Vitreoscilla Hemoglobin

INTRACELLULAR LOCALIZATION AND BINDING TO MEMBRANES

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The obligate aerobic bacterium, Vitreoscilla, synthesizes elevated quantities of a homodimeric hemoglobin (VHb) under hypoxic growth conditions. Expression of VHb in heterologous hosts often enhances growth and product formation. A role in facilitating oxygen transfer to the respiratory membranes is one explanation of its cellular function. Immunogold labeling of VHb in both Vitreoscilla and recombinant Escherichia coli bearing the VHb gene clearly indicated that VHb has a cytoplasmic (not periplasmic) localization and is concentrated near the periphery of the cytosolic face of the cell membrane. OmpA signal-peptide VHb fusions were transported into the periplasm in E. coli, but this did not confer any additional growth advantage. The interaction of VHb with respiratory membranes was also studied. The Kₐ values for the binding of VHb to Vitreoscilla and E. coli cell membranes were 5–6 μM, a 4–8-fold higher affinity than those of horse myoglobin and hemoglobin for these same membranes. VHb stimulated the ubiquinol-1 oxidase activity of inverted Vitreoscilla membranes by 68%. The inclusion of Vitreoscilla cytochrome bo in proteoliposomes led to 2.4- and 6-fold increases in VHb binding affinity and binding site number, respectively, relative to control liposomes, suggesting a direct interaction between VHb and cytochrome bo.

In the years following the discovery of hemoglobin in the Gram-negative bacterium Vitreoscilla in 1986 (1), other hemoglobins and flavohemoglobins have been found in a variety of microbes, indicating the widespread occurrence of Hb-like proteins in the microbial world (2–6). However, at present Vitreoscilla hemoglobin (VHb) remains the most studied of these bacterial hemoglobins, including its potential use in biotechnological applications. The cellular concentration of VHb in Vitreoscilla increases roughly two orders of magnitude (to about 50 nmol heme/g wet weight) when the oxygen concentration of its growth medium falls to a microaerobic level (7). Enhanced biosynthesis of VHb is mediated at the transcriptional level by an oxygen-sensitive promoter that turns on under hypoxic conditions (below ~10% of air saturation) in both its native and recombinant host, Escherichia coli (8–11). The presence of a relatively large cellular concentration of VHb under oxygen-limiting conditions suggests that its primary function is to trap molecular oxygen and facilitate its transfer to the respiratory apparatus to enable Vitreoscilla to survive under these conditions despite its being a strict aerobe.

It has been demonstrated through genetic engineering that the intracellular expression of VHb in various heterologous hosts often results in the enhancement of cell density, oxidative metabolism, engineered product formation, and bioremediation, especially under oxygen-limiting conditions. Some examples of the in vivo effects of VHb include (i) increased cell density in recombinant E. coli and Pseudomonads (8, 12–14), (ii) increased production of α-amyrase in E. coli (15) and cephalosporin C in Acramonium chrysogenum (16), and (iii) enhanced degradation of toxic wastes such as benzoic acid degradation by Pseudomonads (17) and 2,4-dinitrotoluene degradation by Burkholderia (18). Studies conducted so far on biosynthesis, functional characteristics, and genetic regulation of VHb suggest two possible working models to account for the mechanism of VHb action. The first one, called the facilitated diffusion hypothesis (19), implies that the presence of VHb enhances the oxygen flux to one or both of the terminal oxidases (cytochromes bo and bd) under hypoxic conditions. This is supported by the fact that the respiratory activity and ATP production increased in an E. coli strain that contained VHb relative to a control strain lacking VHb (20). The oxygen-binding properties of VHb are presumed to contribute to its postulated function; it has a relatively normal association rate constant (kₐ) for oxygen binding, thus showing a relatively high “avidity” for oxygen, but its rate constant for oxygen dissociation (kₐ) is unusually large (21). The relatively large static equilibrium dissociation constant, Kₐ, which differs 10-fold from the one determined kinetically (22), is 6 μM (equivalent to P₅₀ = 3.3 mm). These oxygen-binding characteristics are consistent with its putative role of sequestering oxygen from the environment and feeding it to the respiratory terminal oxidases. A possible alternative mechanism of action of VHb may be that oxy-VHb influences the activity of some key redox-sensitive component of the cell, which could be a sensor, a regulator, or even an allosteric site of a respiratory enzyme. Such an influence could in turn be transduced into an increase in the efficiency of energy conservation. There is also the possibility that VHb has a totally different function or more than one function. For example, it has recently been demonstrated...
that the related flavohemoglobin from *E. coli* is a nitric-oxide dioxygenase that dioxygenates nitric oxide to form nitrate (23) to protect the cell from this free radical that can be generated by oxidation-reduction systems including the respiratory chain.

The proposed function of VHb as an oxygen carrier suggests that close proximity to the respiratory membrane would enable it to perform its cellular function most efficiently. It was reported previously that ~40% of the VHb expressed in *E. coli* is found in the periplasmic space (24). This result was based on the isolation of periplasmic VHb protein from the recombinant *E. coli* by lysozyme osmotic shock treatment. Further, a phoA-VHb fusion study indicated that the N terminus of VHb may have a transport function. These observations supported the function of VHb as a facilitator of oxygen transfer by placing it closest to the environmental source of oxygen. However, the N-terminal sequence of VHb is not a typical export signal sequence, and it is known that recombinant protein that is overexpressed in *E. coli* often ends up in the periplasm or inclusion bodies or is even secreted from the cell (25). On the other hand, the localization of oxy-VHb near the respiratory apparatus would be preferable if VHb facilitates oxygen delivery by generating sufficient oxygen flux and directly interacting with the respiratory apparatus of the cell. In the homologous mitochondrial protein, cytochrome-c oxidase, the oxygen-reactive sites are oriented in the membrane on the cytoplasmic side (26). Thus, a specific cellular location for VHb may be required for the optimal performance of its cellular function. Because the periplasmic localization of VHb was determined through indirect experimental observations and could be an artifact, we reexamined the question of the cellular localization of VHb by determining the location of VHb inside the cell through electron microscopy. This led to a derivative study of the membrane binding properties of VHb. This paper shows that the location of VHb in *Vitreoscilla* and recombinant *E. coli* is cytoplasmic and concentrated adjacent to the cell membrane, and evidence is provided that because of its membrane association properties, it may perform its cellular function by interacting directly with the respiratory apparatus of the cell.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions—Vitreoscilla sp. strain C1, *E. coli* JM109, and *E. coli* BL21DE3 carrying various recombinant plasmid constructs were used for the experimental work. Recombinant plasmid pUC8:16 carrying *vgb* has been described previously (27). *Vitreoscilla* was routinely grown in PYA medium (1% peptone, 1% yeast extract, and 0.02% sodium acetate, pH 7.8) at 25 °C. *E. coli* strains were grown in Luria Broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.5) at 37 °C, and the oxygen level was adjusted according to the method adopted by Narro et al. (28) and as described previously (29). The plasmid, Bluescript KS(+) (version 3.0), was used for the generation of the *ompA-vgb* gene fusion. *E. coli* BM121DE3 carrying the *ompA-vgb* fusion was induced with 0.1 mM isopropyl-1-thio-

**Immunogold Electron Microscopy of *E. coli* Containing VHb—**Cells were harvested in late log phase for both the high aeration studies (grown at 200 rpm in 500-mL baffled flasks containing 100 mL of medium) and the low aeration studies (grown at 75 rpm in 500-mL flasks containing 300 mL of medium) by centrifugation (5,000 × g at 4 °C), washed twice with Dulbecco’s phosphate-buffered saline (PBS), and resuspended in 0.2% glutaraldehyde plus 4% paraformaldehyde for 20 min. The cells were then hydrated with a graded series of ethanol and embedded in LR white resin (polymerization at 55 °C for 24 h). Ultrathin sections cut with a Reichert Ultracut Ultromicrotome (Leica Reichart Jung, Austria) were picked up on 200-mesh nickel grids. Nonspecific binding sites were blocked with 2% skim milk/0.01% Tween 20 in PBS (blocking buffer). The grids carrying the ultrathin sections were then washed in 0.05% Tween 20 in PBS (washing buffer) and incubated overnight with rabbit anti-VHb antibody (diluted 1:500 in 1:10 diluted blocking buffer) at 4 °C. The grids were then washed in the washing buffer and incubated for 2 h at room temperature with goat anti-rabbit antibody conjugated to 10 nm colloidal gold spheres (diluted 1:20 in 1:10 diluted blocking buffer). This was followed by washing the grids three more times in washing buffer and then blotting dry with filter paper. The sections were then stained in 2% aqueous uranyl acetate for 40 min in the dark followed by a final washing with double-distilled water. The grids thus prepared were examined in a JEOL 1200 EXII transmission electron microscope (TEM, operating voltage 80–80 kV), and random fields were photographed. The prints of the micrographs were then made at a low magnification and scanned for further analysis. Controls included the labeling of each set of samples with the preimmune serum (i.e., normal rabbit serum) instead of anti-VHb serum. *E. coli* cells not expressing VHb were also included as a control for the recombinant *E. coli*.

**Estimation of the Distribution of VHb in the Cytoplasm of *E. coli*—**Prints of the negatives were made and overlaid with transparency sheets. Cell outlines clearly delineating the outer and inner membranes were traced, cut out, and weighed. The gold particles in the cytoplasm and periplasm of each section were counted with the help of an >x5 magnifying glass. The area of each section was computed by comparison with the weight of an area corresponding to 100 square microns cut out from the same transparency sheet. To calculate the probe density in the two cellular compartments, at least 35 sections were analyzed per sample, and the results were subjected to statistical analysis (Graph Pad Prism 3.0) and t tests to test the level of significance. A more detailed description of the immunogold labeling procedure and analytical methods are described by Ramadane et al. (31).

**Construction of *ompA-vgb* Gene Fusion—**An NcoI site was incorporated at the N terminus of *vgb* by PCR using the oligomer HB-P (below). The C-terminal oligomer HB-3 used for amplification incorporated a BamHI fragment. The sequences of these primers are HB-P (5'-GCCATGG-GACAGCCACGAAACACATTAAC-3') and HB-3 (5'-GGATCCGTTTTC-GGCAACACGCACCTGCTGCTG-3'). The restriction sites are underlined.

The amplified product was cloned in Bluescript KS(+) and its complete nucleotide sequence was checked to validate the authenticity of the clone. The NcoI-BamHI fragment was then ligated in frame with the OmpA signal peptide-coding sequences carried on the expression secretion vector pTMN (30). The fusion junction of *ompA* and *vgb* was ascertained through nucleotide sequencing.

**Preparation of Membranes and Proteoliposomes—**Membranes of both *E. coli* and *Vitreoscilla* were obtained by lysozyme treatment of the cells as described previously (32). These were sonicated for 5 min at 25% duty cycle at full power with a Bronson Sonifier Cell Disruptor 35 fitted with a microtip to produce the inverted membrane vesicles. Phospholipids purified from *Vitreoscilla* were used to make liposomes that were produced by suspending 6 mg of the phospholipids in 2.0 ml of 25 mM Tris-Cl, pH 7.2, and sonicating for 1 min at 0 °C. Cytochrome *bo* proteoliposomes were prepared using a modification of a procedure described previously (33) by adding purified cytochrome *bo* to the liposome suspension at 2 mg/ml, adding octyl glucoside at a final concentration of 1.25%, and slowly stirring at 0 °C for 30 min. The suspension was diluted with 20 volumes of the Tris buffer and incubated at room temperature for 20 min. The proteoliposomes were then collected by centrifugation at 120,000 × g for 2 h at 4 °C and stored at −70 °C.

**Purification of VHb and Cytochrome *bo*—**The VHb was purified by a modification of the procedure described previously (34): lysosome lysis of *Vitreoscilla* cells, 45–70% ammonium sulfate fractionation, DEAE-cellulose chromatography, and Sephadex G-100 chromatography. Cytochrome *bo* from *Vitreoscilla* was also purified by a modification of a published method (35). The membranes obtained by lysozyme treatment were extracted with deoxycholate followed by chromatography first on Bio-Gel A (0.5 × 100 cm) and then on DEAE-Sephadex CL-6B.

**Assay for Ubiquinol-1 Oxidase Activity—**The assay medium contained 60 mM Tris-Cl, pH 7.7, 400–500 μM of membrane protein or 0.16 μg of cytochrome *bo* in the proteoliposomes, and 10 mM dithiothreitol in a final volume of 3.85 ml. After a 2-min preincubation at 30 °C, 15 μM ubiquinol-1 was added to start the reaction, which was monitored polarographically for 4 min at 30 °C using a YSI Model 53 oxygen meter. O2 uptake in the absence of membranes was measured similarly to correct for the autoxidation of ubiquinol-1. This assay was performed at normal conditions (100% atmospheric O2 saturation) and hypoxic conditions (18–22% of atmospheric O2 saturation). Hypoxic conditions were produced by bubbling the reaction mixture (minus the ubiquinol-1) in a special test tube containing a long needle connected to a nitrogen gas supply, a tube connected to a vacuum pump, and sealed
with cork and parafilm. Nitrogen gas was bubbled for 5 min, and then the gas phase inside the test tube was evacuated for 5 min. These steps were repeated four times, and then the reaction buffer was transferred using another long needle back to the oxygen monitor to determine the oxygen concentration and start the reaction with ubiquinol-1.

**Determination of Binding of Hbs to Membranes**—This is an adaptation of a procedure that was originally developed for studying the binding of ligands to proteins (36). The Sephadex G-50 used in the original method was replaced by Sephadex G-100, which with its larger pore size excludes membrane fragments but not Hb from the gel. After mixing the Sephadex, the Hb, and membrane fragments and allowing the suspension to settle, the Hb concentration in the supernatant was determined. If the Hb has a binding affinity for the membrane, the Hb concentration in the external gel space will be higher in the presence of the membranes than in their absence. The following equations summarize the procedure used.

\[
\text{Hb} = \text{Hb}_o + \text{Hb}_i \quad \text{(Eq. 1)}
\]

\[
\text{Hb}_o = \text{Hb} - \text{Hb}_i = \text{Hb}(1 - \beta/\alpha) \quad \text{(Eq. 2)}
\]

Hb is the amount of hemoglobin added, Hb, and Hb, are the amounts outside and inside the gel, respectively, \( \alpha \) is the ratio of the concentration of membranes (in terms of protein content) outside the gel to that of the membrane solution added to the dried gel, and \( \beta \) is defined as the ratio of the hemoglobin concentration outside the gel to that of the solution of hemoglobin added. \( \alpha \) is determined by plotting the amount of membrane fragments outside the gel versus the amount of membrane fragments added, as illustrated in Fig. 1 for *Vitreoscilla* membranes, and \( \beta \) is determined as shown in Fig. 2 for control horse hemoglobin.

![Fig. 1. Determination of \( \alpha \) for *Vitreoscilla* membranes. See “Materials and Methods” for experimental details.](image)

Because

\[
\text{Hb}_o = \text{Hb}_{[\text{free}]} + \text{Hb}_{[\text{bound}]} \quad \text{(Eq. 3)}
\]

it can be shown that the concentrations of the bound and free hemoglobin outside the gel are

\[
\begin{align*}
\text{Hb}_{[\text{bound}]} &= \text{Hb}_o - \text{Hb}_{[\text{free}]} = \text{Hb}(\beta - \beta'/\alpha - \beta') \quad \text{(Eq. 4)}
\end{align*}
\]

\[
\text{Hb}_{[\text{free}]} = \beta'(\alpha - \beta'/\alpha - \beta') \quad \text{(Eq. 5)}
\]

where \( \beta' \) is defined as \( \beta \) in the absence of the membranes.

The final operational equation is a modified Scatchard equation,

\[
\frac{1}{R} = \frac{K_d}{N} \frac{\text{Hb}_{[\text{free}]}}{1 + \frac{1}{N}} \quad \text{(Eq. 6)}
\]

where \( R = \text{Hb}_{[\text{bound}]}/\text{mg membrane} \), \( K_d \) = dissociation constant for the hemoglobin binding to the membranes, \( \text{Hb}_{[\text{free}]} \) = concentration of unbound hemoglobin, and \( N \) = the maximum number of binding sites per mg membrane.

Experimentally, 50 mg of the dried Sephadex G-100 was suspended in 1.5 ml of 0.1 M potassium phosphate, pH 7.2, and allowed to swell for 4 h at room temperature, and then 0.5 ml of the buffer solution containing the membranes and/or globins was added. This suspension was incubated for 10 min at room temperature with continuous stirring, the resin was allowed to settle for 10 min, and a 100-µl aliquot of the supernatant was removed. The globin concentration was determined by using heme absorption at \( A_{410} \) (179, 188, and 214 mM cm for equine Hb, Mb, and VHb, respectively), membrane protein using \( A_{280} \).
0.675 ml mg$^{-1}$ cm$^{-1}$, corrected for globin protein when present), and liposome concentration by measuring the inorganic phosphate released after total hydrolysis using the Fiske-Subbarow colorimetric method (37).

**RESULTS**

**Immunolocalization of VHb in Vitreoscilla**—A previous study of VHb indicated that this bacterial hemoglobin is partially transported into the periplasmic space both in its native host, *Vitreoscilla*, and recombinant host, *E. coli* (24). The question of the subcellular location of VHb was addressed further by tracing it in *Vitreoscilla* using immunogold electron microscopy. *Vitreoscilla* cells grown under high and low aeration were probed with an anti-VHb antibody and examined with an electron microscope. *Vitreoscilla* cells cultivated at low aeration had a significantly higher level of labeled VHb (as compared with aerobically grown cells), which was predominantly cytoplasmic (Fig. 3). The standardized results indicated that more than 90% of the VHb was confined to the cytoplasm (Table I), much of it adjacent to the cytoplasmic membranes; of the total signal in the cytoplasm, 57% of the VHb-bound gold particles were found localized within 0.1 μm of the inner membrane (Table II).

**Immunolocalization of VHb in Recombinant E. coli—VHb**

**TABLE I**

|                  | Control* | Anti-VHb |
|------------------|----------|----------|
| No. of cells examined* | 65       | 72       |
| Total no. of gold particles in the cells examined | 16       | 342      |
| No. of gold particles in the cytoplasm (per μm$^2$) | 0.4 (0.13)* | 7.84 (0.76) |
| No. of gold particles in the periplasm (per μm$^2$) | 0.16 (0.11) | 1.13 (0.55) |

* Control sections were probed with normal rabbit serum instead of anti-VHb antibody.

Only cells grown under low aeration conditions are shown in this table. All cells that were in focus on the photographs, including incomplete cells in the field where the periplasmic and cytoplasmic spaces were completely delineated, were analyzed. The t test between the control serum and anti-VHb for the cytoplasm gave p < 0.001 and for the periplasm gave p = 0.087. The t test on *Vitreoscilla* cells grown under high aeration and probed with control rabbit serum versus anti-VHb gave p values of 0.617 for the cytoplasm and 0.327 for the periplasm.

* Standard error of the mean is given in parentheses. The ~1 gold particle found for every 4 control cells is the “noise” or background in the histocytochemistry.

**FIG. 4. Immunolocalization of VHb in E. coli carrying vgb. a, under high aeration probed with anti-VHb antibody; b, under low aeration probed with an anti-VHb antibody; c, cells probed with preimmune serum. Gold particles are indicated by arrow heads, especially in the section marked with a, cytoplasm; p, periplasm; bar, 0.5 μm.**

expressed in *E. coli* through multicopy plasmids usually amounts to 5–10% of the total cellular protein. Because an osmotic shock method used to release periplasmic protein in recombinant *E. coli* indicated the partial transport of VHb into the periplasm, we reexamined the location of VHb in recombinant *E. coli* using immunogold electron microscopy. Cells grown under high and low aeration were probed with gold-labeled anti-VHb. The expression of VHb in *E. coli* led to an almost exclusive cytoplasmic labeling without any significant signal for the presence of VHb in the periplasm (Fig. 4 and Table III). These results coincided with the observations made on *Vitreoscilla* as did the localization of the VHb adjacent to the cytoplasmic membrane: 54% within 0.1 μm of the inner membrane (Table II). Because VHb is so highly expressed in *E. coli*, the immunogold signals were much stronger than in *Vitreoscilla* (Figs. 4 and 3, respectively). Although the primary goal of the immunogold labeling procedure was localization and not quantitation, the efficiency of the labeling can be roughly estimated from the section thickness, 70 nm as indicated by the interference color of the sections, and the gold particle density, 27/μm$^2$. Assuming a concentration of 25 nmol of dimeric VHb/g wet weight of cells, the efficiency was only ~3%. This probably underestimates the efficiency, however, because a large fraction of the slices (~40%) inexplicably had few or no particles, whereas some had over 10 times the number of particles per μm$^2$.

**OmpA-VHb Fusion and Periplasmic Transport**—To establish that VHb can be transported via a full-fledged transport signal and to monitor the effect of accumulation of periplasmic VHb on the physiology of its host, an OmpA-VHb fusion was created, and localization of VHb was monitored through immunogold electron microscopy. In contrast to native VHb, a high level of periplasmic VHb accumulated via OmpA-VHb fusion in *E. coli* (Fig. 5). Growth characteristics and oxygen uptake of *E. coli* carrying the OmpA-VHb fusion were found to be similar to *E. coli* carrying

**TABLE II**

|                    | Percentage of spots within 0.1 μm of cell membrane* |
|--------------------|-----------------------------------------------------|
| *E. coli*          | 54                                                  |
| *Vitreoscilla*     | 57                                                  |

* Only cells showing strong signals (from among the cells considered for Tables I and III) were chosen for this analysis.
For this experiment, horse Mb and Mb to Bacterial Membranes—A plot of 1/R versus 1/Hb[free] is illustrated in Fig. 6 for VHb binding to Vitreoscilla membranes. The $K_d$ is obtained from the slope, and $N$ (the maximum number of VHb molecules bound/mg of membrane protein) is obtained from the intercept. Table IV summarizes the data for the binding of three different Hbs to three different membrane preparations. From these data, VHb has ~4–8 times higher affinity for Vitreoscilla membranes than the control equine Hbs have for these membranes. Surprisingly, VHb also has about the same high affinity for E. coli membranes. To test whether there are binding sites accessible only on the cytosolic side of the plasma membrane, membrane vesicles were prepared from the membranes by sonication, which effectively reverses their topology. However, there was essentially no difference in binding of the VHb to these inside-out membranes compared with the periplasmic side-out membranes (Table IV). Neither of the horse globins showed any significant difference in binding to these two Vitreoscilla membrane preparations.

Although the horse globins have a lower binding affinity than VHb to all three membrane preparations, there seem to be more binding sites for them with $N$ being around 32 nmol/mg of membrane protein for each of them versus only 7 nmol/mg of membrane protein for VHb. This indicates that horse Mb and Hb bind to a number of nonspecific sites with relatively low affinity, whereas VHb interacts with a limited number of higher affinity sites. Likely sites of interaction would be components of the respiratory chain. This possibility was examined using proteoliposomes containing purified Vitreoscilla cytochrome bo, which had been purified previously in our laboratory.

| TABLE III | Distribution of VHb in recombinant E. coli cells as determined by immunogold electron microscopy |
|-----------|----------------------------------------------------------------------------------|
| Growth conditions | Control$^a$ | High aeration$^b$ | Low aeration$^c$ |
| No. of cells examined$^d$ | 59 | 42 | 59 |
| Total no. of gold particles in the cells examined | 86 | 768 | 1316 |
| No. of gold particles in the cytoplasm (per $\mu$m$^2$) | 1.56 (0.002) | 26.9 (2.9) | 23.0 (2.8) |
| No. of gold particles in the periplasm (per $\mu$m$^2$) | 1.37 (0.004) | 2.51 (0.006) | 2.29 (0.46) |

$^a$ Controls are the sections probed with normal rabbit serum instead of anti-VHb antibody. Control values for cells grown under both high and low aeration conditions were similar, and thus only the data for the high aeration cells are shown. The $t$ test between E. coli cells expressing VHb (both high and low aeration) probed with normal rabbit serum and E. coli cells not expressing VHb and probed with both control serum and anti-VHb gave $p$ values ranging from 0.3 to 0.5.

$^b$ High and low aeration sections probed with rabbit anti-VHb antibody. The $t$ test between the control and cells probed with VHb antibody gave $p < 0.001$ for the cytoplasm and $p = 0.114$ and 0.159 for the periplasm for high aeration and low aeration, respectively.

$^c$ All cells that were in focus on the photographs, including incomplete cells in the field where the periplasmic and cytoplasmic spaces were completely delineated, were considered for analysis.

$^d$ Standard error of the mean is given in parentheses.

native VHb, indicating that accumulation of VHb in the periplasm does not confer an advantage over cytoplasmic VHb.

**DISCUSSION**

A model for studying oxygen transfer to living cells is the Vitreoscilla-VHb system, the VHb being synthesized when environmental oxygen becomes limiting. How the presence of VHb enables the bacterium to grow better under oxygen limitation is not conclusively known, but knowledge of its subcellular localization is vital to understanding its function. The ultrastructural studies described here have established the cytoplasmic location of hemoglobin, both in Vitreoscilla and recombinant E. coli overexpressing VHb. Although cells grown under low oxygen synthesize large amounts of VHb in E. coli, little VHb-specific signal was detected in the periplasm, a result in contrast to a previous study, which indicated that ~40% of the VHb in E. coli was located in the periplasm space (24). It might seem that a periplasmic location would have the advantage of locating the VHb closest to the source of environmental oxygen. However, the accumulation of large amounts of oxy-Hb in the relatively small volume of the periplasm may change the redox environment of the periplasm, which may not be favorable to the cell. On the other hand, the presence of VHb in the cytosol may provide an oxygen buffer, and its subcellular localization in close proximity to the cell membrane could facilitate oxygen transfer to the oxygen-binding sites of the terminal oxidases that are oriented toward the cytoplasm (26, 38). Thus, a cytoplasmic localization of VHb in the proximity of respiratory membranes, as demonstrated in the present study, is consistent with its proposed function to feed molecular oxygen to the respiring membranes under oxygen limitation.

Although the detection of VHb in the periplasmic fraction in the 1989 study (24) could have been an experimental artifact of VHb leakage through the membranes during osmotic shock, it is more likely the result of overproduction and extrusion of the VHb, because the extrusion of overproduced cytoplasmic recombinant proteins into the periplasmic space has often been observed (25). Generally, true periplasmic proteins are compartmentalized in the periplasmic space and not divided between there and the cytoplasm. In the work reported here, virtually no VHb was detected in the periplasmic space, but VHb leakage from recombinant E. coli has occasionally been observed while washing harvested cells with buffer. The extrusion of VHb could be a function of growth medium, growth phase, physiological state of the cells, the strain of E. coli bearing the ugb, other genetic determinants, etc.

Based on the finding of VHb in the periplasmic space, it was proposed (24) that the first 16 N-terminal residues of VHb may be an export signal, but this N-terminal sequence does not conform to the usual signal-peptide characteristics, and it was not excised during transport; further, unlike most transport signals, it is important for the protein function. The first 16 residues of VHb are an integral part of the A-helix structure.
FIG. 5. Immunolocalization of the ompA-vgb fusion in *E. coli*. a, probed with anti-VHb antibody 1.5 h after induction; b, probed with anti-VHb antibody 3 h after induction; c, probed with preimmune serum. Arrow heads, gold particles; cy, cytoplasm; p, periplasm; bar, 0.5 μm.

FIG. 6. Plot of 1/R versus 1/VHb[free] for VHb binding to *Vitreoscilla* membranes. The y intercept is 1/N, and the slope is Kd/N.
FIG. 7. Effect of VHb on the ubiquinol-1 oxidase activity of *Vitreoscilla* membranes, *Vitreoscilla* membrane vesicles (sonicated membranes), and *E. coli* membranes under saturating aerobic conditions (236 \(\mu\)M O\(_2\)). VHb (0.7 \(\mu\)M) increased the oxygen uptake of *Vitreoscilla* membranes by 11%, *Vitreoscilla* membrane vesicles (sonicated membranes) by 42%, and *E. coli* membranes by 12%. Values are averages of three individual measurements. Error bars, standard error of the mean.

| Membranes          | Horse Hb\(^a\) | Horse Mb\(^a\) | VHb\(^b\) |
|--------------------|----------------|----------------|-----------|
| *Vitreoscilla*     | \(45 (21) \times 10^{-6}\) | \(21 (18) \times 10^{-6}\) | \(5.4 (2.7) \times 10^{-6}\) |
| *Vitreoscilla*, inverted vesicles | \(30 (18) \times 10^{-6}\) | \(27 (11) \times 10^{-6}\) | \(6.5 (4.3) \times 10^{-6}\) |
| *E. coli*          | \(48 (25) \times 10^{-6}\) | \(23 (11) \times 10^{-6}\) | \(5.6 (3.1) \times 10^{-6}\) |

\(^a\) Maximum number of binding sites (\(N\) values) for horse Hb and Mb binding to *Vitreoscilla* membranes averaged 32 \(\pm\) 9 nmol/mg of membrane protein.

\(^b\) \(N\) values for VHb binding to both *Vitreoscilla* and *E. coli* membranes averaged 7 \(\pm\) 3 nmol/mg of membrane protein.

\(^c\) The \(K_d\) for human Hb binding to human red blood cell membranes is \(1.0 \times 10^{-4}\) M (41).

| Vesicles            | Horse Hb\(^a\) | Horse Mb\(^a\) | VHb\(^b\) |
|---------------------|----------------|----------------|-----------|
| Cytochrome bo proteoliposomes | \(7.0 (2.9) \times 10^{-5}\) | \(3.1 (1.6) \times 10^{-5}\) | \(0.62 (0.36) \times 10^{-5}\) |
| Control liposomes   | \(2.2 (1.5) \times 10^{-5}\) | \(2.1 (1.4) \times 10^{-5}\) | \(1.5 (0.8) \times 10^{-5}\) |

\(^a\) \(N\) values (maximum number of binding sites) for horse Hb and Mb binding to proteoliposomes and control liposomes averaged 0.10 \(\pm\) 0.04 and 0.003 \(\pm\) 0.001 nmol/\(\mu\)mol of phospholipid phosphate, respectively.

\(^b\) \(N\) values for VHb binding to proteoliposomes and control liposomes averaged 0.21 \(\pm\) 0.11 and 0.034 \(\pm\) 0.020 nmol/\(\mu\)mol of phospholipid phosphate, respectively.

\(^c\) Standard error of the mean is given in parentheses.
A VHb mutant lacking the first 14 N-terminal residues is incapable of binding heme. In the present study, VHb was determined to have a cytosolic location, raising the question of whether VHb can be transported into the periplasmic space and whether a periplasmic location is in any way superior to a cytoplasmic localization. The OmpA-VHb fusion study clearly suggested that there is no hindrance to the transport of VHb across the membrane by the structure or folding of the protein per se, because ~50% of the peptide-VHb fusion protein was localized in the periplasm. This enhanced periplasmic localization did not provide any distinct advantage over the cytoplasmic accumulation of VHb under oxygen-limited growth conditions. However, the lack of an effect could be caused by the accumulation of nonfunctional apoprotein in the periplasmic space, which has been observed for E. coli Flavohemoglobin (40).

If the primary function of VHb is to trap and feed oxygen to the membrane-bound terminal oxidases, it may actually associate with the cellular membrane to facilitate this process. Initial evidence for this association was the observation that the respiratory membranes of lysed Vitreoscilla cells retained significant amounts of VHb, and extensive washing of the membrane fragments was required to remove it (35). The study of VHb binding to bacterial membranes in the present work found $K_d$ values that were in the micromolar range, 5.4–6.5 μM (Table IV), which were 4.2–8.3 and 4.1–8.6 times greater than the affinities of the control equine globins for Vitreoscilla and E. coli membranes, respectively. Assuming a value of 50 nmol of VHb heme/g wet weight of bacterial cells (7–9), it can be estimated that there will be about 15,000 molecules of dimeric VHb per bacterial cell. From the N value of 7 nmol of binding sites/mg of membrane protein, it can be estimated that there are roughly four times as many VHb binding sites (69,000) in the membrane of a single bacterial cell as there are VHb molecules in the cell. For a $K_d$ of 6 μM, this would indicate that most of the VHb would be bound (94%). For comparison, the $K_d$ for human Hb binding to human red blood cell membranes is $1.0 \times 10^{-4}$ μM, which was considered physiologically significant (41). Thus, the affinity of VHb for bacterial membranes is likely to be important physiologically and is very probably the reason for the subcellular localization of VHb adjacent to the cellular membrane.

The binding of VHb could involve interactions of residues with membrane lipids and/or specific membrane proteins. In the globin domains of flavohemoglobins, which are structurally similar to VHb, Lys-11 is generally conserved (39). Thus, a hydrophobic N terminus carrying a positively charged residue (Lys-11 in VHb) could play a role in the interaction of VHb with the membrane. Lys-11 is an obvious target for site-directed mutagenesis to test whether it plays any role in the membrane interactions of VHb. Whatever the mechanism by which VHb associates with membranes, binding studies with proteoliposomes implicated an interaction with a specific protein, the cytochrome-bo terminal oxidase. When this protein was incorporated into liposomes, the affinity for VHb increased 2.4 times relative to the control liposomes without the cytochrome, and the number of binding sites for VHb increased 6.1-fold to 0.21 nmol/μmol of liposomal phospholipid phosphate. Using this latter value and assuming a molecular mass of 700 daltons for a phospholipid molecule, there are 4,760 molecules of phospholipid for each binding site. Because the proteoliposomes were made using a cytochrome bo/phospholipid ratio of 1:150 (weight/weight), there will be ~1 cytochrome bo incorporated for every 2,240 phospholipid molecules, about twice as many protein molecules as binding sites. Although these estimates may be fortuitous, previous work has indicated that the cytochrome bo in these proteoliposomes is randomly oriented, i.e. 50% in the inside-out and 50% in the outside-in orientation (33). Thus, there may be preferential binding to one side of the cytochrome. Although the binding experiment with inverted membrane vesicles (Table IV) was inconclusive, VHb did stimulate the ubiquinol-1 respiratory activity of inverted membrane vesicles significantly more than that of periplasmic side-out membranes (Figs. 7 and 8), suggesting a preference for cytosolic side binding.

Although the results of this report are consistent with the proposed role of VHb being to facilitate oxygen transfer to the terminal respiratory apparatus under hypoxic conditions and increase respiratory efficiency, they do not exclude other possible functions for this protein. For example, there is evidence that it can function as a terminal oxidase under some conditions (42). The related flavohemoglobin from E. coli has been...
demonstrated to be a nitric-oxide dioxygenase that dioxygenates this free radical to form nitrate (23). This enzyme is inducible under oxygen-limiting conditions and protects the cell from the nitric oxide, which can be generated by the respiratory chain and other oxidation-reduction systems, probably more so under the more reducing conditions of hypoxia. Whether VHb (in conjunction with its flavoprotein metVHb reductase) has a similar role remains to be tested.

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