Menadione knocks out *Vitreoscilla* haemoglobin (VHb): the current evidence for the role of VHb in recombinant *Escherichia coli*

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Genetically engineering the heterologous bacterial host with the gene (*vgb*) encoding *Vitreoscilla* haemoglobin (VHb) has been found to provide typical advantages in growth and production, and it has generally been assumed that VHb is responsible for this effect. Here, using matched strains of *Escherichia coli* that bear a recombinant R-amylase gene (MK57) or the R-amylase gene and *vgb* (MK79), we examined this assumption. Menadione, which is known to oxidize haem proteins, was tested over a range of concentrations for its effects on growth, R-amylase production, respiration and VHb function in MK57 and MK79. Active VHb accumulated, and VHb was oxidized to the inactive ferric form with the use of menadione at the concentrations of 0.5–10 mM; concentrations that had a much smaller effect on cytochrome oxidase. This decrease in active VHb in strain MK79 was correlated with a reverse in the advantage regarding R-amylase production of MK79 over MK57 seen at a menadione concentration of 0 mM, thus linking the presence of active VHb with the increase in R-amylase production. It is concluded that *vgb*, and not any other *Vitreoscilla* DNA squences on plasmid pMK79, is the source of the advantages in both the growth and product production of α-amylase in this system.

Introduction

The function of globins in higher organisms as faciliators of oxygen tranport and storage are well known but the exact function(s) of microbial haemoglobins still remains obscure. *Vitreoscilla* are filamentous, gliding, strict aerobes but have a tendency to grow in oxygen-poor environments [1]. They have adapted a mechanism, novel for bacteria, to grow under these conditions: they synthesize a bacterial haemoglobin (VHb) in response to critical oxygen concentration [2,3]. VHb was first recognized by Webster and colleagues [4]. The putative function of VHb is to trap oxygen and feed it to the membrane terminal oxidases; the kinetic constants for oxygen binding to VHb reflect this function. VHb has a somewhat greater rate constant for oxygen association (k_{on}) than horse myoglobin, but its rate constant for oxygen dissociation (k_{off}) is over 500 times greater (which accounts for its larger K_d). The larger k_{on} reflects a higher 'avidity' for oxygen, but its much larger k_{off} enables it to release the bound oxygen more readily than myoglobin and almost all other haemoglobins [2]. The VHb gene, *vgb* [5], often improves growth as well as protein and metabolite synthesis in bacteria and fungi [6–12]. In our initial system, genetic engineering with *vgb* resulted in improved production of recombinant R-amylase by *Escherichia coli*, particularly under hypoxic conditions [8,14]. The presence and function of VHb in enhancing respiration and ATP formation has been considered to be the reason for the improvement in this system, as well as others [2,13]. However, this has never been proven and there exist other, albeit unlikely, possibilities such as unknown stimulatory functions carried on *Vitreoscilla* sequences other than *vgb* carried on the recombinant constructs [14].

Menadione (2-methyl-1,4-naphthoquinone; vitamin K_3) has been shown to be an effective electrophilic agent utilized either as an experimental oxidant [15–18] or for various clinical purposes to treat malaria or myopathies [18–21]. Menadione is metabolized by redox and arylation reactions. An electron reduction, interacted with oxyhaemoglobin [22,23] or mediated by flavoenzymes [23], results in the production of the very reactive semi-quinone form, which autoxidizes, yielding redox cycling with oxygen consumption and a rapid drop in the levels of reducing agents.

In the present work menadione was used as an oxidant to examine VHb function. However, it was necessary to find the range of menadione in which only VHb was affected, and with a lesser effect on basal cellular respiration. This was of special interest to us to confirm our previous sodium nitrite study [14].

Key words: α-amylase, menadione, *Vitreoscilla* haemoglobin (VHb).

Abbreviations used: VHb, *Vitreoscilla* haemoglobin; RMF, respiratory membrane fragment.

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Materials and methods

Bacterial strains, growth conditions and chemicals

The parental strain of *E. coli* was JM103 [23]; it was transformed with plasmid pMK57 to yield strain MK57 or plasmid pMK79 to produce strain MK79 [6]. Plasmid pMK57 [6] was created by cloning 3.0 kb of *Bacillus stearothermophilus* DNA containing the R-amylase gene [24] cloned into pUC8 [23]. Plasmid pMK79 was constructed by inserting a 2.3 kb *Vitreoscilla* fragment (Figure 1) containing the 0.6 kb *vgb*, as well as the 1.7 kb C-terminal coding region of *uvrA*, into pMK57 [6]. All cultures were grown at 37 °C with shaking (150 rev./min) in a shake flask bearing one-fifth of their own volume of medium (unless noted otherwise).

Inocula for these experiments were grown from single colonies (overnight followed by 3 h) in LB (initial pH 7.5) [25] containing 100 μg/ml ampicillin (LB-amp). Cells from 3-h cultures were harvested by centrifugation and then washed with, and re-suspended in, ampicillin-free LB at a concentration of 0.5 D_{600} units. Then 0.1 ml of these cells served as the inoculum for 100 ml of ampicillin-free LB in 500 ml Erlenmeyer flasks for shake-flask experiments. Menadione was added to the LB to achieve final concentrations of up to 10 mM. Strains were maintained on LB-amp plates. Medium pH was determined from samples taken periodically from flasks. All chemical and enzymes used throughout this study were purchased from the companies indicated: tryptone, yeast extract and bacto agar were from Difco; lysozyme was from Sigma; lamda DNA cut with *Hin*dIII, *Hin*dIII restriction enzyme buffer, lambda DNA, DNase I, DNase II and RNase were from Promega. All other reagents used in this study were analytical grade.

Figure 1 Confirmation of transformation of *E. coli* with plasmids pMK57 and pMK79

Lane 1, *Hin*dIII-digested lambda DNA; lane 2, plasmid mimiprep from MK57; lane 3, plasmid miniprep from MK79; lane 4, *Hin*dIII commercially precut lambda DNA. All plasmid minipreps were digested with *Hin*dIII. One band of 5.7 kb was found for MK57 and two bands, of 5.7 and 2.2 kb, were found for MK79 after *Hin*dIII cleavage.

Analytical methods

Culture attenuance was measured with a spectrophotometer at D_{600}, with culture samples diluted as necessary with fresh LB to bring the D_{600} to below 0.5 when measured in cells of a 1 cm pathlength. Blanks were LB containing the corresponding concentration of menadione. Plasmid stability was determined by plating serially diluted samples on LB and then transferring 25 colonies at random from each plate to LB-amp plates. Determination of both extracellular and intracellular R-amylase was analysed as described by Liu et al. [26]. VHb levels *in vivo* were calculated by spectral deconvolution by comparing the spectrum of the sample with standard spectra reduced and oxidized in the range 400–500 nm. The reduced standard VHb was obtained by addition of a few grains of sodium dithionite to whole cells. All spectrophotometric determinations were carried out with a Techcomp 8500 II spectrophometer. Whole-cell respiration was determined at 37 °C using a Yellow Springs Instruments model 53 oxygen monitor as described by Khosravi et al. [6] and Crawford and Goldberg [27]; where indicated, sodium succinate was added to a final concentration of 0.25 mM.

To test the effects of menadione on this parameter, suspensions of harvested cells were incubated at menadione concentrations of 0.1–10 mM for 10 min at 37 °C before oxygen uptake was determined. VHb-free respiratory membrane fragments (RMFs) were prepared and assayed for respiratory activity as described by Georgiou and Webster [28]. Before the assay they were incubated for 10 min at 37 °C at menadione concentrations of 0–10 mM; respiration was determined using 0.25 mM NADH as an electron donor and a Yellow Springs Instruments model 53 oxygen monitor.

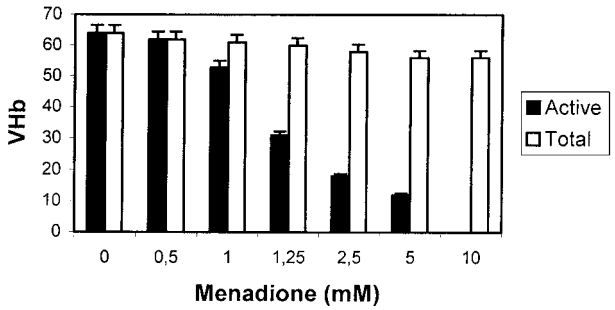

Figure 2 Summary of haemoglobin concentration in MK79 after growth in LB bearing various concentrations of menadione at 150 rev./min and 37 °C for 16 h

VHb measurements (nmol/g of wet weight of cells) were performed without (to measure active VHb; black bars) and with dithionite addition (to measure total VHb; white bars). Values are means ± S.D. from three–five individual measurements.

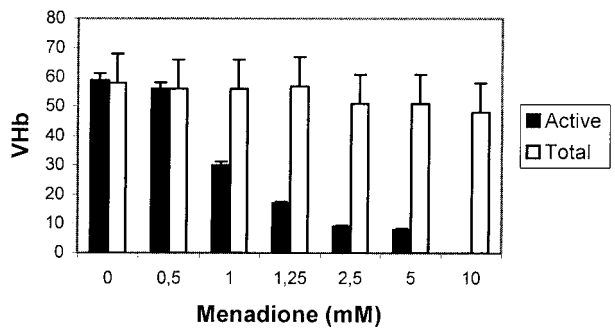

Figure 3 Summary of haemoglobin concentration in MK79 after growth in LB bearing various concentrations of menadione at 150 rev./min and 37 °C for 24 h

VHb measurements (nmol/g of wet weight of cells) were performed without (to measure active VHb; black bars) and with dithionite addition (to measure total VHb; white bars). Values are means ± S.D. from three–five individual measurements.

Results

Effects of menadione on levels of reduced (active) and oxidized (inactive) VHb

In a previous study, Aydin et al. [14] reported that 5 mM nitrite was sufficient to oxidize VHb in MK79, while affecting the cytochromes only a little. Thus, in investigating menadione concentrations that might oxidize VHb without affecting the respiratory chain, we tested a range roughly at this level. From our nitrite study, it was initally expected that any inactivation of VHb by menadione would be due to its oxidation from the active ferrous form to the inactive ferric form. Measurements were therefore performed on the levels of active and inactive VHb in MK79 as a function of menadione concentration (from 0 to 10 mM) in shake-flask cultures. Samples were taken in both the early stationary (16 h) and stationary (24 h) phases. The levels of total VHb at both times decreased as menadione increased to 2.5 mM, and no additional loss was observed at 5 mM (Figures 2 and 3). The decrease was much greater at all concentrations for the 24-h samples (Figure 3). The same trends were observed with the levels of ferrous VHb. For the 10 mM menadione concentration there was no active VHb remaining in the 24-h samples, although the levels of active VHb in the 16-h samples remained constant at around 6–8 nmol/g of cells from 5 to 10 mM (Figures 2 and 3).

For a menadione concentration of 2.5 mM all of the VHb was nearly inactive at 5–10 mM menadione, but total VHb levels were very high for these samples (Figures 2 and 3). Thus menadione did, as expected, increase the fraction of oxidized VHb.

Effects of menadione on growth

At 0 mM menadione, growth of MK79 was similar to or slightly better than that of MK57 in shake flasks. At 2.5 mM menadione, however, MK57 had a slight advantage over MK79, and growth was inhibited for both strains. For MK79 there was a significant effect for 5 mM menadione (Tables 1 and 2).

Effects of menadione on R-amylase production

At 0 mM menadione MK79 produced more R-amylase than MK57 under testing conditions. Reminiscent of our previous results [9], the greatest advantage of MK79 in this regard was for shake-flask growth under oxygen-limited conditions; these trends were reversed above the zero menadione concentration. This was due to a decrease in amylase production for MK79, whereas MK57 suffered a smaller decrease or an increase (Tables 3 and 4).

Effects of menadione on respiration of whole cells and RMFs

Cells of strains MK57 and MK79 were grown in menadione-free LB in shake flasks and harvested after 16 h (early stationary phase). They were then treated with various levels of menadione for 10 min before being assayed for oxygen uptake; both endogenous and succinate-stimulated oxygen uptake were measured. Without succinate there was a decrease in respiration at 2.5 mM menadione for both strains, which remained approximately unchanged for up to 10 mM menadione, the decrease for MK79 being about two or three times that for MK57 (Figure 4). With succinate, the decrease in respiration as a function of menadione concentration was dose-responsive, with the MK79 decrease being about twice that for MK57 (Figure 5). RMFs were tested for respiration following 10-min treatments with the same range of menadione

Table 1 Growth (D_{600}) of strains MK57 and MK79 in LB as a function of menadione concentration at 16 h

Values are means ± S.D. from three–five experiments.

		Growth (D_{600})			
Strain	Menadione concentration . . .	0 mM	1.25 mM	2.5 mM	5 mM
MK79		2.26 ± 0.24	2.20 ± 0.12	1.84 ± 0.18	1.42 ± 0.09
MK57		1.92 ± 0.16	1.89 ± 0.14	1.86 ± 0.11	1.69 ± 0.02

Table 2 Growth (D_{600}) of strains MK57 and MK79 in LB as a function of menadione concentration at 24 h

Values are means ± S.D. from three–five experiments.

		Growth (D_{600})			
Strain	Menadione concentration . . .	0 mM	1.25 mM	2.5 mM	5 mM
MK79		3.41 ± 0.22	3.30 ± 021	2.9 ± 0.11	2.4 ± 0.12
MK57		3.1 ± 0.18	3.16 ± 0.18	3.0 ± 0.16	2.8 ± 0.11

Table 3 α-Amylase production by strains MK79 and MK57 as a function of menadione concentration at 16 h

α-Amylase production is expressed as intracellular plus extracellular levels in terms of mg of starch hydrolysed/min per ml of culture; means ± S.D. are shown.

		α-Amylase production (mg of starch hydrolysed/min per ml)			
Strain	Menadione concentration . . .	0 mM	1.25 mM	2.5 mM	5 mM
MK79		1.36 ± 0.04	1.41 ± 0.08	0.86 ± 0.04	0.74 ± 0.03
MK57		0.98 ± 0.01	1.02 ± 0.04	1.02 ± 0.04	0.86 ± 0.05

Table 4 α-Amylase production by strains MK79 and MK57 as a function of menadione concentration at 24 h

Details are as for Table 3.

		α-Amylase production (mg of starch hydrolysed/min per ml)			
Strain	Menadione concentration . . .	0 mM	1.25 mM	2.5 mM	5 mM
MK79		1.74 ± 0.08	1.62 ± 0.05	1.06 ± 0.02	0.82 ± 0.01
MK57		1.28 ± 0.05	1.3 ± 0.04	1.16 ± 0.02	0.94 ± 0.00

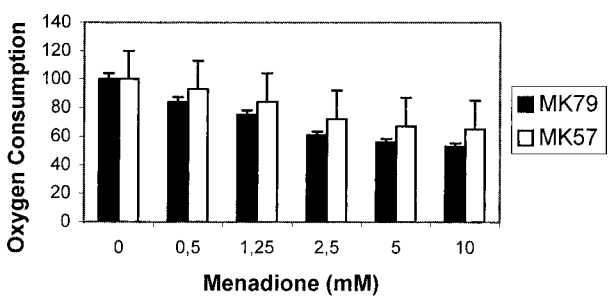

Figure 4 Whole-cell oxygen consumption by MK79 and MK57 after treatment with menadione for 10 min without succinate addition

Cells were grown for 16 h before harvesting. Whole-cell oxygen consumption is given as a percentage of 0 mM menadione controls. Values are means ± S.D. from three individual measurements. 100 % for both MK57 and MK79 represents 0.021 μmol/min per D_{600} of cells.

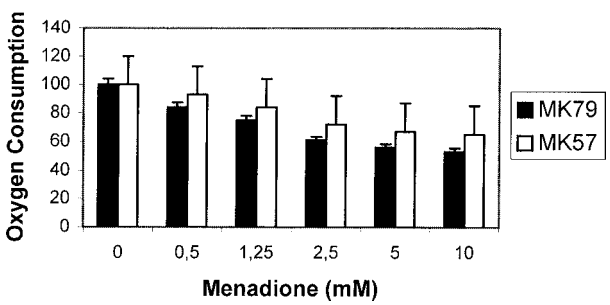

Figure 5 Whole-cell oxygen consumption by MK79 and MK57 after treatments with menadione for 10 min with succinate addition

Cell were grown 16 h before harvesting. Whole-cell oxygen consumption is given as a percentage of 0 mM menadione controls. Values are means ± S.D. from three individual measurements. 100 % for MK57 and MK79 represents 0.061 and 0.066 μmol/min per D_{600} of cells respectively.

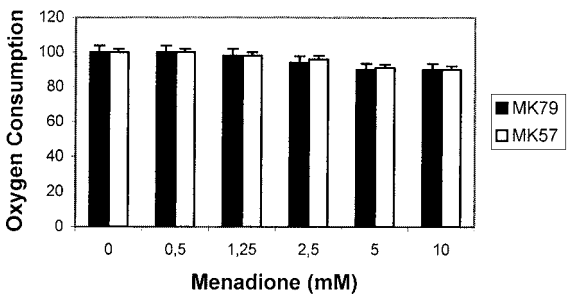

Figure 6 Relative rate of oxygen consumption by MK79 and MK57 RMFs after treatment with menadione for 10 min

Cells were grown for 16 h before harvesting. The substrate was 0.5 mM NADH. The relative rate of oxygen consumption is given as a percentage of the 0 mM menadione controls. Values are means ± S.D. from three individual measurements. 100 % for both MK57 and MK79 represents 0.45 μmol/min per mg of RMF. Calculations based on D_{600} units of cells for each RMF sample gave similar results.

concentrations (Figure 6) with NADH as the electron donor. RMFs of MK79 were slightly more sensitive than those of MK57, but for both strains the RMFs were more resistant to menadione effects than were whole cells. Even 10 mM menadione reduced respiration by only 10–11 % compared with the zero-concentration menadione controls.

Discussion

Menadione, a widely studied quinone, was dictated by its interesting property of acting as an experimental oxidant [15–18] for haem-containing proteins or for various clinical purposes to prevent vitamin K deficiencies. Menadione may be capable of binding and even oxidizing the ferrous haem iron of bacterial flavohaemoglobins, thus preventing oxygen binding [20,29]. Proteins in the strains used in this study that may be subject to the effects of menadion include flavohaemoglobin, cytochromes and VHb; interference with any of them could affect respiration.

In particular, it can be expected that menadione has severe effects on the respiratory chain since menadione is an analogue to bacterial (mena) quinones. However, *in vitro* measurements of oxygen uptake of RMFs in this study suggest that even at 10 mM menadione there is little effect on overall cytochrome activity for both strains.

As shown in this study, the decreased production of α-amylase in response to menadione is due only to the inhibition of VHb when compared with MK57 cells at 1.25–10 mM after 16- and 24-h treatments. It seems that menadione concentration begins to affect cell growth at 1.25 mM, and these negative effects increase with menadione concentrations of up to 10 mM. It should be remembered that *in vitro* measurements of oxygen uptake of RMFs suggest that even above 2.5 mM there is little effect on overall cytochrome activity and little difference between the VHb^+ and VHb^- strains. Therefore, this may be evidence that active VHb is responsible for product yields in the recombinant cells.

Both *in vitro* experiments measuring whole-cell respiration and *in vivo* experiments measuring the effects of menadione concentration on VHb levels in the *vgb*-bearing strain MK79 are consistent with our previous results with nitrite [14], as well as Doeller and Wittenberg's observations of nitrite [30]. Menadione has dose-dependent effects that are up to four times greater for the cell compared with nitrite dose [14]. The whole-cell respiration data for the vgb^- strains (MK57) are roughly consistent with that of respiratory membranes of MK79, suggesting that like the RMF experiments they are detecting effects on cytochromes of the respiratory chain. This observation is supported by the fact that similar results are obtained measuring either endogenous respiration (without succinate) or maximum possible respiration with succinate.

Molecular oxygen is required as a terminal electron acceptor in the aerobic metabolism of carbon compounds. It was found that a concentration of menadione over 2.5 mM inhibits respiration of strain MK79 compared with MK57, presumably due to the inhibition of VHb in addition to inhibition of cytochromes. This is consistent with the inactivation of myoglobin by nitrite, as reported by Doeller and Wittenberg [30] and inactivation of VHb by sodium nitrite reported by Aydin et al. [14]. Inhibitory effects of menadione on VHb accumulation determined from the VHb plus *Vitreoscilla* methaemoglobin measurements are also substantial and exhibit dose-dependant behaviour.

The key result of this work is that experimental MK79 produces more α-amylase than MK57 at zero menadione concentration but not at concentrations above 1.25 mM. This is consistent with all growth data as well as the whole-cell and RMF respiration data and the data concerning the effect of menadione on VHb levels. Specifically there are significant effects on MK79 whole respiration and VHb production at menadione concentrations above 1.25 mM, the same menadione concentration at which the crossover points for cell growth and α-amylase production occur. At this concentration menadione presumably begins to have a negative impact on VHb, and strain MK79 would have the extra burden of replicating a larger plasmid than strain MK57; and if active VHb is oxidized, there is produced a somewhat larger amount of inactive (*Vitreoscilla* methaemoglobin) than occurs without menadione. Taken with the minimal effects of the 2.5 mM menadione on the electron transport chain, this evidence supports the hypothesis that VHb is, in fact, directly responsible for

enhancing recombinant protein production in MK79 and by extension the stimulatory effects in the production of other proteins and metabolites in bacteria engineered to contain *vgb*.

Conclusions

Menadione oxidized VHb relatively selectively, and is a better tool to knock out VHb compared with nitrite oxidation [14]. VHb oxidized with menadione reduced back totally with addition of dithionite whereas oxidized VHb with nitrite did not reduce back at all [14]. It is concluded from the experiment, performed with and without menadione, that I have direct evidence that VHb is responsible for enhanced growth [6–12], biodegration of aromatic compounds [31,32] and the stimulatory effects of other proteins and metabolites in *vgb*-bearing strains [9–12]. As a result, testing α-amylase production with and without menadione confirmed the benefit of VHb in recombinant organisms. In future studies, a better way to prove this point might be to use more molecular approaches, such as using point mutation to inactivate the *vgb* gene, and then assess its effects on α-amylase production compared with wild-type VHb in MK79 cells.

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