Reactions of the *Escherichia coli* flavohaemoglobin (Hmp) with NADH and near-micromolar oxygen: oxygen affinity of NADH oxidase activity

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The soluble flavohaemoglobin (Hmp) of *Escherichia coli*, product of the *hmp* gene, contains haem B and FAD in a single polypeptide of molecular mass 44 kDa. The function of this protein (and of the similar proteins identified in several bacteria and yeast) is unknown, but the observation that the binding of oxygen to haem modulates the reduction level of FAD has suggested that Hmp could act as an oxygen sensor. Here, stopped-flow, rapid-scan spectroscopy has shown that the oxidized protein reacts rapidly with NADH to form an oxygenated species, even when efforts are made to reduce oxygen concentrations to sub-micromolar levels, suggesting a high affinity for this ligand. As is the case at high oxygen concentrations (130 µM), oxygenated species formation was kinetically and spectrally heterogeneous. Between 12 ms and 1 s after mixing, following transient formation of the deoxy form and its reaction with dioxygen, a steady-state level of the oxygenated species was attained. During the oxygenated steady state, the flavin remained largely oxidized, as observed previously at 130 µM oxygen. Hmp is an NADH oxidase; on exhaustion of oxygen by reduction (in < 10 s under these conditions), the oxygenated species disappeared to generate the deoxy Fe(II) haem, whereupon the flavin was reduced. The affinity for oxygen during NADH oxidation was measured by continuous dual-wavelength monitoring of the deoxygenation of oxymyoglobin. The K_m for oxygen was 2.6 µM, much higher than the K_m values determined, using the same method, for the membrane-bound terminal oxidases cytochromes *bo'* and *bd*. These results show that the oxidase activity of Hmp, but not necessarily oxygen binding, would be minimal at oxygen concentrations that limit terminal oxidase function.

Keywords: haemoglobin (bacterial), *Escherichia coli*, Hmp, oxygen affinity, oxidase

INTRODUCTION

The existence of globin-like proteins in micro-organisms is now well-established. The first example of a bacterial haemoprotein shown to have the characteristics of a globin, in terms of amino acid sequence (Wakabayashi *et al.*, 1986) and oxygen-binding characteristics (Orii & Webster, 1986), was a soluble protein from *Vitreoscilla* (reviewed by Webster, 1987), originally referred to as a 'cytochrome *o*' (Webster & Liu, 1974). This protein is

26% identical to lupin leghaemoglobin and is clearly related to globins in higher organisms (Perutz, 1986). The function of *Vitreoscilla* haemoglobin (Vgb) is unclear, but the elevated levels observed in hypoxic conditions (Boerman & Webster, 1982), and the finding that multicopy expression of the cloned *vgb* gene in *Escherichia coli* enhances growth under oxygen-limited conditions (Khosla & Bailey, 1988; Khosravi *et al.*, 1990), suggest that it has an important function in delivering, or perhaps storing, oxygen.

Vasudevan *et al.* (1991) identified a 44 kDa protein in *E. coli* with spectral characteristics similar to Vgb and other globins. This haemoprotein (Hmp), product of the *hmp*

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gene, has an N-terminal haem domain that is 46% identical to Vgb, with conservation of the putative haem ligands and the globin fold characteristic of Vgb and other globins. However, unlike Vgb, Hmp has a C-terminal domain that is homologous to ferredoxin-NADP⁺ reductase and other members of a large family of proteins with highly conserved binding sites for FAD and NAD(P)H (Andrews *et al.*, 1992; Karplus & Bruns, 1994). Hmp may, therefore, be described as a flavohaemoglobin. Consistent with sequence analyses, purified Hmp contains haem B and FAD (Ioannidis *et al.*, 1992; Cooper *et al.*, 1994) and can be reduced with NADH in aerobic solution to yield a moderately stable oxygenated form (Ioannidis *et al.*, 1992). The protein reacts with other ligands, including CO and cyanide; ESR spectroscopy of the nitrosyl complex indicates (Ioannidis *et al.*, 1992) that the haem is attached to the protein through a nitrogenous ligand, probably the imidazole group of histidine-85, as suggested from sequence analyses (Vasudevan *et al.*, 1991).

Despite its globin-like haem domain, Hmp shows the remarkable property of functioning as a soluble oxidase. The decay of the oxygenated form observed by Ioannidis *et al.* (1992) is due not to reversible dissociation of the haem-bound oxygen, but to oxygen reduction (Orii *et al.*, 1992; Poole *et al.*, 1994). In stopped-flow, rapid-scan experiments, the oxidized protein reacts rapidly with NADH in the presence of 130 μ M oxygen to form an oxygenated species, while the flavin remains largely oxidized. With excess NADH and limited oxygen, disappearance of the oxygenated species is accompanied by generation of the deoxy Fe(II) haem and flavin reduction. With limited NADH and excess oxygen, the NADH is consumed and both redox centres are oxidized. Super-oxide anion is a major product of oxygen reduction (Orii *et al.*, 1992; N. Ioannidis & R. K. Poole, unpublished; R. K. Poole, R. D'mello & Y. Orii, unpublished).

We have proposed, on the basis of these experiments (Poole *et al.*, 1994), that Hmp could act as an oxygen sensor in *E. coli* by combining with intracellular oxygen, thus limiting flavin reduction in the aerobic steady state. Lowering of the cytoplasmic oxygen concentration would result in loss of the oxygenated species and extensive reduction of flavin. In view of the ability of Hmp to reduce Fe(III) (Andrews *et al.*, 1992; Eschenbrenner *et al.*, 1994; N. Ioannidis & R. K. Poole, unpublished), such a mechanism might affect the redox status and thus activity of transcriptional regulators such as Fnr (Khoroshilova *et al.*, 1995) or SoxR (Hidalgo & Demple, 1994), both of which contain [Fe-S] clusters. This mechanism might also provide oxygen-modulated reduction of other substrates. However, we have recently shown (Vasudevan *et al.*, 1995) that Hmp is predominantly cytoplasmic, and so any hypothesis that invokes oxygen reaction with Hmp must take into account the ability of Hmp to 'compete' for oxygen with the two terminal quinol oxidases in *E. coli*, cytochromes *bo'* (for an explanation of nomenclature, see Poole & Chance, 1995) and *bd* (Poole, 1994).

Consequently, in this paper, we characterize further the reactions of purified Hmp with NADH at very low

oxygen tensions. We have also determined the K_m for oxygen during the oxidase reaction catalysed by Hmp to allow comparison with the K_m values for cytochromes *bo'* and *bd*, recently determined in one of our laboratories (D'mello *et al.*, 1995, 1996).

METHODS

Organism, growth conditions and purification of Hmp. These procedures have been described in detail before (Vasudevan *et al.*, 1991; Ioannidis *et al.*, 1992). *E. coli* strain RSC521 harbours pPL341, containing the entire *hmp* gene under the control of its own promoter cloned into pBR322. The strain was grown aerobically in Luria-Bertani medium (initial pH 7.0) supplemented with 0.2% (w/v) glucose and ampicillin (35 μ g ml⁻¹). Growth conditions in the 12 l Biostat fermenter were described by Ioannidis *et al.* (1992). Cells were harvested when the OD₆₀₀ of the culture (measured in a Pye-Unicam SP6-550 spectrophotometer, and after appropriate dilution to keep measured values below 0.6 in a 1 cm cuvette) was about 3. Harvested cells, which were deep brown due to over-expression of the flavohaemoglobin, were washed and disrupted in a French pressure cell. Differential centrifugation removed cell debris and membranes, leaving an orange-red 'soluble' fraction. Hmp was purified by anion-exchange chromatography on DEAE-Sepharose CL-6B and gel filtration on Sephacryl S-200 exactly as described by Ioannidis *et al.* (1992). Protein concentrated by ultra-filtration was stored at, or below, -70 °C until used.

Stopped-flow spectrophotometry. The stopped-flow rapid-scan spectrophotometer used (Orii, 1993; Poole *et al.*, 1994) employs a diode array to record 512 absorption spectra over a 208 nm span, each in as little as 1.04 ms. Timing of data collection is with reference to the instant when flow of the mixture stops.

Measurement of oxygen affinity. Oxygenated sperm whale oxy-myoglobin (Sigma, no longer available) was a generous gift from Dr S. Hill (Nitrogen Fixation Laboratory, University of Sussex, UK). The procedure for preparation followed closely that of Appleby & Bergersen (1980) and has been described before by D'mello *et al.* (1994, 1995). The concentration of myoglobin was determined by CO difference spectroscopy using the absorption coefficients cited by Wood (1984). Samples were diluted in potassium phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA, which had been previously degassed with a 1% (v/v) oxygen plus argon gas mixture (BOC). The procedure used the methods and calculations described by Appleby & Bergersen (1980), except that the spectrophotometric measurements were modified as follows. A custom-built (Radley's) glass optical cuvette (1.3 ml) was completely filled with oxy-myoglobin (14 μ M). Ascorbate (1.1 mM) was included to scavenge superoxide, and *o*-dianisidine (0.12 mM) and horseradish peroxidase (6 Sigma units) were added to remove H₂O₂. The cuvette was sealed with a plastic plug having a fine hole through which Hmp and NADH (final concentrations 1.1 μ M and 0.32 mM, respectively) could be introduced. Deoxygenation of the myoglobin was continuously monitored by following the change in absorbance between 575 and 560 nm, using a turbine-driven multi-wavelength time-sharing spectrophotometer (Chance *et al.*, 1975). The absorbance difference (575 minus 560 nm) was plotted against time and used to calculate oxygen consumption rates as free dissolved oxygen concentration declined.

RESULTS

Rapid-scan studies of the Hmp reaction with NADH and oxygen in oxygen-unsupplemented conditions

The ability of Hmp to form an oxygenated compound at low oxygen concentrations was determined in buffers that had been sparged with oxygen-free nitrogen for > 15 min in the reservoir syringes of the stopped-flow apparatus. The oxygen concentration in the apparatus could not be assayed reliably, but an estimation of the residual oxygen concentration under these de-aerated conditions was made as follows. A solution of Hmp (2 μ M final concentration, i.e. after mixing), reduced with NADH (final concentration 0.5 mM), was sparged with nitrogen gas for 10 min. Catalase was added to remove H_2O_2 and the sample was bubbled with nitrogen for a further 5 min to remove oxygen that might have resulted from the activity of catalase. The protein was mixed in the stopped-flow apparatus with an equal volume of nitrogen-bubbled buffer. The experiment was repeated using air-saturated buffer instead of nitrogen-bubbled buffer. In each case, difference spectra were plotted with the deoxy state attained at the end of the reaction as reference. The extent of formation of the oxygenated form, expressed as $\Delta A_{581-563}$, was 0.0186 in aerated buffer and 0.0024 for the de-aerated buffer. By assuming that the K_m for oxygen (2.6 μ M; see later) can be equated with the dissociation constant for oxy Hmp, the free-oxygen concentration under the de-aerated conditions is $2.6 \times 0.0024 / (0.0186 - 0.0024)$ or 0.39 μ M. This is consistent with previous estimates (Orii, 1993) of 'well below micromolar' based on the behaviour of myoglobin in a similar apparatus. Hereafter, the oxygen concentrations in the present experiments will be referred to as 'near-micromolar'.

When purified, Hmp is oxidized and the absolute absorption spectrum has a peak at 403.5 nm (Ioannidis *et al.*,

1992). It is stable in this form, becoming reduced only on addition of NAD(P)H or dithionite, for example. In the experiments reported here, as in our previous work, NADH was used as reductant because the affinity of Hmp for NADH (K_m about 2 μ M) is tenfold higher than that for NADPH (M. Anjum, N. Ioannidis & R. K. Poole, unpublished). A nitrogen-gassed solution of the oxidized form was mixed in the stopped-flow apparatus with a nitrogen-sparged NADH solution in the second syringe to give final concentrations of 3.3 μ M haem and 0.5 mM NADH, as in our earlier work (Poole *et al.*, 1994). The earliest spectrum recorded (1 ms after the mix) exhibited a peak at 407.8 nm which shifted to 409.9 nm at 10 ms and 412.5 nm after 100 ms, with slight gains in intensity (see 0.001–0.1 s in three-dimensional plot; Fig. 1). The changes occurring after 10 s will be described below. NADH contributes to the high absorbance at the shortest wavelengths in Fig. 1, as revealed by scanning below 400 nm (not shown). These changes are similar to those observed with 130 μ M oxygen (Poole *et al.*, 1994) and are attributed to formation of the oxygenated species, despite efforts to remove dissolved oxygen. The same procedure applied under similar experimental conditions was successful in keeping myoglobin in the deoxy state (Orii, 1993). Hmp appears, therefore, to have a high avidity for oxygen.

Difference spectra computed from the rapid-scan data allowed spectral changes occurring during selected time intervals to be visualized. With the 1 ms spectrum as reference (Fig. 2a), the changes in the first 13 ms are characterized by formation of a broad peak centred at 421 nm, but with significant absorbance extending towards longer wavelengths, and an isosbestic point at 407 nm. This result suggests a simultaneous increase in both oxygenated and deoxy forms, since the 421 nm peak is intermediate in position between the absorption maxima of the oxygenated (413 nm) and deoxy (431.5 nm) species (Ioannidis *et al.*, 1992). A similar result was observed in the presence of 130 μ M oxygen (cf. Poole *et al.*, 1994, Fig. 2a). However, the subsequent reaction progress at very low oxygen concentrations was markedly different. When the 12 ms spectrum was used as reference (Fig. 2b), the changes occurring between 12 and 1004 ms showed not only absorbance increases at 418 nm, due to continued formation of the oxygenated form, but a distinct 440 nm trough with an isosbestic point at 430 nm. The similarity of this trough position to that observed in difference spectra with the reduced state as a reference (e.g. CO plus reduced *minus* reduced, trough at 437 nm; Ioannidis *et al.*, 1992) strongly suggests that the oxygenated species is formed from the reduced form during the 12 ms to 1 s interval. Thus, the absorbance change at 440 nm during this period reflects conversion to the oxygenated form of the ferrous haem formed during the flow.

As seen in Fig. 1, the oxygenated form is stable for < 10 s under these oxygen-limited conditions; thereafter it decays to a species with a longer absorption maximum than can be attributed to the deoxy form. Difference spectra, in which the events occurring later than 10 s are plotted with the 10 s spectrum as reference (Fig. 2c), show

Fig. 1. Reaction of Hmp with NADH at near-micromolar oxygen concentration. De-aerated solutions of Hmp and NADH in 50 mM NaCl and 50 mM Tris/HCl buffer (pH 8.0) were mixed in the stopped-flow apparatus at 25 °C, giving final concentrations of 3.3 μ M haem, 0.5 mM NADH and oxygen at near-micromolar concentration (Orii, 1993). Data collection for each spectrum (208 nm) took 1.04 ms.

Fig. 2. Difference spectra obtained during the reaction of Hmp with NADH at near-micromolar oxygen concentration. Experimental conditions were as in Fig. 1. (a) Difference spectra at 1, 3, 4, 5, 7, 11 and 13 ms, in the order indicated by the arrow, with the 1 ms spectrum as reference. (b) Difference spectra at 12, 16, 21, 25, 31, 51, 102 and 1004 ms, in the order indicated by the arrows, with the 12 ms spectrum as reference. (c) Difference spectra at 10, 20, 30.5, 50.5, 71.2, 100.4, 203 and 500.7 s, in the order indicated by the arrows, with the 10 s spectrum as reference.

an absorbance increase at 438 nm (the deoxy form) and a trough at 412 nm (loss of the oxygenated form). Isosbestic points were observed at 424 and 455 nm.

Fig. 3 shows the absorbance changes recorded in Figs 1 and 2 as a function of time with either logarithmic (a) or linear (b) time scales. Comparison of these kinetic profiles with those obtained in the presence of a high concentration of oxygen (cf. Ioannidis *et al.*, 1992, Fig. 1b) reveals the following. (1) A steady-state condition is attained within 200 ms of mixing in both cases, and the extent of formation of the oxygenated species (417.7 nm) is also similar in each case. (2) During the steady state, the level of the deoxy form (439.7 nm) is lower at near-micromolar oxygen than in 130 μ M oxygen. (3) Significantly, under both conditions, the redox state of the flavin (466.9 nm) does not change in the interval between the first recording and the loss of the oxygenated form (~ 10 s in Figs 1 and 3), whereupon the signal decreases in amplitude, indicative of reduction.

Fig. 3. Time courses for the reaction of Hmp with NADH at near-micromolar oxygen concentration. Experimental conditions were as in Fig. 1. (a) 1, 431.1 nm; 2, 469.8 nm; 3, 412 nm; 4, 403.4 nm. (b) 1, 417.7 nm; 2, 466.9 nm; 3, 439.7 nm; 4, 402.6 nm. Solid lines fitted to the symbols were obtained according to either a two-exponential (417.7 nm) or a three-exponential (439.7 nm) equation with the kinetic parameters given in Table 1.

Table 1. Apparent kinetic parameters for reduction of the flavohaemoglobin with NADH in the presence of a near-micromolar concentration of oxygen

Reciprocal relaxation times (s^{-1}) for the phases shown are given at four wavelengths. Standard errors for each kinetic parameter derived from the curve fitting are shown in parentheses. For details, see text.

	Wavelength (nm)			
	417.7	439.7	466.8	562.0
$1/\tau_1$	63.5 (0.04)	129.5 (0.115)	36.2 (0.0217)	99.6 (0.0068)
$1/\tau_2$	15.4 (0.012)	103.4 (0.106)	2.8 (0.0115)	6.4 (0.0055)
$1/\tau_3$		18.1 (0.012)	0.048 (0.0106)	0.10 (0.0099)
$1/\tau_4$			0.004 (0.0297)	0.006 (0.0134)

The absorbance increases for the oxygenated form formation are best explained by two exponential terms under both conditions. Table 1 lists the reciprocal relaxation

Fig. 4. Reaction of Hmp with NADH at near-micromolar oxygen concentration. De-aerated solutions of Hmp and NADH were mixed in the stopped-flow apparatus as described in the legend to Fig. 1, except that the final concentration of Hmp was 13 μ M haem. (a) Absolute spectra recorded at: 1, 1 ms; 2, 8 ms; 3, 21 ms; 4, 51 ms; 5, 20 s; 6, 1807 s. (b) Absorbance changes at: A, 562.0 nm; B, 582.8 nm; C, 625.9 nm; D, 466.8 nm. Solid lines fitted to the symbols were obtained according to a four-exponential equation with the kinetic parameters given in Table 1.

times for the 417.7 nm change at near-micromolar oxygen concentrations; these values, 63.5 s⁻¹ for the fast phase and 15.4 s⁻¹ for the slower phase, should be compared with the corresponding parameters for 130 μ M oxygen, namely 111.2 s⁻¹ and 17.7 s⁻¹, respectively, calculated from Poole *et al.* (1994). Thus, only the initial phase is sensitive to oxygen concentration. The most significant quantitative difference is found between the traces for the deoxy form (437.6 nm at high oxygen concentrations and 439.7 nm at low oxygen concentrations). The former is represented by a single exponential term ($1/\tau_1 = 168$ s⁻¹; Poole *et al.*, 1994), whereas the latter is represented by three terms, i.e. $1/\tau_1 = 129.5$ s⁻¹, $1/\tau_2 = 103.4$ s⁻¹ and $1/\tau_3 = 18.1$ s⁻¹ (Table 1). This result suggests that, at 130 μ M oxygen, the level of the oxygenated form stays unchanged after it is attained, but at low oxygen concentrations the deoxy form is generated transiently, giving way to the oxygenated form by reaction with oxygen (see Fig. 2b).

More stringent oxygen limitation was achieved by using a

Fig. 5. Difference spectra obtained during the reaction of Hmp with NADH at near-micromolar oxygen concentration. Experimental conditions were as in Fig. 4. (a) Difference spectra at 8, 11, 16 and 21 ms, with the 8 ms spectrum as reference. (b) Difference spectra at 21, 30, 42 and 51 ms, with the 21 ms spectrum as reference. (c) Difference spectra at 0.05, 0.1, 0.2, 0.51, 1.01, 2.02, 5.05, 10.2 and 20 s, with the 0.05 s spectrum as reference. (d) Difference spectra at 20, 50.4, 101, 203, 508 and 1807 s, with the 20 s spectrum as reference. Labelled arrows indicate wavelengths of features referred to in the text. Unlabelled arrows show the direction of change of broad spectral regions.

higher protein concentration (13 μ M haem), which also permitted observation in the α/β regions of the spectrum. Formation of the oxygenated species was still evident in the 1 ms spectrum (Fig. 4a). Up to 51 ms after the start of the reaction, the absolute spectra revealed little change in the level of the oxygenated form and little flavin reduction. By about 20 ms (scan 3 in Fig. 4a), the β -band had shifted to 558 nm, characteristic of the deoxy form (Ioannidis *et al.*, 1992), and flavin reduction was evident from the trough below 500 nm. Fig. 4(b) presents these absorbance changes on a logarithmic time scale. The subsequent spectral changes were complex, suggesting multiple reaction steps, and were further analysed by plotting time difference spectra (Fig. 5). Between 8 and 21 ms (Fig. 5a), the time difference spectra revealed an absorbance increase centred at 560 nm and a small absorbance decrease centred

Fig. 6. Oxygen affinity of the NADH oxidase activity of Hmp measured by deoxygenation of oxymyoglobin. (a) Progress of deoxygenation measured at 560 minus 575 nm in the dual-wavelength spectrophotometer. Additions are shown. (b) Oxygen dependence of oxidase activity. Experimental conditions are described in the text.

at about 490 nm. The breadth of the band suggests formation of both oxygenated and deoxygenated forms, as revealed in the Soret region (Fig. 2). The peak height was larger than the trough depth, attributable to the reduction of haem B without accompanying FAD reduction. Between 21 and 51 ms (Fig. 5b), the absorbance decrease centred around 470 nm was the main change, indicating the beginning of reduction of FAD. From 50 ms to 20 s (Fig. 5c), the absorbance increased with a maximal change at 560 nm but extending to 600 nm. The absorbance decrease at 470 nm (flavin reduction) continued. Finally, in the 20–1807 s interval (Fig. 5d), a small portion of the oxygenated form (decreasing absorbance at 583 nm and about 540 nm) was converted into the deoxy form (560 nm), also accompanied by further FAD reduction. In accordance with these complex changes, the absorbance changes at both 562.0 nm for haem B and 466.8 nm for FAD are each simulated by four exponential terms. The reciprocals of relaxation times (Table 1) for the 466.8 nm trace were lower than those for the 562.0 nm trace at each phase, suggesting that haem B is reduced prior to FAD. This may reflect rapid intramolecular electron transfer from FAD to haem, as invoked in an earlier model to explain the predominantly oxidized state of FAD during the oxygenated steady state.

Oxygen affinity of Hmp during NADH oxidation

Fig. 6(a) shows the kinetics of deoxygenation of oxymyoglobin resulting from the consumption of 'free' dissolved oxygen by Hmp with NADH as reductant. Spectra recorded before and after the deoxygenation (not shown) revealed that conversion of oxymyoglobin to the ferric form, rather than the deoxygenated form, made a negligible contribution to the absorbance changes measured, as we have observed previously (D'mello *et al.*, 1994, 1995). The addition of dithionite on completion of Hmp-catalysed deoxygenation resulted in a negligible further

change in absorbance (Fig. 6a). The rates of oxygen consumption in a typical experiment are plotted against oxygen concentration in Fig. 6(b). The K_m was $2.6 \pm 0.16 \mu\text{M}$ and the $V_{\max}$, expressed as a turnover number, was $18 \pm 0.48 \text{ min}^{-1}$ (each a mean of four separate determinations).

DISCUSSION

Proteins having haem domains that show clear homologies to the well-characterized oxygen-carrying globins of multicellular organisms have been described in several micro-organisms. Small proteins (12.5–15 kDa) that resemble superficially the well-characterized *Vitreoscilla* globin have been reported in protozoa (Takagi, 1993) and the cyanobacterium *Nostoc* (Potts *et al.*, 1992). Larger, two-domain or chimaeric proteins that resemble Hmp have been reported in yeasts (Iwaasa *et al.*, 1992; Zhu & Riggs, 1992) and several bacteria, including *Alcaligenes eutrophus* (Probst *et al.*, 1979; Cramm *et al.*, 1994) and *Erwinia chrysanthemi* (Favey *et al.*, 1995). The function(s) of none of these proteins is known. The reactions of microbial globins with oxygen have received scant attention, even though the ligand-binding characteristics should yield important clues to function(s). Most, but not all (e.g. Favey *et al.*, 1995), of the above microbial globins have been shown to bind oxygen. Apart from work on Vgb (Webster, 1987), only the yeast globin has been extensively studied in this respect. Oshino *et al.* (1973a) showed that the oxygenated form of yeast haemoglobin could be detected in intact cells of *Candida mycoderma* and that the oxygen concentration producing half-oxygenation of isolated globin *in vivo* was $0.015\text{--}0.02 \mu\text{M}$. The dissociation rate constant was 17 s^{-1} (pH 7.0, 23 °C), very similar to the values of animal globins and leghaemoglobin (Oshino *et al.*, 1973b).

A striking feature of Hmp is its ability to act as an oxidase, i.e. Hmp not only binds oxygen, but reduces it to superoxide (Oriei *et al.*, 1992; M. Anjum, N. Ioannidis & R. K. Poole, unpublished; R. K. Poole, R. D'mello & Y. Oriei, unpublished). Vgb is also an oxidase, being first described as 'a soluble cytochrome *o*' ('*o*' for oxidase; Webster & Liu, 1974). Thus, an important aspect of understanding the physiological function of Hmp (and presumably its homologues in other organisms) must be to determine the ability of Hmp to 'compete' for oxygen with other oxygen-consuming enzymes. In *E. coli* (and probably the other enteric bacteria in which Hmp-like proteins have been tentatively found; see Andrews *et al.*, 1992), there are two such oxidases, cytochrome *bo'* and cytochrome *bd*. The former is a member of the haem-copper super-family of membrane-bound terminal oxidases that includes mitochondrial cytochrome *c* oxidase. Cytochrome *bo'* is a proton pump, whose synthesis is markedly affected by growth conditions (Cotter *et al.*, 1990) such that it is present maximally in cells grown with 'high aeration'. Recent determinations (D'mello *et al.*, 1995) of the affinity of cytochrome *bo'* for oxygen, using the same sensitive technique as described in the present work, have revealed K_m values between 0.016 and

0.35 μ M. The alternative oxidase, cytochrome *bd*, is structurally unrelated to cytochrome *bo'*, in having a haem-haem binuclear centre where oxygen is reduced, and is synthesized under microaerobic conditions. Several measurements of its affinity for oxygen (e.g. Rice & Hemphling, 1978) have suggested that its affinity for oxygen is much higher than that of cytochrome *bo'*, suiting it to function as a scavenger of oxygen. Recently, we have used the deoxygenation of oxyleghaemoglobin to demonstrate a remarkably high affinity for oxygen of around 5 nM (D'mello *et al.*, 1996), unprecedented for a terminal oxidase. It is tacitly assumed, though not formally proven, that the oxygen-consuming sites of both oxidases are on the inner, cytoplasmic face of the membrane, although some experimental evidence with *Azotobacter vinelandii* is consistent with the view that cytochrome *bd* might reduce oxygen on the outer side of the membrane (Jones, 1977; D'mello *et al.*, 1994). Thus, even the lower affinity oxidase, cytochrome *bo'*, has a K_m well below that of Hmp. If Hmp (Vasudevan *et al.*, 1995) and the oxygen-consuming site of cytochrome *bo'* are in the cytoplasm, and assuming that access of oxygen to the cytoplasm is unrestricted (Unden *et al.*, 1995) and that there are no significant intracellular gradients of oxygen concentration, the oxidase activity of Hmp is unlikely to make a major contribution to cellular oxygen uptake. Reinforcing this view are the relative intracellular concentrations of the oxidases and Hmp. Cotter *et al.* (1990) have calculated that the concentrations of cytochromes *bo'* and *bd* are 2–300 and 200–600 molecules per cell, respectively, according to growth conditions, and are readily measured in intact cell suspensions. In contrast, Hmp is undetectable in intact cells or crude extracts, unless over-expressed from plasmid-borne *hmp* (Vasudevan *et al.*, 1991) or induced by paraquat (M. Anjum, N. Ioannidis & R. K. Poole, unpublished). We conclude, therefore, that under normal growth conditions Hmp does not contribute substantially to cellular uptake of oxygen in *E. coli*.

It is striking, however, that even the use of nitrogen-sparged reactants and relatively high protein concentrations do not prevent the formation of the oxygenated species of Hmp when the ferric form is mixed with NADH in the stopped-flow apparatus. Under the experimental conditions employed here, the oxygen concentration at the instant of initiating the reaction is estimated to be near-micromolar (Orii, 1993), declining thereafter as a consequence of the oxidase activity of Hmp. We are not aware of any estimates of the intracellular concentration of oxygen in *E. coli*, but it is reasonable to suppose that Hmp will be substantially oxygenated *in vivo*.

This conclusion is immediately relevant to our previous proposal (Poole *et al.*, 1994) that Hmp may act as an oxygen sensor. In that model, occupancy of the haem by oxygen is envisaged to control the steady-state reduction level of FAD and the transfer of electrons from FAD to an (unknown) acceptor. In the presence of oxygen, the oxygenated complex is formed and the flavin is predominantly oxidized; in the absence of oxygen, e.g. as a result of oxygen consumption by Hmp, the flavin is predom-

antly reduced and allows Hmp to act as a reductase for, perhaps, Fe(III). The results presented in this paper demonstrate that a key element in this model, namely the control of flavin oxidoreduction by oxygen binding, is operative at or below micromolar concentrations of oxygen. The data do not, however, distinguish between models that invoke (a) rapid electron withdrawal from FAD by intramolecular electron transfer or (b) conformational changes in the protein elicited by occupancy of ligand at the haem and subsequent effects on flavin chemistry (Cooper *et al.*, 1994). It is interesting to note that in the FixL protein of *Rhizobium meliloti*, Gilles-González *et al.* (1995) have shown that the presence of any ligand that gives a high-spin state of the haem endows the protein with kinase activity, which initiates a cascade of reactions culminating in nitrogen fixation. Oxygen binds to FixL, giving a low-spin complex that prevents induction of nitrogen fixation. The effects of other ligands on the reductase activities of Hmp remain to be established.

Detailed kinetic studies of oxygen binding and reduction by Hmp remain to be performed. The present data reveal that an early stage in the formation of the oxygenated species is sensitive to oxygen concentration and that persistence of the oxygenated form at low oxygen concentrations involves reduction of Hmp by NADH, followed by oxygenation on a time scale that can be observed using the rapid-scan technique. The kinetics of oxygen binding to, and dissociation from, Hmp are important objectives of future work.

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