Redox-mediated interactions of Vhb (Vitreoscilla haemoglobin) with OxyR: novel regulation of Vhb biosynthesis under oxidative stress

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The bacterial haemoglobin from Vitreoscilla, Vhb, displays several unusual properties that are unique among the globin family. When the gene encoding Vhb, vgb, is expressed from its natural promoter in either Vitreoscilla or Escherichia coli, the level of Vhb increases more than 50-fold under hypoxic conditions and decreases significantly during oxidative stress, suggesting similar functioning of the vgb promoter in both organisms. In this present study we show that expression of Vhb in E. coli induced the antioxidant genes katG (catalase–peroxidase G) and sodA (superoxide dismutase A) and conferred significant protection from oxidative stress. In contrast, when vgb was expressed in an oxyR mutant of E. coli, Vhb levels increased and the strain showed high sensitivity to oxidative stress without induction of antioxidant genes; this indicates the involvement of the oxidative stress regulator OxyR in mediating the protective effect of Vhb under oxidative stress. A putative OxyR-binding site was identified within the vgb promoter and a gel-shift assay confirmed its interaction with oxidized OxyR, an interaction which was disrupted by the reduced form of the transcriptional activator Fnr (fumurate and nitrate reductase). This suggested that the redox state of OxyR and Fnr modulates their interaction with the vgb promoter. Vhb associated with reduced OxyR in two-hybrid screen experiments and in vitro, converting it into an oxidized state in the presence of NADH, a condition where Vhb is known to generate H2O2. These observations unveil a novel mechanism by which Vhb may transmit signals to OxyR to autoregulate its own biosynthesis, simultaneously activating oxidative stress functions. The activation of OxyR via Vhb, reported in the present paper for the first time, suggests the involvement of Vhb in transcriptional control of many other genes as well.

Key words: bacterial haemoglobin, oxidative stress, OxyR, protein–protein interaction, Vitreoscilla haemoglobin (Vhb), Vitreoscilla.

INTRODUCTION

Haemoglobins present within the microbial world have challenged the common perception that haemoglobins and myoglobins are mainly tailored for oxygen storage and transfer functions. In the last decade a great deal of structural and functional diversity among microbial haemoglobins has been discovered [1–3]. The haemoglobin from the bacterium Vitreoscilla [4], Vhb, the first haemoglobin discovered in a prokaryote, displays a classic globin fold but has unusual structures in both proximal and distal haem pockets [5,6]. It has been proposed that its unique structural organization and ability to remain in different conformational states may allow it to perform multiple functions. Of the currently known globins, Vhb is especially useful for engineering the energy metabolism of various heterologous hosts [7–9] and represents a versatile tool for a variety of biotechnological applications [2,9]. Proteomic and microarray analyses of recombinant Escherichia coli expressing Vhb have indicated that the presence of Vhb in E. coli significantly affects the expression of hundreds of genes, including up- and down-regulation of genes involved in energy metabolism, central intermediary metabolism and cellular processes [10,11]. However, the molecular mechanism by which Vhb is able to exert such diverse effects on its host metabolism is currently unknown.

Vhb exists predominantly as a homodimer [5,12] and binds oxygen reversibly with an oxygen association rate constant similar to eukaryotic haemoglobins; its rate of oxygen dissociation, however, is unusually high [12,13]. Under hypoxic conditions the level of Vhb increases more than 50-fold in Vitreoscilla and in recombinant E. coli expressing Vhb under control of its native promoter [14,15]. This led to the proposal that one of its functions is to facilitate oxygen flux to the respiratory apparatus of its host under oxygen-limiting conditions [12,15,16]. This is supported by the observations that Vhb remains localized and concentrated near the periphery of the cytosolic face of the cell membrane in both Vitreoscilla and E. coli [17], improves the oxygen uptake of its host [18] and specifically interacts with cytochrome o [17,19] and phospholipids of cell membranes [20].

Vhb displays structural characteristics for lipid attachment that are similar to flavohaemoglobins and it exhibits a relatively high propensity towards phospholipid binding in vitro [20]. It has been noted that when Vhb binds lipids there in a several fold decrease in its oxygen affinity, and a commitment increase in oxygen release, compared with lipid-free Vhb [20]; this might allow Vhb to deliver oxygen directly to the respiratory apparatus of cells for efficient utilization. These lipid-binding effects suggest that interaction of Vhb with other molecules may mediate Vhb function in diverse ways. Correspondingly, Vhb has been shown to stimulate oxygenase activity [21–23], serve as an alternate

Abbreviations used: ArcA, aerobic respiration control A; Cpr, catabolic repressor protein; Fnr, fumurate and nitrate reductase; Kat, catalase–peroxidase; LB, Luria–Bertani; metVHb, Vitreoscilla methaemoglobin; ROS, reactive oxygen species; RT, reverse transcription; Sod, superoxide dismutase; vgb, gene encoding Vitreoscilla haemoglobin; Vhb, Vitreoscilla haemoglobin.

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terminal oxidase [24], modulate the redox status of cells [25] and be involved in nitric oxide detoxification [26,27] and protection from oxidative stress [28,29].

Regardless of how many processes VHb may affect, its expression probably increases the amount of oxygen within the cell. This is presumably related to its interaction with the terminal respiratory oxidase [17,24] and may explain the improved growth and bioproduticity of a variety of heterologous hosts engineered to express VHb [7,9]. High oxygen concentrations in a cell are known to result in generation of ROS (reactive oxygen species) and potentially lethal damage to membranes and DNA [30,31]. On the other hand, in several cases the presence of VHb provides the ability to cope better with oxidative stress by altering the status of antioxidant enzymes. For example, VHb-expressing Enterobacter aeruginosa cells have been found to have elevated levels of catalase activity and become more tolerant to oxidative stress [28]. VHb-bearing Streptomyces lividens cells exhibit significant up-regulation of katA (catalase–peroxidase A) and sodF (superoxide dismutase F), two antioxidant enzymes [32], and VHb expressed in the plant Arabidopsis endogenes has been correlated with higher levels of antioxidants, such as ascorbate, and higher tolerance to photodamaging damage [33].

The involvement of VHb in the antioxidant systems of heterologous cells might be a common phenomenon that can be correlated with the observation that the biosynthesis of catalase in Vitreoscilla increases when there is an increase in VHb level [34]. How VHb maintains the fine balance between sustaining oxidative stress management using antioxidant enzymes. For example, VHb-expressing Enterobacter aeruginosa cells have been found to have elevated levels of catalase activity and become more tolerant to oxidative stress [28]. VHb-bearing Streptomyces lividens cells exhibit significant up-regulation of katA (catalase–peroxidase A) and sodF (superoxide dismutase F), two antioxidant enzymes [32], and VHb expressed in the plant Arabidopsis endogenes has been correlated with higher levels of antioxidants, such as ascorbate, and higher tolerance to photodamaging damage [33].

The experiment of VHb in the antioxidant systems of heterologous host cells. Studies on VHb in its native host, Vitreoscilla, have been hampered due to the unavailability of a suitable and stable gene transfer system. Although transformation of Vitreoscilla with E. coli plasmid vectors was successfully attempted [35], the recombinants were found to be unstable after subsequent transfer. Therefore the present study was undertaken to understand the molecular mechanism of VHb function(s) in the context of oxidative stress management using E. coli as a model system. As vgb (the gene encoding VHb) is expressed efficiently in E. coli, through its natural promoter, and the transcriptional response of the vgb promoter to oxygen availability in E. coli and Vitreoscilla is similar [14,15], an insight into the function of VHb in E. coli can be extended to its native host Vitreoscilla and possibly to other heterologous hosts as well.

EXPERIMENTAL

Bacterial strains, plasmids and culture conditions

The Vitreoscilla C1 strain, described previously [4,14], was utilized for monitoring the effect of oxidative stress on vgb transcription and VHb biosynthesis in its native host. E. coli strains JM109 and BL21 DE3 were used for routine cloning and expression of recombinant proteins. E. coli AB1157 and its sodA-negative derivative PN132 [36] were a gift from Professor James Imlay (School of Molecular and Cellular Biology, University of Illinois at Urbana Champaign, IL, U.S.A) and E. coli NC4963 and its oxyR-negative strain NC4112 were a gift from Professor Herb Schellhorn (Department of Biology, McMaster University, Hamilton, Ontario, Canada); the strain NC4112 displays enhanced sensitivity towards H₂O₂ and other oxidants [37]. The recombinant plasmid pUC8:16, was used as a source of VHb and has been described previously [38]. Details of the bacterial strains, oligonucleotide primers used for PCR amplification for wild-type and mutant genes and the oxyR antisense RNA sequence are provided in Supplementary Tables S1 and S2 (available at http://www.BiochemJ.org/bj/426/bj4260271add.htm).

E. coli strains were grown in LB (Luria–Bertani) or Terrific broth at 37°C with shaking at 180 rev./min unless otherwise indicated. When required, ampicillin and kanamycin (Sigma–Aldrich) were added at concentrations of 100 μg/ml and 30 μg/ml respectively. Vitreoscilla strain C1 was grown in PYA medium (1% peptone, 1% yeast extract and 0.02% sodium acetate, pH 7.8) at 30°C with shaking at 180 rev./min [34]. The growth profiles of E. coli strains NC4936 and NC4112, carrying either pUC8:16 or pUC19 (New England Biolabs), and the control strains transformed with pUC8:16 or pUC19, were checked at sublethal concentrations of H₂O₂ by monitoring the attenuation at 600 nm and compared with growth without H₂O₂ addition.

RT (reverse transcription)–PCR and protein expression analysis

To monitor the level of vgb transcription under oxidative stress, 1% inocula from overnight cultures of Vitreoscilla and E. coli JM109 bearing plasmid pUC8:16 were added to 20 ml of LB broth and grown to a D₆₀₀ of 0.5. At this point, 1 mM H₂O₂ was added and cells were harvested 6 h and 12 h later for E. coli, or 10 h and 18 h later for Vitreoscilla, and used for RNA isolation [with an RNeasy Mini Kit (Qiagen) and RNase-free DNase I (Qiagen) according to the manufacturer’s instructions]. RT–PCR was performed with the One-Step RT–PCR Kit (Stratagene) using vgb-specific primers. RT–PCR of 16S rRNA served as the control for analysis of RT–PCR products.

VHb content was determined by SDS/PAGE (12% gels) of total cell lysates, followed by Western blotting using polyclonal anti-VHb antibodies [17] and densitometric analysis of the VHb band with Alphamager 3500 (Alpha Innotech), or by using CO-difference spectra of whole cells [38]. The fraction of total VHb that was bound to haem was determined by measuring the amount of VHb protein through densitometric analysis (which measures both holo and apo forms of VHb) and comparing it with the VHb content determined by CO-difference spectral analysis (which measures only the haem-bound form of VHb). These measurements indicated that 80–85% of VHb is associated with haem and thus may be in the functional state.

Cloning and expression of genes encoding OxyR, Fnr and their redox-sensitive mutants

oxyR and fnr were cloned from the genome of E. coli by PCR amplification using primers designed on the basis of their respective sequences. The OxyR-C199S mutant has been described previously and carries a serine residue at position 199 resulting in a mutant OxyR that remains trapped in a reduced form [39]. Fnr-D154A, reported previously [40], is a mutated form of Fnr where Asp-154 has been replaced by an alanine residue and remains oxidized. These two mutants were generated by site-directed mutagenesis using the PCR-based overlap extension method as described previously [41]. Amplified gene sequences were authenticated by nucleotide sequencing, cloned into expression vector pET28c (Novagen) and then expressed in E. coli strain BL21 DE3 as His₆-tagged proteins. Recombinant OxyR, Fnr and their mutants were isolated and purified from cell extracts using metal-affinity columns (Qiagen) according to the manufacturer’s instructions.
Gel-shift assays and competitive binding of transcriptional activators with vgb promoter

The 250 bp vgb promoter fragment (−150 to +100 of vgb) was end-labelled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs). The labelled promoter (3.8 pM) was then incubated with 0.2–0.5 μM purified wild-type or mutant OxyR protein at 20°C for 20 min in buffer A [25 mM Tris/HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2 and 5% (v/v) glycerol] containing 5 μg/ml herring sperm DNA. Unlabelled promoter was added at a concentration of 8 pM per assay when required for competition with the labelled vgb promoter. Samples were analysed for DNA–protein complex formation on TBE (Tris/borate/EDTA)-buffered polyacrylamide gels at 4°C. The VHb–OxyR complex, along with samples of VHb and oxidized OxyR (0.5 μM each) that had been pre-incubated in buffer A, was followed by incubation for an additional 10 min at 25°C. Conversely, increasing concentrations of OxyR (0–500 nM) were added to a pre-incubated mixture of labelled vgb promoter and Fnr (50 nM each) in buffer A followed by further incubation for 10 min at 25°C. Samples of each reaction mixture were electrophoresed on 6% polyacrylamide gels at 4°C; the gels were transferred onto to filter paper (3MM, Whatman) and dried for autoradiography.

For the competitive DNA-binding assay, increasing concentrations of Fnr (0–100 nM) were added to a mixture of labelled vgb promoter fragment and oxidized OxyR (0.5 μM each) that had been pre-incubated in buffer A; this was followed by incubation for an additional 10 min at 25°C. Conversely, increasing concentrations of OxyR (0–500 nM) were added to a pre-incubated mixture of labelled vgb promoter and Fnr (50 nM each) in buffer A followed by further incubation for 10 min at 25°C. Samples of each reaction mixture were electrophoresed on 6% polyacrylamide gels at 4°C; the gels were transferred onto to filter paper for autoradiography following standard protocols.

OxyR antisense constructs and strains

In order to substantiate the role of OxyR in the regulation of VHb production, oxyR was cloned in the antisense configuration, adjacent to vgb in pUC8:16, creating pUC8:16T. Both pUC8:16 and pUC8:16T were transformed into E. coli strains AB1157 and PNI132 (the latter is deficient in Sod function) to produce strains PNI132-(pUC8:16), PNI132-(pUC8:16T), AB1157-(pUC8:16) and AB1157-(pUC8:16T) (see Supplementary Table S1). The efficiency expression and function of the oxyR antisense sequence was indicated by the complete absence of catalase activity in both PNI132-(pUC8:16T) and AB1157-(pUC8:16T), in contrast with the presence of considerable catalase activity in the other four strains.

Yeast two-hybrid experiments

Yeast two-hybrid screen experiments were performed using the DupLex Yeast two-hybrid kit (Origene Technologies). Saccharomyces cerevisiae strain EGY48 was used as host for the transformation of bait, prey and reporter plasmids and vectors pEG202, pSH18-34 and pJG4-5 were used for cloning the relevant genes (see Supplementary Table S1). Plasmids pUC8:16 and pNKD1 [38] were used as sources of vgb. The E. coli strain JM109 was used as the host strain for all subcloning and maintenance of plasmids, and was the source of DNA for fir and oxyR. All clones were verified by restriction digestion and agarose gel electrophoresis, as well as by sequencing at the University of Illinois at Chicago Core Facility. All sequences were examined and found to be without error and in the correct reading frame. β-Galactosidase activity was used to quantify each interaction according to the method described previously [42].

rifampicin resistance assay and exposure to H2O2

rifampicin was dissoloved in 50% (v/v) ethanol by heating slightly in a microwave followed by vortexing. Cultures of PNI132, PNI132-(pUC8:16), PNI132-(pUC8:16T), AB1157, AB1157-(pUC8:16) and AB1157-(pUC8:16T) were grown by inoculating 5 ml of LB, or LB containing ampicillin for plasmid-bearing strains, with isolated colonies and incubating at 37°C with shaking at 220 rev./min for approx. 19 h, or until 10-fold dilutions yielded a Doo of 0.4 (such stationary-phase cultures of AB1157 and one of its Sod-deficient derivatives have been used successfully previously to monitor the sensitivity of these strains to ROS [43]).

RESULTS

Down-regulation of VHb biosynthesis in Vitreoscilla and E. coli under oxidative stress

Biosynthesis of VHb is regulated similarly, via an oxygen-sensitive promoter, at the transcriptional level in Vitreoscilla and E. coli [14]; 80–85% of VHb is associated with haem (as described in the Experimental section) and is therefore in a functional state. To explore the VHb biosynthesis response to oxidative stress, we first examined the content of VHb in Vitreoscilla and VHb-expressing E. coli in the presence of sublethal concentrations of H2O2. CO-difference spectra (results not shown) and densitometry of total protein profiles (Figures 1A

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and 1B) of VHb-expressing cells revealed a 40–50% reduction in VHb content in both Vitreoscilla and E. coli. RT-PCR analysis indicated that there was a further substantial reduction in the level of VHb transcripts in both Vitreoscilla and VHb-expressing E. coli (Figures 1C and 1D).

VHb relieves oxidative stress in an OxyR-dependent manner

We next undertook further studies to gain an insight into the precise mechanism of the VHb down-regulation under oxidative stress using E. coli as a model system due to the availability of well-defined mutant strains. Strains NC4936 (wild-type for oxyR) and NC4112 (oxyR-negative) were transformed either with pUC8:16, to enable vgb expression from its native promoter, or a control plasmid vector pUC19, which does not carry vgb. Growth of NC4936, either expressing or not expressing VHb, was tested in the presence of increasing concentrations of H₂O₂ (Figure 2A). Although VHb levels decreased under oxidative stress, NC4936 cells expressing VHb were able to grow better than non-VHb-expressing counterparts under these conditions, especially in the presence of high concentrations of H₂O₂ (Figures 2A and 2C). Interestingly, the VHb-expressing oxyR mutant (NC4112 VHb) displayed 2–3-fold higher VHb levels (Table 1) but significantly slower growth compared with its counterpart not expressing VHb (Figures 2B and 2C). These results suggest that the VHb-mediated protection from H₂O₂-induced oxidative stress relies on the presence of OxyR.

The involvement of OxyR with VHb with VHb-mediated protection against H₂O₂ was confirmed by the experiments shown in Figure 3(A). Cell survival after a 15 min exposure to H₂O₂ was greatly enhanced by the presence of VHb but the protective effect of VHb was eliminated by the additional presence of oxyR antisense RNA. The same effect was observed for Sod-positive (AB1157) and Sod-negative (PN132) strains, indicating that the protective effect of VHb is independent of Sod but not of OxyR. A further experiment was performed to assess the extent of damage caused by superoxide ions produced during normal aerobic growth in the various E. coli strains (Figure 3B). This is measured by assessing the rate of mutation to rifampicin resistance (i.e. the increase in the proportion of rifampicin-resistant cells) [36]. As expected, the rate is negligible in the wild-type strain (AB1157), but high in the SOD-deficient strain (PN132), where the presence of VHb has a large protective effect. This is probably due to a direct effect of VHb on superoxide degradation, independent of the VHb induction of sodA expression as described below.

OxyR controls VHb levels

Further investigation of the connection between VHb and OxyR included measurement of VHb levels in E. coli strains NC4963 (wild-type for oxyR) and NC4112 (oxyR-negative) transformed with vgb. In stationary-phase cells, the VHb level of NC4112 (vgb) was increased 2.2-fold compared with NC4963 (vgb). Similarly, stationary-phase cells of E. coli strain AB1157-Δ(pUC8:16T) (vgb plus oxyR antisense) had VHb levels approx. 4-fold that found in AB1157-Δ(pUC8:16) (Table 1). Subsequent re-examination of the upstream control region of vgb indicated a site with near-identity to the consensus OxyR-binding motif identified previously by Tartaglia et al. [45]. As shown in Figure 4(A), this putative OxyR-binding site overlaps the Fnr- Crp- (catabolic repressor protein) and ArcA (aerobic respiration control A)-binding sites identified previously within the vgb promoter [46–48]. The possible involvement of OxyR in regulation of VHb production was then assessed via a gel-shift assay, which provided direct evidence that OxyR, but not a non-specific E. coli protein mixture, binds the vgb promoter (Figure 4B).

Binding of OxyR with the vgb promoter is modulated by its redox state

It is known that OxyR is activated by the formation of an intramolecular disulfide bond and activity of OxyR in vivo is determined by the balance between oxidative stress generated by cellular oxidants and the redox environment [39]. The presence of large amounts of VHb in a cell is expected to result in significant changes in the redox state of cells under specific environmental conditions. Therefore we determined whether the interaction of OxyR with the vgb promoter is modulated by its redox state. To test this possibility we utilized the gel-shift assay with

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**Table 1** Effect of oxyR absence on the cellular level of VHb in E. coli

<table>
<thead>
<tr>
<th>Strain used</th>
<th>Cellular level of VHb</th>
<th>Strain used</th>
<th>Cellular level of VHb</th>
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<td>Effect of oxyR antisense RNA</td>
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<td>Effect of oxyR deletion</td>
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<td>E. coli AB1157 (wild-type)</td>
<td>VHb level (nmol per g (wet weight) of cells)</td>
<td>Relative VHb expression value (%)</td>
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<td>E. coli NC436 (oxyR wild-type)</td>
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<td>E. coli AB1157 (pUC8:16T)</td>
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<td>E. coli NC436 (pUC8:16)</td>
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<tr>
<td>E. coli NC4112 (ΔoxyR)</td>
<td>774 ± 217</td>
<td>E. coli NC4112 (control)</td>
<td>n/a</td>
</tr>
</tbody>
</table>

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Regulation of VHb biosynthesis by OxyR

Figure 2 Growth profiles and VHb contents of *E. coli* wild-type and OxyR mutant under oxidative stress with or without VHb expression

(A) Growth trajectories of OxyR wild-type *E. coli* NC4936 transformed with either control (pUC19) plasmid or the VHb-expressing plasmid (VHb) in the presence of 0 mM, 1 mM or 3 mM H₂O₂.

(B) Growth trajectories of OxyR-mutant *E. coli* NC4112 carrying the VHb-expressing pUC8:16 plasmid (VHb) or the non-expressing control pUC19 plasmid in the presence of 0 mM, 1 mM or 2 mM H₂O₂.

(C) Western blot analysis of VHb content in strains NC4112 (ΔOxyR) and NC4936 (WT) bearing the VHb-expressing pUC8:16 and grown in the presence of 1 mM H₂O₂.

Figure 3 Effect of VHb on survival of Sod-mutant *E. coli* under oxidative stress

(A) Wild-type *E. coli* AB1157 (AB) and its Sod-mutant derivative PN132 (PN) were exposed to 5 mM H₂O₂ for 15 min as described in the Experimental section. Survival of cells was determined by counting colony-forming units. (B) Frequency of mutation to rifampicin resistance in strains AB1157 (AB) and PN132 (PN). Cells were plated on LB medium containing 100 μg/ml rifampicin in order to count the number of rifampicin-resistant cells and LB medium (plus ampicillin for plasmid bearing strains) to measure total number of cells. pUC8:16, VHb-expressing plasmid. All values are means ± S.D. for three independent experiments.

Figure 4 OxyR function at the vgb promoter

(A) The transcriptional control region of the vgb promoter showing the position of the putative OxyR-binding region (indicated by asterisks beneath the sequence). Bold and italicized bases in this region indicate the positions of the OxyR-binding site consensus identified by Tartaglia et al. [45]. All except the most left-hand C (which is G in the consensus) are identical to the consensus in both identity and position (and there is a G immediately adjacent to the unconserved C). Other motifs, which are putative binding sequences for other regulators are underlined and identified beneath the sequence. (B) Binding of OxyR to the vgb promoter region as determined by gel-shift assay. Lane 1, 3.8 pM ³²P-labelled vgb promoter; lane 2, labelled promoter and 0.2 μM OxyR; lane 3, labelled promoter and 0.2 μM OxyR; lane 4, labelled promoter and 0.3 μM OxyR; lane 5, labelled promoter and 0.4 μM OxyR; lane 6, labelled promoter and 0.5 μM OxyR; lane 7, labelled promoter, 0.5 μM OxyR and 6 μM unlabelled promoter; and lane 8, labelled promoter and 5 μg of non-specific protein mixture.
wild-type OxyR and an OxyR mutant (OxyR-C199S) that does not reversibly form the disulfide bond and thus cannot be activated into the oxidized state. Unlike wild-type OxyR, OxyR-C199S was unable to interact with the vgb promoter (Figures 5A and 5B).

**VHb interacts directly with OxyR and modifies its redox state**

It has been shown previously that VHb is relatively more auto-oxidizable than eukaryotic haemoglobins and the products of its auto-oxidation are metVHb (Vitreoscilla methaemoglobin) and H₂O₂ [49]. Therefore it is possible that VHb itself may transmit signals for OxyR activation. To explore this possibility, we tested whether VHb was able to interact directly with OxyR. Initial experiments using a yeast two-hybrid assay showed a strong interaction between VHb and OxyR (Figure 6A). In order to describe the VHb–OxyR interaction in more detail we next performed an experiment where VHb was allowed to interact with fully oxidized OxyR prepared after H₂O₂ exposure and OxyR-C199S, which exists only in the reduced state. Protein–protein interactions of these proteins in equimolar concentrations (5 μM) were analysed by native PAGE followed by Western blotting using anti-VHb antibodies (Figure 6B). A distinct complex of VHb with OxyR-C199S, which exists only in the reduced form, was detected with the reduced state OxyR-C199S but not with the fully oxidized form of OxyR.

The role of VHb in altering the redox state of OxyR was examined further by studying the redox status of OxyR in the presence of VHb and H₂O₂. The fully reduced form of a His₆-tagged wild-type OxyR was incubated with VHb and NADH, separately or in combination, or with H₂O₂, and conversion from reduced into oxidized OxyR was monitored by non-reducing SDS/PAGE after Western blotting using anti-His₆ polyclonal antibodies (as it has been described previously [50]) (Figure 6C). In the presence of H₂O₂, OxyR was rapidly converted into the oxidized state. A similar change in the redox state of OxyR was observed in the presence of VHb and NADH (the condition that generates H₂O₂), whereas no change in OxyR was observed when it was allowed to interact with either VHb or NADH alone. This change in the redox state of OxyR mediated by VHb and NADH was prevented in the presence of catalase, suggesting that the superoxide ion released by the oxidase activity of VHb may be responsible for OxyR oxidation.

**Fnr interrupts interaction of OxyR with the vgb promoter**

As VHb biosynthesis is positively regulated via Fnr and the vgb promoter carries an Fnr-binding site [46,48], which overlaps the putative OxyR site, we tested whether OxyR competes with Fnr for promoter binding. When Fnr and OxyR were allowed to interact individually with the vgb promoter, gel-shift assays demonstrated specific binding of both transcriptional regulators (Figures 5C and 5D). A competitive binding assay was then used to assess the interactions of these two transcriptional regulators at the vgb promoter. When the vgb promoter was allowed to interact initially with OxyR followed by addition of Fnr, gel-shift analysis indicated gradual displacement of OxyR with increasing concentration of Fnr. In contrast, when the vgb promoter was allowed to interact initially with Fnr, followed by addition of 200 nM to 5 μM OxyR, no displacement of Fnr was observed. These results suggested that OxyR interacts with the vgb promoter only when Fnr is not operative.

**VHb expression induces transcription of genes encoding antioxidant enzymes**

As VHb interacts with reduced OxyR and converts it into the oxidized state, which is known to positively regulate the expression of many genes involved in the oxidative stress response [51], we checked the status of several genes that encode antioxidant enzymes (katE, katG and sodA) in E. coli over-expressing VHb. Transcript analysis of VHb-expressing cells revealed distinct increases in the expression of katG and sodA.
Regulation of VHb biosynthesis by OxyR

Figure 6 Protein–protein interactions between VHb and OxyR

(A) Two-hybrid screen showing binding between VHb and wild-type E. coli OxyR. Values are means ± S.D. for four independent measurements. Cells bearing the prey vector with a vgb insert and bait vector with no insert (Control) were used to check that VHb alone does not result in activation; they have no detectable β-galactosidase activity. (B) VHb associates with reduced OxyR (OxyR-C199S) but not oxidized wild-type OxyR. Lane 1, purified VHb; lane 2, VHb and wild-type OxyR (oxidized with 0.5 μM H₂O₂); lane 3, purified OxyR; lane 4, VHb and reduced OxyR (OxyR-C199S). The samples were subjected to native PAGE and anti-VHb antibodies were used to detect the interaction. (C) VHb-mediated redox change in OxyR. Either reduced or oxidized OxyR wild-type protein (at 5 μM) was incubated with or without VHb (5 μM), NADH (100 μM) and catalase (328 units) as indicated. The samples were subjected to native PAGE and anti-His6 antibodies were used to detect the change in redox state.

compared with control cells (Figure 7A). In addition, when lysates of control and VHb-expressing cells were analysed on gels using zymography, a catalase-specific band appeared in cells expressing VHb but not in control cells (Figure 7B). These results substantiate further that there is an increase in the antioxidant activities of VHb-expressing cells.

DISCUSSION

ROS are generated primarily as by-products of intense respiratory activities and aerobic metabolism of cells. VHb-expressing bacteria display enhanced oxygen uptake and have a more oxidized cytoplasm than their non-VHb carrying counterparts [18,25]. In addition, VHb is more auto-oxidizable than eukaryotic haemoglobins and the products of this auto-oxidation are metVHb and H₂O₂ [49]. Nevertheless, VHb has been found to provide protection from oxidative stress in several cases, although the mechanism by which it is able to do so has until now been unknown [28,29]. The present study has uncovered key aspects of this mechanism, especially the interaction between VHb and the oxidative stress regulator OxyR, an apparently interconnected regulatory mechanism which involves regulation of VHb biosynthesis and a more general induction of the host cell protective response to the toxicity of ROS.

OxyR is known to be both a positive and negative regulator of transcription, with the potential for acting positively only when oxidized but able to act negatively in either the oxidized or reduced form [51,52]. The levels of both VHb and vgb transcription are reduced under oxidative stress in both Vitreoscilla and E. coli. In the present study we observed distinct increases in VHb content in OxyR-mutant E. coli (2–3-fold), or in wild-type E. coli expressing oxyR antisense RNA (4-fold), suggesting that OxyR is acting as a negative regulator of vgb transcription. The vgb promoter carries binding sites for the global transcription regulators Fnr, ArcA and Crp, which have been shown to regulate vgb transcription in response to oxygen availability; Fnr, in conjunction with Crp,

Figure 7 Transcription levels of antioxidant genes in E. coli expressing VHb

(A) Transcript level of antioxidant genes in VHb-expressing cells. Levels of genes encoding antioxidant functions in VHb-expressing E. coli (katE, katG and sodA) were determined by RT–PCR after exposure to 2 mM H₂O₂ as described in the Experimental section. The 16S rRNA transcript was used as a control. (B) Zymographic analysis of catalase activity in VHb-expressing E. coli. Control and VHb-expressing E. coli cells were analysed through zymography on native PAGE as described in the Experimental section.
A model for the interactions between VHb and OxyR and their possible functions

The model indicates protein–protein interactions between VHb and OxyR and DNA–protein interactions between the vgb promoter and OxyR and Fnr, and how these interactions might be related to the control of VHb biosynthesis and the involvement of VHb in the response to oxidative stress. See the Discussion section for further details.

Figure 8 A model for the interactions between VHb and OxyR and their possible functions

has been found to up-regulate transcription of VHb several fold under hypoxia [48]. The putative OxyR-binding site within the vgb promoter overlaps the Fnr-, ArcA- and Cpr-binding sites, suggesting that multiple circuits may work to exert fine-level control of vgb expression in response to different oxygen levels and environmental stimuli.

Aerobic metabolism under high aeration may result in significant superoxide stress and is known to activate OxyR, whereas the same conditions inactivate Fnr. Gel-shift assays indicate that OxyR binds with the vgb promoter only in its oxidized form and in the absence of Fnr. In the presence of higher VHb levels reactive oxygen within the cell may increase due to higher oxygen levels and respiratory activities and/or the redox status of cell may change. This, in turn, may work to disrupt the association of Fnr with the vgb promoter and activate OxyR to alleviate the situation by down-regulating VHb biosynthesis. The 2–4-fold negative control exerted by OxyR on VHb production could serve to allow VHb to reach a level that can provide oxygen availability for maximum benefit without exerting stress on cells owing to production of H$_2$O$_2$ (see below) or diversion of cell resources to produce an unnecessary VHb excess. OxyR, however, does not appear to down-regulate vgb by dislodging Fnr from the vgb promoter, although Fnr appears to dislodge OxyR, presumably when VHb levels need to be increased. This type of regulatory combination of OxyR and Fnr has also been observed in the case of two E. coli superoxide stress responsive genes, yhiA and katG [53,54].

Despite having a relatively low VHb content under conditions of oxidative stress, VHb-expressing cells displayed significant protection from H$_2$O$_2$, demonstrated by enhancement of both growth rates (Figures 2A and 2B) and survival (Figure 3A). The dependence of VHb on OxyR for relieving oxidative stress and the induction of the antioxidant system in the presence of VHb [32,33] suggest a close correlation between VHb and OxyR in controlling this function. VHb associates only with the reduced state of OxyR and is able to convert it into oxidized OxyR in vitro, but only in the presence of NADH, conditions under which VHb has been shown to generate H$_2$O$_2$ [48]. VHb may then regulate the redox state of OxyR directly by transmitting the H$_2$O$_2$ stress signal. This hypothesis is also supported by our observation of the stimulation by VHb of transcription of E. coli katG, which is known to be induced by H$_2$O$_2$-activated OxyR [54].

The protective effect of VHb found in several heterologous hosts, where VHb is not regulated under its native promoter, might also be due to activation of OxyR by VHb and subsequent induction of the antioxidant genes. Indeed, it has been demonstrated that the expression of VHb activates antioxidant systems in various heterologous hosts, e.g. the kat and sod genes in E. aerogenes [29] and S. lividens [32], and the level of ascorbate, a component of the antioxidant system, in Aradiopsis [33].
mechanism may also be involved in the parallel increases in catalase and VHb, which occur in *Vitreoscilla* [34].

Up-regulation of sodA transcription in VHb-expressing cells indicates that, in addition to its involvement in the response to H$_2$O$_2$, VHb may aid in protection from the superoxide ion. It has been reported that the sodRS regulon, to which sodA belongs, is also triggered by H$_2$O$_2$ [55]. In addition, the results of the experiment in Figure 3B, which was performed in a Sod-mutant strain, indicate VHb has a direct role in protection against superoxide. One possible mechanism for this effect is the reaction of metVHb with superoxide to yield oxygenated Fe$^{2+}$-VHb [56]. The apparent ability of VHb to affect the activities of both the sodRS and oxyR regulons may indicate a broader ability of VHb to affect, either directly or indirectly, the activities of other transcription factors, as is also suggested by the changes in expression of a large number of genes during VHb overexpression in different heterologous hosts reported previously [10,11].

**Implications of OxyR regulation of VHb biosynthesis: a proposed model**

OxyR is known to process different redox-related signals into distinct transcriptional processes [39]. vgb has an oxygen-responsive promoter that is ‘crowded’ with binding sites for the redox-sensitive transcriptional regulators Fnr, ArcA and Crp, and these sites overlap the OxyR-binding site. This indicates that regulation of vgb expression may occur in response to different redox signals, either independently or in coordination with each other. On the basis of results in the present study, we propose a mechanism for the regulation of VHb biosynthesis via OxyR (Figure 8). This mechanism may also provide an insight into the protective effect of VHb observed under oxidative stress in different hosts, as well as in *Vitreoscilla* itself.

Under hypoxic conditions transcription of vgb is up-regulated several fold by Fnr [46], presumably facilitating oxygen availability and enhancing respiratory activity. This, in turn, may generate elevated levels of superoxide due to the ability of VHb to produce H$_2$O$_2$ in the presence of NADH [48]. This may be detrimental to cells, particularly during externally imposed oxidative stress. Accumulation of VHb within the cell may also lead to close association of VHb with reduced OxyR so that the superoxide released by VHb is readily available to activate OxyR, in some way more efficiently or more sensitively than by direct oxidation of OxyR by H$_2$O$_2$. A subsequent conformational change due to the change in the redox state of OxyR may disrupt the OxyR–VHb association and allow OxyR binding to the vgb promoter. Simultaneously, the oxidizing conditions created due to the accumulation of VHb may disrupt the iron–sulfur cluster of Fnr and thus binding with the vgb promoter. This would help clear the region on the promoter to allow OxyR binding to down-regulate VHb production and thus reduce the level of VHb-generated superoxide. A redox change in OxyR mediated by VHb may also allow OxyR to activate its regulon and thus provide more widespread protection from the oxidative stress.

*Vitreoscilla* is an obligate aerobe and may require a sufficient amount of VHb to sustain its aerobic metabolism under the low oxygen conditions of its natural habitat [4,12]. The down-regulation of VHb biosynthesis by OxyR may optimize the beneficial effects of VHb by keeping its levels high enough to provide sufficient oxygen for the cell while acting to minimize the level of VHb-generated H$_2$O$_2$ due to aerobic metabolism. A role for the VHb–OxyR association in protecting the aerobic metabolism of its native host by inducing antioxidant genes may also be relevant in this respect. Our proposed model also explains the protective effect of VHb against oxidative damage observed in several heterologous hosts [28,32,33].

**AUTHOR CONTRIBUTION**

Arvind Anand and Brian Duk performed the experiments on protein–protein interactions, DNA–protein interactions and the genetic regulation studies of VHb by OxyR. Sandeep Singh contributed to the experiments on protein–protein interactions of VHb and OxyR spectral studies. Meltem Akbas performed the analysis of VHb cellular content in recombinant cells. Benjamin Stark, Dale Webster and Kanak Dikshit conceived the ideas, planned and designed experimental strategies, analysed the data and prepared the manuscript.

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**REFERENCES**


47 Webster, D. A. (1975) The formation of hydrogen peroxide during the oxidation of reduced nicotinamide adenine dinucleotide by cytochrome o from Vitreoscilla. J. Biol. Chem. 250, 4995–4998


54 Orli, Y. and Webster, D. A. (1977) Oxygenated cytochrome o (Vitreoscilla) formed by treating oxidized cytochrome with superoxide anion. Plant Cell Physiol. 18, 521–526
Redox-mediated interactions of VHb (Vitreoscilla haemoglobin) with OxyR: novel regulation of VHb biosynthesis under oxidative stress

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Table S1 Plasmids and strains used in the present work

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Description and source</th>
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<tr>
<td>EGY48</td>
<td>S. cerevisiae strain; Origene Technologies</td>
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<tr>
<td>pSH18-34</td>
<td>Origene Technologies</td>
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<tr>
<td>pEG202</td>
<td>Bait vector; Origene Technologies</td>
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<td>pJGA-5</td>
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<tr>
<td>AB1157</td>
<td>E. coli wild-type for Sod; [27]</td>
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<tr>
<td>AB1157-(pUC8:16)</td>
<td>AB1157 transformed with pUC8:16; present work</td>
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<tr>
<td>AB1157-(pUC8:16T)</td>
<td>AB1157 transformed with pUC8:16T; present work</td>
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<tr>
<td>PN132</td>
<td>Sod-negative derivative of AB1157; [27]</td>
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<tr>
<td>PN132-(pUC8:16)</td>
<td>PN132 transformed with pUC8:16; present work</td>
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<tr>
<td>PN132-(pUC8:16T)</td>
<td>PN132 transformed with pUC8:16T; present work</td>
</tr>
<tr>
<td>pUC8:16</td>
<td>vgb expression vector; [38]</td>
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<tr>
<td>pUC8:16T</td>
<td>pUC8:16 plus oxyR in the antisense orientation; present work</td>
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<td>NC4963</td>
<td>E. coli wild-type; [37]</td>
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<td>NC4112</td>
<td>oxyR-negative mutant of nc4963; [37]</td>
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Table S2 Primers used for cloning and mutation of E. coli Fnr and OxyR

<table>
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<th>Protein</th>
<th>Primers</th>
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<tr>
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<tr>
<td>Fnr-D154A mutant</td>
<td>5′-AATCAAGGCAGCTAGCAGCATGA-3′ and 5′-GATCCGCGGGACCTGTTGA-3′</td>
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<tr>
<td>E. coli OxyR</td>
<td>5′-GATACATATGATCCGTTTAGAGT-3′ and 5′-GACGGATCCCTAGCCGACAGGTTACGCGTAGACC-3′</td>
</tr>
<tr>
<td>OxyR-C199S mutant</td>
<td>5′-GATACATATGATCCGTTTAGAGT-3′ and 5′-GACGGATCCCTAGCCGACAGGTTACGCGTAGACC-3′</td>
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