Oxygen Inhibition of Globin Gene Transcription and Bacterial Haemoglobin Synthesis in *Vitreoscilla*

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A soluble dimeric haemoprotein, structurally and functionally similar to plant and animal haemoglobins, is found in the Gram-negative aerobic bacterium *Vitreoscilla* sp., strain C1. *Vitreoscilla* haemoglobin (VtHb) increases in concentration when the cells are exposed to hypoxic conditions. The globin part of VtHb is encoded by a single gene (vgb). An RNA transcript, approximately 500 bases long, specific for vgb was detected after Northern hybridization. The relative amount of this mRNA increased in cells grown at low levels of oxygen. Two enzymes important for haemoglobin function are δ-aminolaevulinic acid synthase (ALAS), which is necessary for haem biosynthesis, and NADH-methaemoglobin reductase, which is necessary to keep VtHb in the physiologically functional ferrous state. An increase in ALAS specific activity under hypoxic conditions preceded the increased haem production. Cellular reductase content also increased when the VtHb increased in cells grown under hypoxic conditions. The ratio of cellular reductase activity to VtHb content remained relatively constant in cells grown under a variety of conditions. The data suggest that in *Vitreoscilla* the transcription of the globin gene and the biosynthesis of two enzymes important for VtHb function are regulated by oxygen.

INTRODUCTION

Haemoglobins have generally been considered to occur exclusively in eukaryotic organisms. However, Wakabayashi *et al.* (1986) reported the sequence of a haemoglobin in *Vitreoscilla*, a Gram-negative bacterium. This haemoglobin (VtHb) is a cytoplasmic, dimeric haem protein that exists primarily in a stable oxygenated form, similar to that of oxymyoglobin (oxyMb) and oxyhaemoglobin (oxyHb) of higher organisms, during aerobic growth and respiration. Alignment of the amino acid sequence of VtHb with other haemoglobins revealed considerable sequence similarity at many critical conserved regions; in particular there was a 24% sequence identity with yellow lupin leghaemoglobin. The biosynthesis of VtHb increases dramatically when oxygen availability is low, and therefore it was suggested that the function of this protein is to ensure an adequate supply of needed oxygen to the respiring membranes of *Vitreoscilla*, which is an obligate aerobe (Wakabayashi *et al.*, 1986).

The taxonomy proposed by Woese *et al.* (1984) places the genus *Vitreoscilla* in a subdivision of the purple photosynthetic bacteria; this classification has been supported by studies from several laboratories (Nichols *et al.*, 1986; Reichenbach *et al.*, 1986; Strohl *et al.*, 1986a). The cytochromes in *Vitreoscilla* have been analysed by Strohl *et al.* (1986b) and in this laboratory (Georgiou & Webster, 1987). The latter study identified *b*-, *c*-, and *d*-type cytochromes in the membrane. The latter two types were present in very small quantities, and some or all of the *b*-type cytochromes could be ascribed to the cytochrome o terminal oxidase by its carbon

**Abbreviations:** ALA, δ-aminolaevulinic acid; ALAS, δ-aminolaevulinic acid synthase; DO, dissolved oxygen; metHb, methaemoglobin; oxyHb, oxyhaemoglobin; oxyMb, oxymyoglobin; PYA, peptone/yeast extract/acetate medium; vgb, *Vitreoscilla* globin gene; VtHb, *Vitreoscilla* haemoglobin; VtmetHb, *Vitreoscilla* methaemoglobin.

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monoxide binding properties. Most of the cellular haem in this organism, however, is present in the cytosol as VtHb [Note: VtHb was called a (soluble) cytochrome o in all references prior to 1986].

By using a synthetic oligonucleotide as a hybridization probe, we isolated the Vitreoscilla globin gene (vgb) from a Vitreoscilla genomic library constructed in the broad host range cosmid vector, pVK102, and subsequently subcloned it in Escherichia coli, using pUC8, as a 1.4 kb HindIII–SalI fragment of the Vitreoscilla genome (Dikshit & Webster, 1988). E. coli cells containing the vgb gene exhibited difference spectra characteristic of oxyHb and produced up to eight times more VtHb than Vitreoscilla. Khosla & Bailey (1988a) have cloned the vgb gene into pUC19, and they also found that it was expressed at a high level. Further subcloning and spectroscopic analysis of the 1.4 kb HindIII–SalI genomic fragment revealed that the vgb gene is expressed in E. coli using its own promoter, indicating that the manner of promoter recognition is similar in both organisms. Hybridization studies indicated that the vgb gene is present in a single copy on the Vitreoscilla genome and is transcribed by an RNA transcript approximately 500 bases long. For this report we studied the expression of the vgb gene during the growth cycle of Vitreoscilla and under different levels of oxygen. Our results provide evidence that oxygen plays an important role in the expression of this gene. The control of haem production was also examined in this study by focusing on δ-aminolaevulinic acid synthase (EC 2.3.1.37) (ALAS), the first enzyme in the biosynthetic pathway of haem. Additionally, we examined the effect of oxygen on another enzyme essential for haemoglobin function, NADH-methaemoglobin reductase. Methaemoglobin (metHb) is incapable of binding oxygen, and the reductase, formerly called NADH-cytochrome o reductase (Gonzales-Prevatt & Webster, 1980), reduces the ferric iron in Vitreoscilla metHb (VtmetHb) to the physiologically active ferrous form of VtHb.

METHODS

Bacterial strains and plasmids. Vitreoscilla sp., strain C1, and the E. coli strains and plasmids used in this study and for cloning of the vgb gene have been described previously (Lamba & Webster, 1980; Maniatis et al., 1982; Dikshit & Webster, 1988).

Media and growth conditions. E. coli strains and Vitreoscilla were grown under conditions described previously (Lamba & Webster, 1980). To determine the effect of oxygen on the appearance of vgb specific RNA, total RNA was isolated from Vitreoscilla cells grown under different oxygen levels. Exponentially growing culture (1 ml) was inoculated into each of two 100 ml flasks containing 50 ml of standard 1% (w/v) PYA medium (1% peptone, 1% yeast extract, 0.02% sodium acetate, pH 7.8). One flask was grown in air (20% v/v, oxygen) at room temperature and the other was grown in a closed desiccator in which the atmosphere was maintained at 95% (v/v) nitrogen and 5% (v/v) oxygen. Cells were harvested at the mid-exponential growth phase and used for RNA isolation. For the ALAS and NADH-VtmetHb reductase determinations the cells were grown in PYA with experimental variations (e.g. shaking rate, medium concentration) noted in the appropriate section. Cells were harvested using a Sorvall GSA rotor at 13000 g for 30 min and the cell paste was stored at −20 °C for the ALAS, NADH-VtmetHb, and haem determinations. For more precise control of dissolved oxygen (DO) and to obtain larger quantities of cells in the earlier stages of the growth cycle needed for some of the ALAS determinations, the cells were grown in 25 l of medium in a New Brunswick Scientific IF-40 industrial fermenter. DO was monitored using an ABEC model 316-36 DO probe and recorded on a model 40 DO recorder or model 80 DO controller (New Brunswick Scientific). The controller was set to control both aeration and agitation when DO was maintained at a constant level. DO is expressed as a percentage of the oxygen dissolved relative to normal atmospheric concentration (236 μM at 30 °C); thus a DO value of 10% atmospheric corresponds to 24 μM-dissolved oxygen. An Ingold model 465 pressurized pH probe recorded pH on a PH-22 pH controller (New Brunswick Scientific). For fermentation experiments the standard medium was used, except that the final pH 7.5 was adjusted with gaseous ammonia. The incubation temperature was 30 °C and the agitation was 200 r.p.m. in all cases except when a constant DO was maintained. A 4% (v/v) inoculum was used. Samples were taken at 8–12 h intervals and harvested in a Sharples air driven continuous flow centrifuge. The wet weight yield was determined and cells were frozen at −20 °C for haem and ALAS determinations.

Isolation of RNA and Northern blot analysis. All glassware used in the RNA isolation was baked overnight at 160 °C; solutions and plasticware (Eppendorf tubes and pipette tips) were treated with 0.2% (w/v) diethylpyrocarbonate and autoclaved for 40 min. Cells were suspended at approximately 10⁸ cells ml⁻¹ in TE (10 mM-Tris, 1 mM-EDTA, pH 8-0) containing 5% (v/v) Triton X-100 and 2 mg lysozyme ml⁻¹ and lysed by placing the tubes in boiling water for 3–5 min. Cell debris and chromosomal DNA were removed by centrifugation, and the supernatant was extracted three or four times with buffered phenol (until a clear interface was visible), once with chloroform/isoamyl alcohol (24:1, v/v), and precipitated with 2 vols ethanol and 0.1 vol.
done in duplicate or triplicate and each growth curve, etc, was run at least twice. Representative results are shown.

described previously (Gonzales-Prevatt and Promega Biotech and used as instructed by the supplier. RQ1 DNAase was from Promega Biotech. For

Bradford (1976) with bovine serum albumin as a standard. The NADH-metHb reductase assay was performed as
described previously (Boerman

[15 mCi mmol-1 [LY-~~PI~ATP obtained from ICN Radiochemicals was used. Omnifluor and [2,3-14C]succinate

about 500 bases long (Dikshit

was obtained from Research Products, Dowex 50W-X4 from J. T. Baker, and Coomassie (Brilliant) Blue R-250 from Bio-Rad. The peptone, Hy Case SF was from Sheffield Chemical, and the yeast extract was purchased from

labelling DNA, [LY-~~PI~ATP obtained from ICN Radiochemicals was used. Omnifluor and [2,3-14C]succinate

for ALAS by the isotopic method of Ebert et al. (1970) modified by using the reaction mixture described by

The incubation mixture contained glycine (100 mM), MgCl2 (10 mM), pyridoxal phosphate (0-3 mM), Tris/HCl pH 7-5 (50 mM), ATP (10 mM), coenzyme A (0-4 mM), EDTA (10 mM), succinic thiokinase

for 16 h at 42 °C. Filters were washed for 15 min in 2 × SSC containing 1% SDS (1 × SSC is 0:15 m-NaCl, 0:015 m-sodium citrate, pH 7-0), twice at room temperature then twice at 42 °C and subjected to autoradiography.

ALAS determinations. Cell free extracts were prepared by suspending 1 g of cells in 20 ml 0-5 m-sucrose/0-01 m-potassium phosphate pH 7-0, adding 5 mg lysozyme and incubating at 34 °C for 60 min. All subsequent operations were at 0 to 4 °C. The spheroplasts were centrifuged in a Sorvall SS-34 rotor at 12000 g for 30 min then resuspended in 40 ml 50 mM-Tris/HCl, pH 7-5. The spheroplasts were disrupted by sonication using a Branson Sonifier Cell Disruptor for 15 s at setting 6 (approx. 200 w), pulsed at 50% duty. The disrupted spheroplast suspension was then centrifuged in an SS-34 rotor at 6000 g for 15 min and the supernatant was saved and assayed for ALAS by the isotopic method of Ebert et al. (1970) modified by using the reaction mixture described by

Potassium phosphate buffer (0-1 M), Tris/HCl pH 7-5, MgCl2 (1-5 mM), Tris/HCl pH 7-5, MgCl2 (1-5 mM), coenzyme A (0-4 mM), EDTA (10 mM), succinic thiokinase

was washed with 5-0 ml TCA (1%, w/v) and centrifuged as before. To the pooled supernatants, 3-0 ml 1-0 m-sodium acetate pH 4-8, 1 μmol carrier 6-aminolaevulinic acid (ALA), and 50 μmol carrier succinate were added and the volume was brought to 20 ml. The mixture was then applied to a Dowex 50W-X4 50-100 mesh column

(0.6 cm) previously equilibrated with 0-1 M-sodium acetate buffer, pH 3-9. The [2,3-14C]succinate was eluted

% was subjected to electrophoresis on 1-2% agarose gels containing 2-2 M-formaldehyde and transferred to nitrocellulose paper as described by Maniatis et al. (1982). RNA was immobilized on the paper by baking at 80 °C under vacuum for 2 h. Dot blot and Northern blot filters containing RNA samples were prehybridized at 42 °C in 50% (v/v) deionized formamide, 1% SDS, 1 m-NaCl, and 10% (w/v) dextran sulphate. The 1-4 kb HindIII–SalI fragment containing the entire vgb gene was nick-translated using [α-32P]dATP (Maniatis et al., 1982), denatured by boiling, and added along with denatured tRNA (10 μg ml-1) to the prehybridization buffer; hybridization was carried out for 16 h at 42 °C. Filters were washed for 15 min in 2 × SSC containing 1% SDS (1× SSC is 0-15 m-NaCl, 0-015 m-sodium citrate, pH 7-0), twice at room temperature then twice at 42 °C and subjected to autoradiography.

Haem and NADH-VtmetHb reductase determinations. Haem b content was determined using the method described previously (Boerman & Webster, 1982). Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. The NADH-metHb reductase assay was performed as described previously (Gonzales-Prevatt & Webster, 1980) using VtmetHb as acceptor. All determinations were done in duplicate or triplicate and each growth curve, etc, was run at least twice. Representative results are shown.

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase and DNA polymerase were obtained from BRL and Promega Biotech and used as instructed by the supplier. RQ1 DNAase was from Promega Biotech. For labelling DNA, [α-32P]dATP obtained from ICN Radiochemicals was used. Omnifluor and [2,3-14C]succinate

were obtained from New England Nuclear. Glycine, Tris, ATP, coenzyme A, pyridoxal phosphate, EDTA, succinic thiokinase and lysozyme were all obtained from Sigma. Triton X-100 was obtained from Research Products, Dowex 50W-X4 from J. T. Baker, and Coomassie (Brilliant) Blue R-250 from Bio-Rad. The peptone, Hy Case SF was from Sheffield Chemical, and the yeast extract was purchased from Fidco.

RESULTS
Transcription of globin mRNA under hypoxic conditions

The vgb gene is present in a single copy on the Vitreoscilla genome and the RNA transcript is about 500 bases long (Dikshit & Webster, 1988). The effect of hypoxic growth conditions on the amount of this transcript in Vitreoscilla cells was tested by growing cells in 20% and 5% oxygen, isolating total cellular RNAs, and hybridizing them to a labelled DNA probe prepared from the 1-4 kb restriction fragment containing the vgb gene (Dikshit & Webster, 1988). Dot blots of total
RNA isolated from cells grown under 5% oxygen and harvested in the late exponential phase showed substantially more globin transcript than those from cells grown under 20% oxygen (Fig. 1a). This increased amount of specific transcript in cells grown under relative hypoxic conditions was present at all stages of the growth cycle (Fig. 1b). When the radioactivities of the Northern blots in Fig. 1(b) were measured with a scintillation counter, the maximal ratio of radioactivity of the RNA from cells grown in 5% oxygen to that from cells grown in 20% oxygen was 5.9 and occurred at 10 h.

**NADH-VtmetHb reductase activity in cells containing different levels of VtHb**

The increase of cellular haemoglobin and NADH-VtmetHb reductase during the growth cycle of *Vitreoscilla* under standard conditions is shown in Fig. 2. The major increase of cellular
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haemoglobin and the parallel increase in cellular NADH-VtmetHb reductase occurred when the DO fell to about 10% of atmospheric (i.e. about 24 µM), which results when the aeration rate cannot keep up with the steadily increasing oxygen consumption due to cellular respiration. The time of the fall of the DO depends on experimental conditions, including shaking rate, medium concentration, and inoculum size (Boerman & Webster, 1982). The cellular content of haemoglobin in Vitreoscilla can also be experimentally varied simply by changing shaking rate and medium concentration (Boerman & Webster, 1982). Cells were grown in media varied from 0.25%–2.0% (1.0% being standard) at shaking rates fixed at 50–250 r.p.m. and the cells assayed for both VtHb and NADH-VtmetHb reductase. Increases in VtHb were generally paralleled by increases in the reductase; the VtHb : reductase ratio at different shaking rates and media concentrations varied only between about 1 and 3.

ALAS activity under different conditions of oxygenation

The assay for ALAS required both succinic thiolokinase and EDTA for maximal activity (Table 1). EDTA inhibits ALA dehydratase, the next enzyme in haem biosynthesis (Granick &
Mauzerall, 1958). The activity of this enzyme would result in an apparent decrease in ALA formed, by condensing two molecules of ALA to form porphobilinogen. The effects of succinic thiokinase and EDTA were additive, and both were included in our standard assay for ALAS. There was no significant difference in the ALAS activity in crude extracts prepared by sonication alone or by digestion with lysozyme (Table 1), but the lysozyme method was more suitable for preparing larger numbers of extracts at the same time and was used throughout the course of this work. The ALAS assay was linear up to 0.3 ml of cell extract.

In one set of experiments performed to investigate the effects of oxygen on haem biosynthesis in *Vitreoscilla*, the effect of different aeration rates [0.2, 0.4 and 1.0 l air min⁻¹ (l culture medium)⁻¹] on ALAS and the haem content of the cells was examined. An example of the results from this type of experiment is shown in Fig. 3(a). The DO had fallen below 10% of atmospheric at around 16 h for all three aeration rates, and the ALAS activity was at or near maximal, about 0.3 units (mg protein)⁻¹, for all three. It continued to increase slightly at the two lower aeration rates. Maximum biomass (7–7.5 g l⁻¹) was attained at around 24 h for high aeration rate, 48 h for medium, and roughly 72 h for low (Fig. 3b). Likewise, maximal haem content attained was roughly the same for all three aerations, 40–60 nmol (g wet weight)⁻¹, but also occurred earlier at
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Table 2. Cellular content of haem and ALAS in Vitreoscilla grown at controlled DO

<table>
<thead>
<tr>
<th>Controlled DO (% of atmospheric)</th>
<th>Time (h)</th>
<th>Haem content (nmol g⁻¹)</th>
<th>ALAS activity (nmol h⁻¹ mg⁻¹)</th>
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<td>75</td>
<td>20</td>
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<td>11</td>
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<td>10</td>
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<td>53</td>
<td>0.075</td>
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higher aeration rates (Fig. 3b). To summarize, the higher aeration rates produced higher initial cell growth and earlier attainment of maximum cell yield, but they also led to earlier attainment of stationary phase and death phase.

In another type of experiment, summarized in Table 2, the DO was decreased in a stepwise fashion from 75% to a final 10% of atmospheric. The DO controller varied aeration and/or agitation rates to maintain the DO at the selected level. The ALAS activity of the cells was barely detectable (0.016 units mg⁻¹) after growth at a DO of 75% of atmospheric but increased after each stepwise reduction in the DO, reaching a maximal value after 12 h at a DO of 10% of atmospheric and declining thereafter. The haem content similarly was very low in cells grown at a DO of 75% of atmospheric and increased after each reduction of the DO, reaching its maximal rate of increase at a DO of 10% of atmospheric and its maximal value after around 30 h at this DO. The initial increase in ALAS between 40 and 52 h of culture and the continued increase in haem content between 40 and 70 h occurred even though the cells had reached stationary phase at this time.

DISCUSSION

The availability of the cloned vgb gene made it possible to study certain aspects of the regulation of the biosynthesis of VtHb in Vitreoscilla. Specifically, the transcription data (Fig. 1) show that the amount of VtHb present in Vitreoscilla growing at different concentrations of oxygen is regulated at the level of transcription and that the level of vgb specific mRNA is elevated at low concentrations of oxygen. Although the amount of vgb message does increase during the growth cycle (Fig. 1b), the amount of specific transcript was always higher in cells grown under hypoxic conditions at all stages of the growth cycle. Since it is known that the DO falls during the growth cycle (Boerman & Webster, 1982) it is likely that it is the decreased oxygen concentration that is primarily, if not entirely, the cause of increased vgb transcription.

Another protein that is synthesized in response to hypoxia is erythropoietin, the hormone that stimulates red blood cell production. The expression of erythropoietin mRNA is markedly increased by hypoxia, and there is evidence that in this mammalian system the oxygen sensor is a haem protein (Goldberg et al., 1988). If the oxygen sensor in Vitreoscilla is a haem protein, VtHb itself is a likely candidate: it binds oxygen co-operatively, which leads one to speculate that in the deoxy conformation, for example, it could stimulate its own production but be inactive in the oxy conformation. Whatever the sensor molecule we still know nothing at present about the number of steps that intervene between it and the direct control of transcription of vgb.

VtHb is more autoxidizable than other haemoglobins and myoglobins, which may be due to a more open haem pocket. This is supported by infra-red spectroscopic studies which indicated that the oxygen is more loosely bound (Choc et al., 1982) and the fact that it has a very high off rate constant (5.6 x 10⁻³ s⁻¹) (Orii & Webster, 1986). The cellular concentration of NADH-VtmetHb reductase, which is necessary to keep the haem prosthetic group in the functional ferrous state, increased during the growth cycle, parallel to the increase in VtHb that
accompanied the falling level of DO (Fig. 2). Under a variety of growth conditions with varying oxygenations the ratio of haemoglobin to reductase remained fairly constant, but not constant enough to be able to state that regulation of the two proteins is tightly co-ordinated. The regulation of the reductase by oxygen may be by a different mechanism than that which regulates VtHb.

The synthesis of ALAS also increased at low oxygen levels, reaching maximal values when the DO fell to about 10% of atmospheric, and this was followed by a rise in haem content. There was generally a considerable lag between the derepression of ALAS synthesis and haem biosynthesis, perhaps because of the relatively low ALAS activity in *Vitreoscilla* (see Table 2).

Haem content only increased substantially when the DO was decreased to 10% of atmospheric even though ALAS activity had increased more than tenfold earlier (at a DO of 25% of atmospheric). The relatively low haem content of cells grown at DO values of 25% and 50% of atmospheric may be due to an inhibition of the expression of ALAS activity in vivo, an inhibition of some other step(s) in the haem biosynthetic pathway, or a high haem degradation rate. Free-living (laboratory cultured) cells of *Bradyrhizobium japonicum* grown under hypoxic conditions also exhibited a tenfold increase in cellular haem content, and a similar increase in the activities of the first two enzymes of haem biosynthesis, ALAS and ALA dehydratase (EC 4.2.1.24) (Avissar & Nadler, 1978). It was proposed that decreased oxygen tension may also play a role in inducing syntheses necessary for leghaemoglobin formation and bacterial differentiation in soybean root nodules. Di- and trisulphides can convert inactive ALAS to the active form in *Rhodopseudomonas* (Tuboi & Hayasaka, 1972; Sandy *et al.*, 1975), and it was postulated that oxygen inhibits conversion of the enzyme to the active form by decreasing cellular trisulphide. Cystathionase and a regulator-activator protein have also been implicated in this conversion (Oyama & Tuboi, 1979). Whether *Vitreoscilla* contains active and inactive forms of ALAS is not known but this would not explain the low haem production in cells grown at DO values of 25% and 50% of atmospheric, which had relatively high ALAS activity (Table 2) unless inactive enzyme was somehow converted to active enzyme in our in vitro assay.

When *vgb* is cloned in *E. coli* it is transcribed under the control of its own promoter and is highly expressed, up to seven times the maximum level observed in *Vitreoscilla* (Dikshit & Webster, 1988; Khosla & Bailey, 1988a). Since the haem for this is furnished by the host there must be derepression of the haem biosynthetic pathway in *E. coli* under these conditions, but it is not known how this is regulated. Since the haemoglobin is in the physiologically active ferrous form (Dikshit & Webster, 1988) a protein with VtmtHb reductase activity must be present in *E. coli*. It has been suggested that VtHb functions as an oxygen storage-trap to supply oxygen to the terminal oxidases under hypoxic conditions (Wakabayashi *et al.*, 1986). Recombinant *E. coli* cells containing *vgb* grew faster and to greater cell densities than cells containing a comparable plasmid, and the VtHb increased the rate of oxygen use when DO was less than 5% of atmospheric (Khosla & Bailey, 1988b).

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