm$ S.D. for four experiments, 358 ± 30 nmol/min per mg of protein) and the apparent K_m in Figure 1(C) was 16 μM (mean $\pm$ S.D. for four experiments, 15 ± 6 μM). These K_m values are significantly higher than that reported previously in the presence of ascorbate (3 μM [7]) and measured by oxymyoglobin deoxygenation. In the absence of exogenous FAD (Figure 1C), the V_{max} was unchanged (mean $\pm$ S.D. for four experiments, 345 ± 10 nmol/min per mg of protein) but the apparent K_m value was approx. 90 μM O_2 (mean $\pm$ S.D. for four experiments, 80 ± 20 μM).

Although Hmp binds and reduces O_2 at the haem [6–8], it has previously been shown to reduce cytochrome *c* [26] in a CO-insensitive process, demonstrating that electrons can exit from Hmp at the level of FAD. To determine whether the presence of exogenous FAD might catalyse O_2 consumption by such a mechanism, cyanide was used to inhibit haem reactions. Cyanide binds to ferric Hmp with a K_d of approx. 3 μM; at 100 μM it abolishes NO oxygenase activity [13]. The addition of 100 μM cyanide inhibited Hmp-catalysed O_2 consumption in the absence



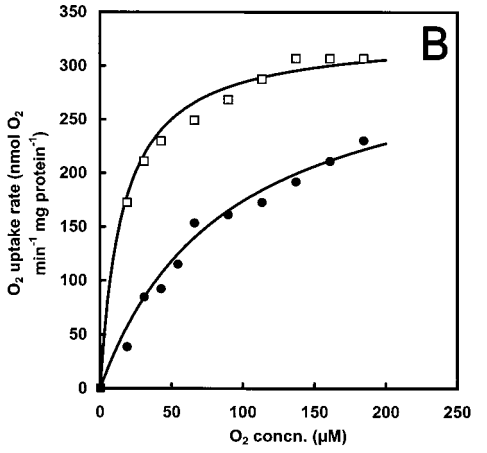


Figure 1 Oxygen consumption kinetics by purified Hmp and the effects of exogenous FAD

(**A**) The reaction mixtures contained 50 mM Mops buffer, pH 7.0, 50 mM NaCl, 0.37 mg of Hmp and initially approx. 200 μM O_2. NADH (500 μM) was added at the time indicated. The experiment was performed without (trace 1) and with (trace 2) 15 μM FAD. (**B**, **C**) The calculated rates of O_2 consumption plotted as a function of O_2 concentration (**B**) and as double-reciprocal plots (**C**).

of NO after a lag (Figure 2, trace 1). However, the subsequent addition of FAD markedly stimulated O_2 consumption, indicating flavin-catalysed electron transfer to O_2. In the absence of Hmp, O_2 consumption, even after FAD addition, was negligible (Figure 2, trace 2).

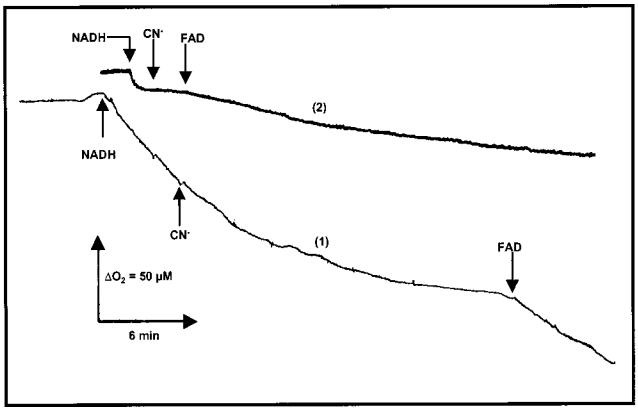


Figure 2 Oxygen consumption kinetics by purified Hmp and the effects of cyanide and exogenous FAD

The reaction (trace 1) contained 50 mM Mops buffer, pH 7.0, 50 mM NaCl, 0.034 mg of Hmp and initially approx. 200 μM O_2. NADH (500 μM), cyanide (100 μM) and FAD (15 μM, all final concentrations) were added at the times indicated. In trace 2, Hmp was omitted.

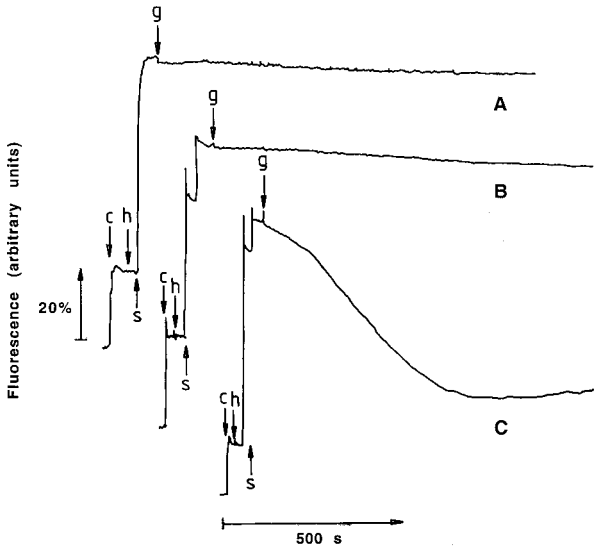


Figure 3 H_2O_2 production by cell-free extracts from wild-type cells (A), an *hmp* knockout mutant (B) and cells overexpressing Hmp (C)

Points of addition of cell extract (at the arrow labelled c), HRP (h), scopoletin (s) and glucose (g) are shown. Each assay contained approx. 0.2 mg of cell extract protein. The results are typical of three experiments.

Products of Hmp-catalysed O_2 reduction

One-electron reduction of O_2 yields the superoxide anion, which is a major product of the Hmp-catalysed oxidase reaction *in vivo* [8] and *in vitro* [7,8,26]. However, superoxide will dismutate naturally in solution and many proteins exhibit some catalase-like contamination. Such peroxide formation via superoxide and its dismutation, or the direct reduction of O_2 to give peroxide, should each result in the consumption of NADH equivalent to two-electron reduction of O_2. We determined whether the consumption of NADH during O_2 reduction was commensurate with the accumulation of peroxide by adding limiting amounts of NADH (25–75 μM, final concentrations) to a buffer initially saturated with air. The reaction was allowed to proceed for

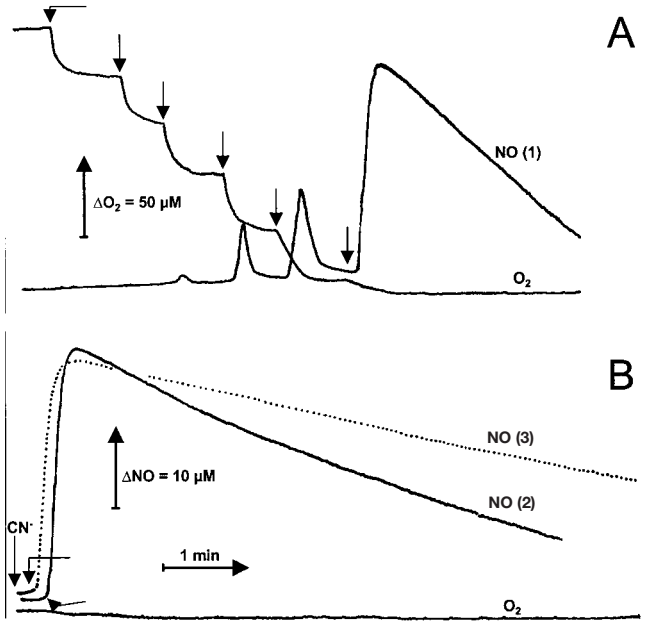


Figure 4 Dependence of NO oxygenation on O_2 concentration and anoxic NO removal

The reaction mixture contained 0.05 mg Hmp and 500 μM NADH in 50 mM Mops buffer, pH 7.0, with 50 mM NaCl. At each arrow in (**A**) an anoxic solution of NO was added (final concentration 36 μM). O_2 (labelled trace) and NO (trace 1) were monitored continuously and simultaneously with electrodes. The NO electrode trace has not been corrected for the 4 s lag behind the O_2 trace. (**B**) After the exhaustion of O_2, a further addition of NO (final concentration 36 μM) was made (bottom arrow, trace 2). After the disappearance of NO (full trace not shown), cyanide (final concentration 100 μM) was added, followed by NO (final concentration 36 μM, trace 3). Bars show the calibration of the O_2 and NO electrodes with air-saturated and NO-saturated solutions respectively. The NO calibration was performed anoxically.

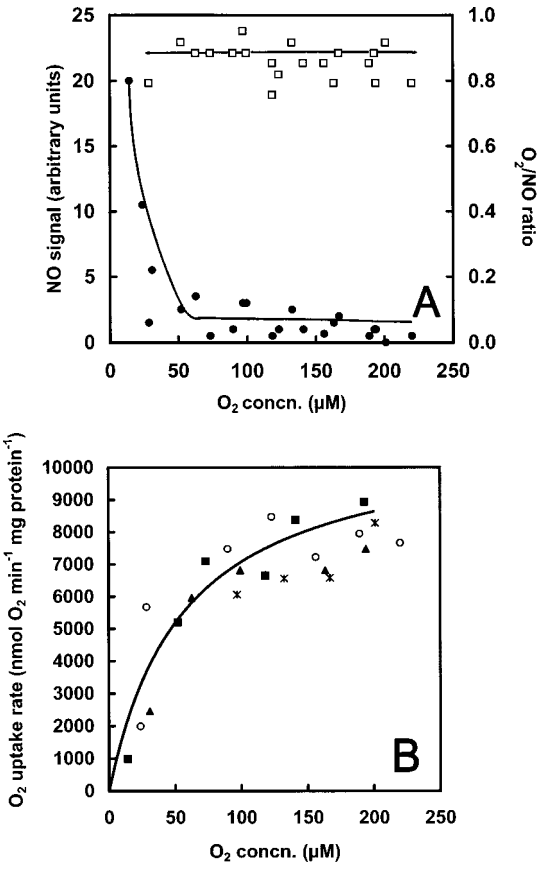


Figure 5 Dependence of NO oxygenation on O_2 concentration

Experiments were conducted as in Figure 4 with 0.05 mg of Hmp. (**A**) The magnitude of the NO signal (●) and the ratio of O_2 consumed to NO added (□) are plotted as a function of the O_2 concentration at which NO additions were made. (**B**) Rates of NO-stimulated O_2 uptake on the addition of 36 μM NO, measured as a function of O_2 concentration. The fitted line corresponds to a K_m for O_2 of 57 μM. Results from four separate experiments indicated by the symbol types are shown.

several minutes after the oxygen uptake rate had slowed, to ensure that oxygen uptake had ceased. In the absence of FAD, the ratio was 1.17 ± 0.17 (mean $\pm$ S.D. for 10 determinations), whereas with FAD added at 15 μM the ratio was 1.06 ± 0.14 (mean $\pm$ S.D. for 14 determinations), or approx. 2 electrons per O_2. This is indicative of H_2O_2 accumulation, although such experiments do not permit the initial product to be determined, because of the possibility of spontaneous dismutation of superoxide.

If O_2 is reduced to superoxide anion that does not dismutate but accumulates, the effect of adding superoxide dismutase (SOD) should be to inhibit O_2 consumption by 50 %, because the dismutation produces 0.5 O_2 for each O_2 initially reduced. The addition of SOD to a sample of Hmp consuming O_2 in the presence of excess NADH inhibited O_2 consumption by 30–50 %, indicative of superoxide formation with some spontaneous dismutation (results not shown).

If O_2 is reduced to superoxide anion that totally dismutates, or if O_2 is reduced directly to peroxide, then the addition of catalase should yield O_2 equivalent to 50 % of that initially consumed. The addition of catalase to a sample of Hmp consuming O_2 in the presence of excess NADH resulted in O_2 evolution accounting for approx. one-third of that consumed, suggesting substantial peroxide accumulation but indicating also that some superoxide was present.

To confirm the accumulation of peroxide as a result of Hmp activity *in vivo*, peroxide generation was assayed directly in extracts supplemented with glucose (because NADH interfered with fluorimetry). Peroxide generation (fluorescence quenching) was almost undetectable in extracts from *hmp* mutant RKP4600 [11] and its isogenic wild-type strain VJS676. However, the addition of glucose to extracts from strain RSC2057, which overexpresses Hmp, was followed by extensive production of peroxide (Figure 3).

Effects of O_2 concentration on NO removal by Hmp

Because exogenous FAD catalyses haem-independent electron transfer to O_2, and because the accumulation of superoxide and peroxide is a normal consequence of O_2 metabolism by Hmp, we omitted ascorbate, exogenous FAD and superoxide-scavenging agents in the studies reported below on NO oxygenase activity.

Addition of a solution of NO (36 μM final concentration immediately after each addition) (Figure 4A) immediately and markedly stimulated O_2 consumption from a basal activity of 150–450 nmol of O_2/min per mg of Hmp protein. Further additions of NO at successively lower O_2 concentrations in the chamber each resulted in accelerated O_2 uptake followed by a decline in rate to pre-NO levels after approx. 60 s. At O_2 concentrations near or above 100 μM, the NO electrode registered small transient responses significantly lower than expected

from calibration of the apparatus with anoxic NO solutions in the absence of Hmp. Indeed, at the highest O_2 concentrations the additions of NO were undetectable by the NO electrode. However, at lower O_2 concentrations, the size of the NO signal progressively increased and the NO-stimulated initial rate of O_2 uptake was diminished (Figure 4A). At the lowest O_2 concentrations at which NO could be added (e.g. approx. 10 μM in Figure 4A), the NO-stimulated initial rate of O_2 uptake was further decreased and the magnitude of the NO signal increased to 80% of that expected from anoxic calibration with NO solutions (Figure 4A, scale in Figure 4B, and Figure 5A). On exhaustion of detectable O_2, the NO signal decayed slowly (Figure 4A, trace 1). The ratio of O_2 uptake to NO added was 0.93 ± 0.11 (mean $\pm$ S.D. for 44 determinations) over the range 30–220 μM O_2 (Figure 5A). This is consistent with the stoichiometry for NO oxygenase activity (1.0) [13,15,16].

Measurement of the initial rate of O_2 uptake after adding NO (Figure 4A) over a range of O_2 concentrations (four experiments are shown in Figure 5B) revealed the dependence of NO oxygenase activity on O_2 concentration. The data are best fitted by a K_m of 47 ± 10 μM (mean $\pm$ S.D. for six experiments) and a V_{max} of 8000–11000 nmol of O_2/min per mg of protein. These results were unaffected by the addition of SOD to the reactions (results not shown).

To determine whether the progressive loss of oxygenase activity (Figures 4 and 5) as O_2 tension declined was due to the inactivation of Hmp by added NO, the following experiment was conducted. On exhaustion of O_2 from solution, after successive additions of NO, the electrode chamber contents were re-aerated by removing the top cap and the experiment was repeated without further supplementation of the solution with Hmp but after adding NADH and making successive NO additions, as in Figure 4(A). The patterns of NO and O_2 disappearance were as shown in Figure 4(A). In two such experiments, in the first cycle of NO additions, the O_2-to-NO ratio was 0.92 ± 0.077 (mean $\pm$ S.D. for ten determinations); in the second the O_2-to-NO ratio was 0.98 ± 0.067 (mean $\pm$ S.D. for nine determinations). The dependence of NO-stimulated O_2 consumption rates on O_2 concentration was also similar to that shown in Figure 5(B). Thus the declining effectiveness of NO removal at lower O_2 tensions (Figures 4A, 5A and 5B) was not attributable to the irreversible inactivation of Hmp by NO.

Essentially similar results were obtained in the presence of 15 μM added FAD (results not shown) with regard to the patterns of NO removal from solution determined polarographically, and for the K_m for oxygen of NO oxygenase activity. The O_2-to-NO ratio observed during NO oxygenase activity was 1.06 ± 0.13 (mean $\pm$ S.D. for 28 determinations) and the apparent K_m for O_2 of the oxygenase activity was 60 ± 7.4 μM (mean $\pm$ S.D. for six determinations).

When NO solutions were added to oxic buffer in the absence of Hmp, an O_2 uptake associated with a non-enzymic reaction of NO with O_2 to give nitrite [27] was observed. The ratio of O_2 consumption to NO added was approx. 0.4 and was not dependent on O_2 concentration in the range 30–220 μM O_2 (results not shown).

NO consumption under anoxic conditions

Hmp has previously been demonstrated to exhibit NO reductase activity under anoxic conditions. To determine the extent to which this activity contributes to the decay of the NO signal after exhaustion of O_2 to undetectable levels (Figure 4A), NO was added again as shown in Figure 4(B) (trace 2). The NO signal decayed slowly, the initial rate giving an apparent turnover number of 0.20 s^{-1}. On decay of the NO signal to the baseline level (results not shown), cyanide (100 μM final concentration) was added to inhibit haem-catalysed NO reduction, followed by NO (Figure 4B, trace 3, dotted). The rate of disappearance of NO was significantly slower than in cyanide-untreated conditions, demonstrating the anoxic, cyanide-sensitive NO reductase activity of Hmp. Correction of the anoxic rate of NO decay shown in trace 2 of Figure 4(B) for the initial rate that persisted in the presence of cyanide gave a turnover number of 0.11 s^{-1}. In four experiments, similar measurements gave a turnover number of 0.15 ± 0.07 s^{-1} (mean $\pm$ S.D.).

DISCUSSION

The Hmp purification used here yields protein with a prosthetic group content and flavin-to-haem ratio greater than those in previous reports. In comparison with our previous preparations [23], the FAD content has been increased 1.4–2.1-fold and the haem is increased 2.7–3.6-fold in protein that has the expected 1:1 FAD-to-haem ratio. The prosthetic group content is 0.57 mol of haem per mol of protein and 0.58 mol of FAD per mol of protein, which compares very favourably with the 0.1 and 0.01 mol of haem and FAD respectively reported [15] in azide-treated preparations. Gardner et al. [17] recently described a preparation with 0.28 and 0.42 mol fractions of FAD and haem respectively. The content of prosthetic groups was not described by Hausladen et al. [16]. Despite the relatively high FAD content of the Hmp preparations used here, exogenous FAD markedly increased the apparent affinity of our preparation for oxygen in the absence of NO. However, the K_m value must be interpreted with caution in view of the accumulation of superoxide and peroxide during the assays, a situation avoided by Poole et al. [7]. In this work we elected not to add superoxide scavengers owing to their possible interference in the NO metabolism reactions. The low affinity for O_2 in the absence of NO (Figure 1) is expected to limit the accumulation of peroxide or superoxide in cells during growth under the microaerobic conditions favoured by *E. coli*. Superoxide production by Hmp has been demonstrated previously by showing that the superoxide-sensitive Φ (*sodA–lacZ*) fusion is up-regulated *in vivo* when cells overexpress plasmid-borne *hmp* [8]. Furthermore, purified Hmp reduces Fe(III) in a SOD-sensitive manner [8]. Those results and the production of peroxide in Hmp-overexpressing cells reported here strongly suggest that Hmp synthesis must be tightly regulated to prevent intracellular oxidative stress. Indeed, basal levels of *hmp* expression are low [9] both aerobically and anaerobically.

The finding that the oxygenase reaction is insensitive to SOD is in agreement with the observation [16] that the product yields (predominantly nitrate) were unaffected by SOD, whereas Gardner et al. [15] reported partial inhibition of NO oxygenase activity by MnSOD. Despite the presence of high equimolar concentrations of haem and flavin in this preparation, we found that FAD increased the V_{max} of O_2 consumption in the presence of NO (results not shown). A loss of NO oxygenase activity during purification and a requirement for FAD were noted previously [15,17].

Under aerobic conditions, Hmp has a high affinity for NO. The addition of 36 μM NO resulted in rates that were linear for a significant proportion of the reaction (Figure 4A), which is consistent with the recently determined K_m of 0.28 μM NO [17]. Others have addressed the O_2 concentrations required for oxygenase activity. Cells grown with minimal aeration had decreased NO consumption activities relative to cells grown with vigorous shaking under air [15]. Gardner et al. [28] described inducible

NO-consuming activities that protect aconitase (an [Fe–S] protein) from NO and attributed these to Hmp. The aerobic activity was cyanide-sensitive but identity with Hmp was not proved (e.g. through the use of *hmp* mutants). Aconitase was protected at O_2 concentrations (approx. 17 μM) at which nitrate and nitrite formation in growing cultures was negligible. This suggests that the O_2-mediated decomposition of NO that is expected to yield these ions (e.g. by Hmp action) cannot fully account for aconitase protection [28]. Further evidence of the limited effectiveness of Hmp in protecting metabolic functions at low O_2 tensions comes from the demonstration of enhanced sensitivity to NO of cell respiration [13,29]. Recently, a K_m for O_2 of 100 μM has been determined with purified Hmp supplemented with FAD when assayed at saturating NO (1 μM) and NAD(P)H [17]. Even at 0.1 μM NO, the K_m for O_2 is 35 μM and in intact cells a K_m of 60 μM has been reported [17]. However, on each addition of NO in the experiment of Figure 4(A), the first half of the reaction proceeded linearly, even when the oxygenase activity was initiated by adding NO at O_2 concentrations well below the K_m determined here and by others [17]. The reason for this is not known and will require further investigation.

The finding that 35–100 μM O_2 is required for half-maximal oxygenase activity raises the question of whether Hmp provides effective NO removal under microaerobic conditions. However, under anoxic conditions, Hmp exhibits NO reductase activity [16–18], confirmed here. The rate obtained from a direct determination of the rate of NO loss from solution by using an NO electrode (0.15 s^{-1}) is in reasonable agreement with that determined by membrane-inlet mass spectroscopic analysis of NO and N_2O (0.25 s^{-1}), made with an earlier preparation of Hmp and at a higher (310 μM) initial NO concentration [18]. These values are also very similar to that (0.14 NO s^{-1} per haem) obtained by Gardner et al. [17]. More significantly, the V_{max} measured at saturating NO, O_2 and NADH concentrations but in the presence of exogenous FAD and diothiothreitol at 37 °C by those authors considerably exceeded (670 s^{-1}) the value obtained by determination of NO disappearance (Figure 4), for which a V_{max} of approx. 10 s^{-1} was obtained. Our results indicate that NO reduction might account for NO consumption under anoxic conditions at rates that are at least approx. 1 % of the O_2-dependent oxygenase activity. In the presence of cyanide, NO disappearance was significantly slower than in cyanide-untreated conditions, which might have been due to the abstraction of H from NADH, yielding N_2O (E. Atsipapa and M. N. Hughes, unpublished work). An alternative explanation is that H_2O_2 generated during the aerobic phase of the experiment is decomposed, yielding O_2 that, as it forms, combines with NO and is therefore not detected by the O_2 electrode.

Like all indigenous bacteria of the gut, *E. coli* must be able to grow anaerobically; however, its ability to use O_2 is thought to provide ecological advantages, for example close to epithelial cells, where O_2 can pass from the blood through the epithelium to the microbial populations attached to it [30,31]. Dioxygen diffuses readily across biological membranes and it is generally assumed that no significant concentration gradient exists across respiring bacterial membranes, although the intracellular oxygen concentration has not been measured in *E. coli* (or any other bacterium). In accord with the need for growth in low-oxygen environments, the oxygen-reducing oxidases of *E. coli* have high affinities for the ligand, with K_m values typically in the submicromolar or nanomolar range [32]. It is not clear whether other microorganisms that contain flavohaemoglobins, such as *Erwinia*, *Bacillus*, *Alcaligenes* and yeasts, enjoy environments where the high oxygen requirements of NO oxygenase activity are met during challenge by NO and nitrosative stress.

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