Hydroxylated naphthoquinones as substrates for *Escherichia coli* anaerobic reductases

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We have used two hydroxylated naphthoquinol menaquinol analogues, reduced plumbagin (PBH_2, 5-hydroxy-2-methyl-1,4-naphthoquinol) and reduced lapachol [$LPCH_2$, 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinol], as substrates for *Escherichia coli* anaerobic reductases. These compounds have optical, solubility and redox properties that make them suitable for use in studies of the enzymology of menaquinol oxidation. Oxidized plumbagin and oxidized lapachol have well resolved absorbances at 419 nm ($\epsilon = 3.95\ mM^{-1}\cdot cm^{-1}$) and 481 nm ($\epsilon = 2.66\ mM^{-1}\cdot cm^{-1}$) respectively (in Mops/KOH buffer, pH 7.0). PBH_2 is a good substrate for nitrate reductase A ($K_m = 282 \pm 28\ \mu M$, $k_{cat} = 120 \pm 6\ s^{-1}$) and fumarate reductase ($K_m = 155 \pm 24\ \mu M$, $k_{cat} = 30 \pm 2\ s^{-1}$), but not for DMSO reductase. $LPCH_2$ is a good substrate for nitrate reductase A ($K_m = 57 \pm 35\ \mu M$, $k_{cat} = 68 \pm 13\ s^{-1}$), fumarate reductase ($K_m = 85 \pm 27\ \mu M$, $k_{cat} = 74 \pm 6\ s^{-1}$) and DMSO reductase ($K_m = 238 \pm 30\ \mu M$, $k_{cat} = 191 \pm 21\ s^{-1}$). The sensitivity of enzymic $LPCH_2$ and PBH_2 oxidation to 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide inhibition is consistent with their oxidation occurring at sites of physiological quinol binding.

INTRODUCTION

Escherichia coli, when grown anaerobically with fumarate, nitrate or DMSO as respiratory oxidant, develops a respiratory chain terminated by a membrane-bound oxidant-specific menaquinol–oxidant oxidoreductase [1]. These enzymes are menaquinol–fumarate oxidoreductase (FrdABCD) [2,3], menaquinol–nitrate oxidoreductase (NarGHI) [4,5] and menaquinol–DMSO oxidoreductase (DmsABC) [6–8] respectively. The operons encoding these enzymes have been cloned, sequenced and genetically modified, and the enzymes they encode have been overexpressed and assembled to high levels in the *E. coli* cytoplasmic membrane. FrdABCD, NarGHI and DmsABC are therefore excellent model systems for the study of biological electron transfer. Each enzyme comprises a catalytic subunit containing either a flavin (FrdABCD) or molybdenum cofactor (NarGHI and DmsABC) prosthetic group, an [Fe-S] cluster containing electron-transfer subunit, and two (FrdABCD) or one (NarGHI and DmsABC) membrane anchor subunits. The catalytic and electron-transfer subunits comprise a membrane-extrinsic dimer anchored to the *E. coli* cytoplasmic membrane by the membrane anchor subunit(s). In each enzyme, substrate reduction occurs within the catalytic subunit, whereas menaquinol (MQH_2) oxidation occurs within the membrane anchor subunit(s). Large numbers of site-directed mutants have been constructed in the three enzymes (FrdABCD [9–14], NarGHI [5,15–17], DmsABC [18–22]) with the aim of understanding their cofactor ligation, redox chemistry and enzymology.

The enzymology of the anaerobic reductases has been characterized using reducing substrates that donate electrons specifically to the MQH_2-binding site of each enzyme, or non-specifically at some point between this site and the site of substrate reduction. Both types of substrate are typically used when evaluating site-directed mutants [5,12,18], as this allows evaluation of the effect of the mutants on the substrate-reducing active site and on the overall pathway of electrons through the enzymes. For example, in mutants of the catalytic subunit of DmsABC (DmsA-C38), a non-specific reducing substrate [reduced Benzyl Viologen ($BV^{\bullet+}$)] is still able to donate electrons to the molybdenum cofactor, whereas a specific reducing substrate (dimethylnaphthoquinol, $DMNH_2$) demonstrates that electron transfer through the physiological electron-transfer pathway is blocked [18,19].

In studies of the membrane-bound bacterial anaerobic reductases, a typical specific reducing substrate used is a naphthoquinol MQH_2 analogue such as $DMNH_2$ or menadiol [23–26]. These compounds have intense overlapping absorbances in the UV wavelength range in both their reduced and oxidized forms, requiring the use of dual-wavelength spectrophotometry to obtain accurate results [24,27]. Further complications arise because of the overlapping protein absorbance at 280 nm; for example, the two wavelengths used in studies of $DMNH_2$ and menadiol are 270/290 nm and 260/280 nm respectively [28]. Improved MQH_2 analogue substrates for the anaerobic reductases would have characteristics that include the following: (i) well-resolved visible optical absorbances; (ii) relatively high solubility in aqueous solutions; (iii) kinetic parameters (K_m, k_{cat}) indicative of effective partitioning into the hydrophobic membrane environment; (iv) efficient enzymic oxidation.

In this paper, we identify two hydroxylated naphthoquinols as MQH_2 analogues and demonstrate their use as substrates for the three membrane-bound anaerobic reductases of *E. coli* that are described above. These compounds, reduced plumbagin (PBH_2, 5-hydroxy-2-methyl-1,4-naphthoquinol) and reduced lapachol [$LPCH_2$, 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinol]

Abbreviations used: $BV^{\bullet+}$, reduced Benzyl Viologen; DmsABC, *Escherichia coli* dimethyl sulphoxide reductase; DMN, 2,3-dimethyl-1,4-naphthoquinone; $DMNH_2$, reduced DMN; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; FrdABCD, fumarate reductase; lapachol, 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone; LPC, oxidized lapachol; $LPCH_2$, reduced lapachol; MQ, menaquinone; MQH_2, reduced MQ; NarGHI, nitrate reductase A; PB, oxidized plumbagin; PBH_2, reduced plumbagin; plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone; TMAO, trimethylamine-*N*-oxide.

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have optical, solubility and kinetic properties that make them amenable for use in studies of MQH_2 oxidation by *E. coli* anaerobic reductases.

EXPERIMENTAL

Bacterial strains and plasmids

The bacterial strains and plasmids used here are shown in Table 1. pVA700 overexpresses NarGHI in the presence of isopropyl thiogalactoside. pFRD84 constitutively overexpresses FrdABCD under anaerobic and microaerobic conditions. pDMS170 constitutively overexpresses DmsABC under anaerobic conditions. On pVA700, the *narGHJI* operon is under the control of the *tac* promoter [5]. On pFRD84 and pDMS170, the *frdABCD* and *dmsABC* operons respectively are under the control of the *fnr* promoter [6,31].

Bacterial cell growth

LCB79/pVA700

Cells were grown microaerobically overnight in 2-litre batches at 30 °C on Terrific Broth [32]. A 10 % inoculum of a stationary-phase culture was used, and 0.2 mM isopropyl thiogalactoside was added to induce overexpression of the *narGHJI* operon.

HB101/pFRD84

Cells were grown overnight in 2-litre batches at 37 °C under microaerobic conditions on Terrific Broth. A 1 % inoculum of a stationary-phase culture was used.

HB101/pDMS170

Cells were grown anaerobically for 48 h at 37 °C on a glycerol/fumarate medium as previously described [33]. Where appropriate, ampicillin and streptomycin were included in the growth medium at a concentration of 100 μg/ml.

Isolation of cytoplasmic membranes

Cells were harvested, washed and membranes prepared by French pressure cell lysis and differential centrifugation [21] in 100 mM Mops/KOH/5 mM EDTA, pH 7.0. The buffer used during the French pressing step contained 0.2 mM PMSF. The isolated membranes were resuspended in buffer and subjected to a second ultracentrifugation step. Finally, membranes were resuspended in 100 mM Mops/KOH/5 mM EDTA and frozen in liquid nitrogen before being stored at −70 °C until use.

Table 1 Bacterial strains and plasmids

pDMS170 was generated by ligating the 4.8 kbp *Eco*RI/*Sal*I fragment from pDMS223 [21] into pBR322 that had previously been cut with *Pvu*II and *Nru*I and self-ligated to destroy these sites. Prepared by Dr. C. A. Trieber (Department of Medical Microbiology and Immunology, University of Alberta, Alberta, Canada).

Strain/plasmid	Description	Source
Strain		
HB101	*supE44 hsdS20* ($r_B^- m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* Strep^R	Laboratory collection
LCB79	*AraD139* Δ(*lac*IPOZYA-*argF*) *rpsL thi* ϕ*79* (*nar-lac*) Strep^R	[29]
Plasmid		
pVA700	pJF119EH Amp^R (*narGHJI*)$^+$	[5]
pDMS170	pBR322 Amp^R (*dmsABC*)$^+$	Dr. C. A. Trieber
pFRD84	pBR322 Amp^R (*frdABCD*)$^+$	[30]

Quantification of the anaerobic reductases

EPR spectra at 12 K of membrane samples were recorded after ferricyanide oxidation or dithionite reduction using a Bruker ESP300 EPR spectrometer equipped with an Oxford Instruments ESR-900 flowing helium cryostat. Relative spin concentrations were determined by double integration of spectra recorded under non-saturating microwave power with 1 mM CuEDTA as standard [34]. For NarGHI and FrdABCD, the concentration of the single [3Fe-4S] cluster was determined [13,35]. For DmsABC, the total concentration of [4Fe-4S] clusters was determined and the enzyme concentration estimated assuming that there are four [4Fe-4S] clusters per DmsABC trimer [36].

Optical spectroscopy

Optical spectra were recorded using a Hewlett–Packard HP8453 single-beam diode array spectrophotometer. Quinol/quinone spectra were recorded in a 100 mM potassium phosphate buffer, pH 7.7, containing 5 mM EDTA. For kinetic measurements, data were recorded using a Varian DMS100S dual-beam spectrophotometer.

Enzyme assays

Assays were carried out in N_2-saturated 100 mM Mops/KOH/5 mM EDTA, pH 7.0, using 4 ml acryl cuvettes (Sarstedt 67.738) with machined Teflon stoppers constructed in the Faculty of Medicine's Biomedical Workshop (University of Alberta). These had an 11 mm plug that inserted into the acryl cuvettes, creating an effective seal against oxygen diffusion. Holes suitable for insertion of Hamilton syringe needles were bored through the stoppers. The combination of the acryl cuvettes and the Teflon stoppers maintained stable anaerobiosis during the kinetics experiments described here. Mixing of the cuvette contents was achieved by inserting a micro stir bar (2 mm × 7 mm; Fisher 14511-67) and inversion. Assays were carried out as follows: reduced quinol was prepared by exposing an acidified (to a final concentration of 0.18 M HCl) 20 mM stock solution in ethanol to metallic zinc powder in a 2 ml glass HPLC vial with a septum and cap. For NarGHI, FrdABCD and DmsABC assays, the buffer (100 mM Mops/KOH/5 mM EDTA, pH 7.0) was supplemented with 4 mM potassium nitrate, 20 mM potassium fumarate and 70 mM trimethylamine *N*-oxide (TMAO) respectively. After transfer of the N_2-saturated buffer to the cuvette (3.31 ml volume), the stopper was inserted, and quinol was added at a range of concentrations up to approx. 0.3 mM. No gas phase was present above the reaction solution in the cuvette. The reaction was initiated by addition of enzyme. For PBH_2, the absorbance was followed at 419 mm, and for $LPCH_2$ it was followed at 481 nm. These wavelengths allowed the formation of PB and LPC (i.e. the oxidized forms) to be followed. In the absence of enzyme, no significant PBH_2 or $LPCH_2$ oxidation was observed.

Determination of PB and LPC absorption coefficients

Absorption coefficients (ϵ) for PB and LPC were determined by recording spectra at a range of concentrations. For each analogue, ϵ was determined from the slopes of plots of quinone concentration against absorbance.

Determination of PB and LPC midpoint potentials

Midpoint potential ($E_{m,7}$) was measured by rotating-disk voltammetry at a glassy carbon (GC-20; Tokai) electrode in a three-electrode cell (reference electrode Ag/AgCl; auxillary Pt wire). Potentials versus Ag/AgCl were subsequently converted into potentials versus the standard hydrogen electrode. Rotation rates were controlled by a Princeton Applied Research model 636 RDE and were between 4000 and 6000 rev./min. Potential scan rates between 2 and 50 mV/s were controlled by a Bioanalytical Systems CV-27 potentiostat, and current was outputted to an XY recorder. Values of $E_{m,7}$ were determined from at least three different rotation rates and three potential scan rates. The glassy carbon electrode was freshly polished on polishing cloth (Buehler) with 0.05 μm alumina powder (Buehler) slurried in deionized water. After being polished, electrodes were rinsed with and sonicated in deionized water. Quinones were initially dissolved in ethanol and diluted with 100 mM Mops/KOH/5 mM EDTA buffer, pH 7.0 to final concentrations of 2 mM for PB and 4 mM for LPC. All solutions were purged with N_2 before use.

Protein determination

Protein concentrations were estimated by a modification of the Lowry procedure [37] using a Bio-Rad BSA protein standard.

Materials

LPC and PB were purchased from Aldrich and Sigma respectively. Zinc powder was also obtained from Aldrich or Matheson, Coleman and Bell. Mops was from Fisher Biotech.

RESULTS

Identification of hydroxylated naphthoquinone MQH_2 analogues

There are a number of commercially available hydroxylated naphthoquinones that in their reduced forms are potential substrates for the *E. coli* anaerobic reductases. These include lawsone (2-hydroxy-1,4-naphthoquinone), juglone (5-hydroxy-1,4-naphthoquinone), PB and LPC. Lawsone and juglone were not selected for further study as they do not have a methyl or phytyl (3-methyl-2-butenyl) side chain at the 2- or 3-positions on the naphthoquinone bicycle. Figure 1 shows the structures of PB and LPC in comparison with menaquinone-1 (the physiological quinone in anaerobically grown *E. coli* is menaquinone-8 [38]).

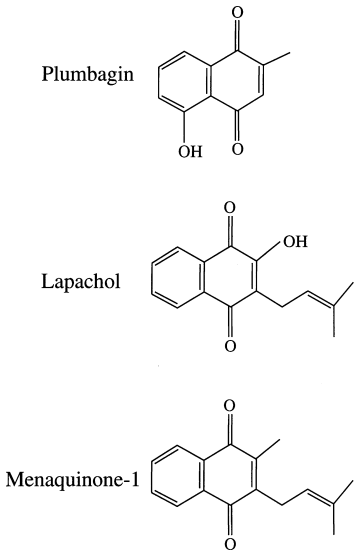

Figure 1 Structures of hydroxylated naphthoquinones in comparison with menaquinone-1

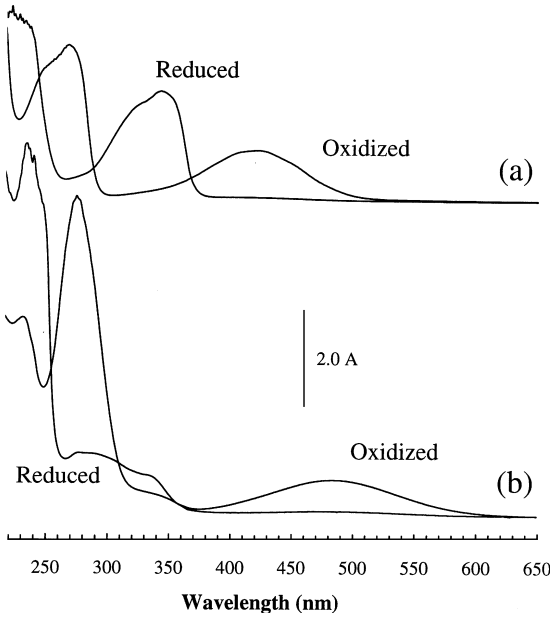

Figure 2 UV–visible absorption spectra of PB/PBH_2 (a) and $LPC/LPCH_2$ (b)

Concentrations of 0.2 mM in 100 mM potassium phosphate buffer, pH 7.7, were used. Reduced quinols were prepared as described in the Experimental section. Spectra were recorded at ambient temperature (23 °C) using a Hewlett–Packard 8453 diode array spectrophotometer.

Optical spectroscopy of PB/PBH_2 and $LPC/LPCH_2$

Figure 2 shows optical spectra of 0.2 mM PB (spectrum a) and LPC (spectrum b) recorded at between 200 and 650 nm in 100 mM potassium phosphate buffer, pH 7.7. PBH_2 shows intense absorbance at 341 nm and PB absorbs at 419 nm. There is a slight overlap between the reduced and oxidized absorbances in the 341 nm range, suggesting that the well-resolved oxidized absorbance at 419 nm is suitable for studying the kinetics of enzymic PBH_2 oxidation. LPC shows a well-resolved absorbance at 481 nm, and an intense absorbance at 275 nm, which has some overlap with the absorbance of the reduced form. The 275 nm absorbance also potentially overlaps with the absorbance of protein at approx. 280 nm, suggesting that the 481 nm absorbance of LPC is the most suitable for studying the enzymic oxidation of $LPCH_2$.

Absorption coefficients were determined for PB and LPC from plots of absorbance against concentration in the 0–0.5 mM range (results not shown) in 100 mM Mops/KOH/5 mM EDTA, pH 7.0. The ϵ_{419} for PB is 3.95 $mM^{-1} \cdot cm^{-1}$, and the ϵ_{481} for LPC is 2.66 $mM^{-1} \cdot cm^{-1}$. In both cases the quinone is soluble at concentrations above 0.5 mM in the buffer system used here for kinetic assays (100 mM Mops/KOH/5 mM EDTA, pH 7.0). However, in experiments carried out using buffer containing no EDTA, the analogues sometimes precipitated, probably as a result of the formation of an insoluble analogue–Zn^{2+} complex. In the presence of EDTA, the quinones are consistently soluble in both their oxidized and reduced forms.

Table 2 Kinetic parameters of anaerobic reductases with PBH_2 and $LPCH_2$ as substrates

Enzyme concentration is given in nmol/mg of membrane protein and as a percentage of total membrane protein. n.d., Not determined.

	Enzyme concentration		PBH_2			$LPCH_2$		
Enzyme	nmol/mg	% of membrane protein	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
NarGHI	1.30	28.7	282 ± 28	120 ± 6	0.43 ± 0.04	57 ± 35	68 ± 13	1.56 ± 0.89
FrdABCD	2.67	32.3	155 ± 24	30 ± 2	0.20 ± 0.01	85 ± 27	74 ± 6	0.95 ± 0.35
DmsABC	0.37	5.2	n.d.	n.d.	n.d.	238 ± 30	191 ± 21	0.81 ± 0.07

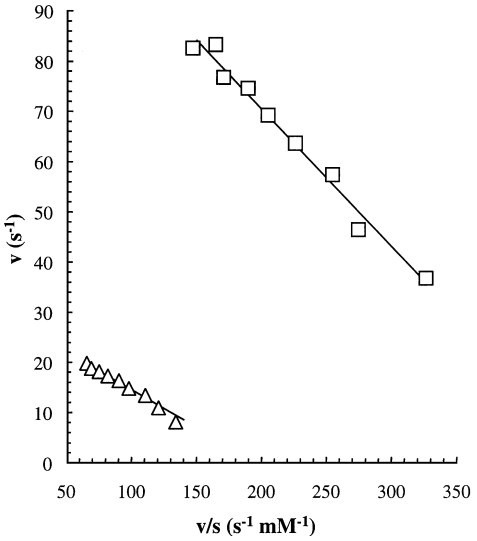

Figure 3 Determination of kinetic parameters for PBH_2 oxidation by NarGHI and FrdABCD

Rates of PBH_2 oxidation were determined by monitoring the initial rate of change in A_{419} using a Varian DMS100S dual-beam spectrophotometer. □, Nitrate-dependent oxidation (K_m = 272 μM, k_{cat} = 125 s^{-1}); △, fumarate-dependent oxidation (K_m = 148 μM, k_{cat} = 29 s^{-1}).

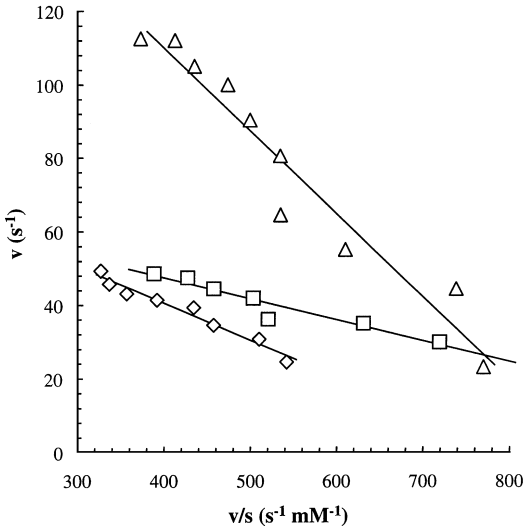

Figure 4 Determination of kinetic parameters for $LPCH_2$ oxidation by DmsABC, NarGHI and FrdABCD

Rates of $LPCH_2$ oxidation were determined by monitoring the rate of change in A_{481} using a Varian DMS100S dual-beam spectrophotometer. △, TMAO-dependent oxidation (K_m = 225 μM, k_{cat} = 200 s^{-1}); □, nitrate-dependent oxidation (K_m = 57 μM, k_{cat} = 70 s^{-1}); ◇, fumarate-dependent oxidation (K_m = 100 μM, k_{cat} = 91 s^{-1}).

Determination of kinetic parameters for PBH_2 and $LPCH_2$ with the anaerobic reductases

Because of the ease with which enzyme-enriched membrane preparations can be obtained, *E. coli* NarGHI, DmsABC and FrdABCD were selected as enzyme systems to evaluate PBH_2 and $LPCH_2$ as MQH_2 analogues. The concentration of the enzymes was determined by EPR spin quantification. For NarGHI and FrdABCD, the [3Fe-4S] cluster concentration was determined, and the enzyme concentration was assumed to be the same as the [3Fe-4S] cluster concentration (assuming a single [3Fe-4S] cluster per NarGHI [35] or FrdABCD [13]). In the case of DmsABC, the [4Fe-4S] cluster concentration was determined, and the enzyme concentration was assumed to be 25 % of that of the cluster concentration (assuming four clusters per DmsABC [20,21]). Table 2 shows the results of the quantification for preparations enriched in NarGHI, FrdABCD and DmsABC. NarGHI and FrdABCD both constitute approx. 30 % of their respective membrane preparations, whereas DmsABC comprises 5 % of its membrane preparation.

Kinetic parameters for PBH_2 and $LPCH_2$ as substrates for the three enzymes were determined by constructing Eadie–Hofstee plots in the presence of saturating concentrations of oxidizing substrate. Potassium nitrate (4 mM), potassium fumarate (20 mM) and TMAO (70 mM) were used for NarGHI, FrdABCD and DmsABC respectively. These concentrations were based on studies of the reductase reactions carried out with $BV^{\bullet+}$ as substrate, which yield K_m values of 0.33 mM for nitrate [39], 0.25 mM for fumarate [40] and 20.2 mM for TMAO [7] for NarGHI, FrdABCD and DmsABC respectively. TMAO was chosen as the substrate for DmsABC because this compound has been used as a substrate for this enzyme in routine studies of the wild-type and mutant forms [18–22].

Figure 3 shows representative Eadie–Hofstee plots for PBH_2-dependent reduction of nitrate by NarGHI and fumarate by FrdABCD, and Table 2 shows summaries of the K_m, k_{cat} and k_{cat}/K_m data obtained from three independent determinations. Surprisingly, PBH_2 is a substrate for NarGHI and FrdABCD, but not for DmsABC (see the Discussion). The k_{cat}/K_m parameters suggest that PBH_2 is a better substrate for NarGHI than it is for FrdABCD.

Figure 4 shows Eadie–Hofstee plots for $LPCH_2$ oxidation by the three enzymes. With this substrate, significant rates of enzyme activity are detected with all three enzymes. However, in the case of NarGHI, the standard error of the K_m is high compared with

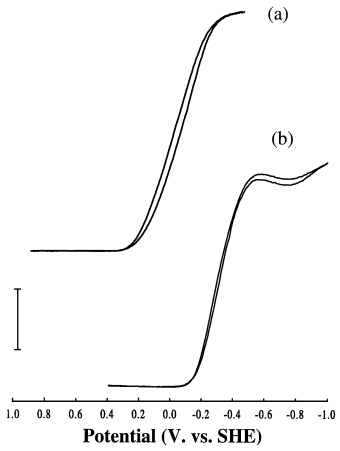

Figure 5 Determination of E_m for PB and LPC at pH 7.0 by rotating-disk voltammetry

Curve (a) 2.0 mM PB; rotation rate = 6000 rev./min; potential scan rate = 10 mV/s. Curve (b) 4.0 mM LPC; rotation rate = 5000 rev./min; potential scan rate = 2 mV/s. The vertical line represents currents of 50 μA and 30 μA for curves (a) and (b) respectively. SHE, standard hydrogen electrode.

its absolute value. The estimated K_m is 57 μM, towards the low end of the concentration range used in the assays, and it is likely that residual oxygen dissolved in the buffer is a factor in the observation of a high standard error. Statistically, the lapachol assay performed better with FrdABCD and DmsABC.

Comparison of the kinetic parameters determined with the three enzymes used here is complicated by the dependence of these parameters on the reaction occurring at the site of substrate (nitrate, fumarate or TMAO) reduction. The k_{+2} is determined by the rate of reaction at this second site, and this parameter has a significant effect on the K_m. Despite this, it is clear that NarGHI has the highest K_m and k_{cat} with PBH_2 as substrate, whereas DmsABC has the highest K_m and k_{cat} with $LPCH_2$ as substrate. Overall, the k_{cat}/K_m values for $LPCH_2$ as substrate for the three enzymes are higher than they are for PBH_2, suggesting that $LPCH_2$ is an overall better substrate.

Midpoint potentials of PB and LPC

In order to assess why PBH_2 is not a substrate for DmsABC, we determined $E_{m,7}$ for both PB and LPC by rotating-disk voltammetry. Representative results of these experiments are presented in Figure 5. At pH 7.0, the rotating-disk current-potential curves (voltammograms) for PB (curve a) and LPC (curve b) both exhibit steady-state current plateaus and appear near-ideal. The constant slope observed during the rising part of the voltammograms implies single two-electron transfers at the scan rates employed here (2–10 mV/s). The slight hysteresis between the reductive and oxidative scans is probably due to quinone species adsorbed on to the glassy carbon electrode surface. A value for $E_{m,7}$ can be estimated by measuring the potential where the current equals half of the plateau current. We thus obtain $E_{m,7}$ values of -40 and -310 mV versus standard hydrogen electrode for PB and LPC respectively. We note that the value of $E_{m,7}$ for LPC obtained does not agree with a reported value (-179 mV) obtained by redox titration [41,42] (see the Discussion). We believe the discrepancy derives from the differences in methodology and solution composition between the two studies.

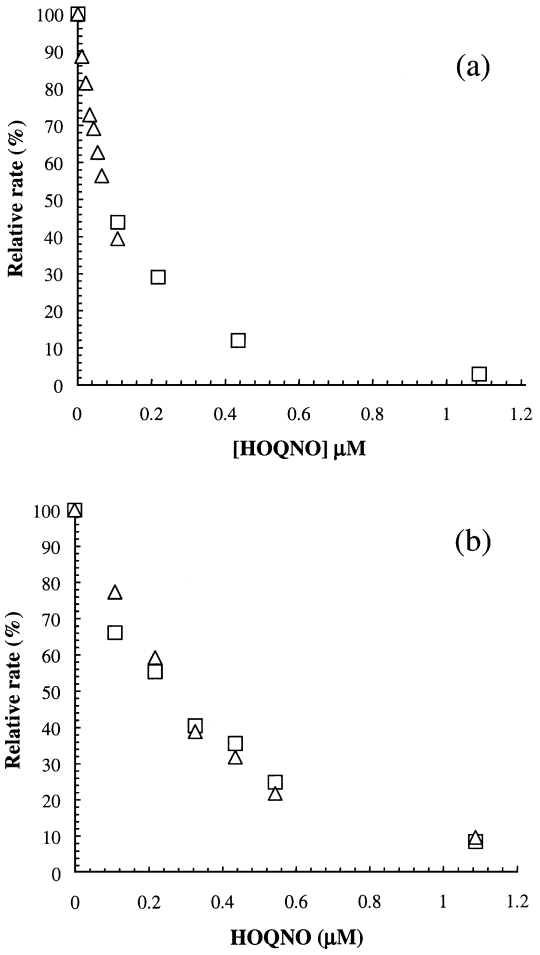

Figure 6 Effect of HOQNO on $LPCH_2$ and PBH_2 oxidation

(**a**) Effect of HOQNO on TMAO-dependent $LPCH_2$ oxidation by DmsABC. The concentration of $LPCH_2$ used was 140 μM. Triangles and squares represent data points from two separate experiments with different ranges of HOQNO concentration. The I_{50} was estimated to be approx. 0.08 μM. (**b**) Effect of HOQNO on fumarate-dependent oxidation of $LPCH_2$ (□) and PBH_2 (△). The concentration of $LPCH_2$ used was 140 μM and the concentration of PBH_2 was 280 μM. The I_{50} for HOQNO inhibition of both $LPCH_2$–fumarate and PBH_2–fumarate activities was estimated to be 0.24 μM.

Specificity of PBH_2 and $LPCH_2$ oxidation at MQH_2-binding sites

We studied the specificity of $LPCH_2$ and PBH_2 oxidation at the MQH_2-binding sites of DmsABC and FrdABCD by using the potent MQH_2-analogue inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) [22,43]. Figure 6(a) shows the effect of increasing HOQNO concentration on the rate of TMAO-dependent $LPCH_2$ oxidation of membranes enriched in DmsABC. It is clear that the I_{50} for HOQNO at the concentration of $LPCH_2$ used is approx. 0.08 μM. Figure 6(b) shows the effect of HOQNO on fumarate-dependent $LPCH_2$ and PBH_2 oxidation by FrdABCD-enriched membranes, and in this case, the I_{50} appears to be approx. 0.24 μM for both substrates. These results are entirely consistent with $LPCH_2$ and PBH_2 oxidation occurring at sites at which HOQNO competes for binding with high affinity, namely the MQH_2-binding sites of DmsABC and FrdABCD. The inhibitory effects of HOQNO on NarGHI using menadiol and duroquinol as quinol substrates have been shown to be complex (A. Magalon, R. A. Rothery, D. Lemesle-

Meunier, C. Frixon, J. H. Weiner and F. Blasco, unpublished work), so the inhibitory effect of HOQNO was not explored with this enzyme.

DISCUSSION

We have developed assays for the reactions catalysed by NarGHI, FrdABCD and DmsABC of *E. coli* using PBH_2 and $LPCH_2$ as quinol analogue substrates. These quinols have properties that make them useful substrates for the characterization of wild-type and mutant enzymes: (i) they are both soluble at relatively high concentrations in aqueous solution (> 0.5 mM); (ii) they have well-resolved optical absorbances at visible wavelengths in their oxidized forms; (iii) they have K_m values that are within their range of aqueous solubility. In addition to the identification and application of these quinols to the anaerobic reductases, we have demonstrated that they can be readily reduced (using zinc) and stored in acidified ethanol. This method of reduction does not rely on excess reductants such as $NaBH_4$ or dithionite [43,44], and therefore the assay mixtures used here require the presence of only quinol, oxidant (nitrate, fumarate or TMAO) and enzyme in the cuvettes used. The use of Teflon plugs to convert disposable acryl cuvettes into anaerobic cuvettes has the additional advantage of eliminating the high cost of stoppered glass optical cuvettes, as well as the inherent risk of breakage.

That $LPCH_2$ appears to be a closer structural analogue of MQH_2 (Figure 1) than PBH_2 is supported by the kinetic parameters presented in Table 2. $LPCH_2$ is a good substrate for NarGHI, FrdABCD and DmsABC, whereas PBH_2 does not appear to work with DmsABC. Both NarGHI and FrdABCD have been shown to interact with ubiquinol and analogues thereof [12,45], and it is therefore tempting to suggest that PBH_2 behaves as a ubiquinol rather than an MQH_2 analogue. However, the $E_{m,7}$ determined here for PBH_2/PB (-40 mV) is much closer to that reported for MQH_2/MQ (-74 mV) [26] than that reported for UQH_2/UQ ($+112$ mV). It is also notable that, despite the similarities in structure, the $E_{m,7}$ of $LPCH_2$/LPC (-310 mV) is 236 mV lower that that reported for MQH_2/MQ. It is clear that the overall similarity in structure between $LPCH_2$ and MQH_2 is one of the most significant factors in determining the ability of the former quinol to donate electrons to the anaerobic reductases at their quinol-binding sites. On the basis of the data presented here, there are clearly significant differences between the quinol-binding specificities of DmsABC from those of NarGHI and FrdABCD.

Our estimate of the $E_{m,7}$ of LPC differs significantly from that reported by Ball [42]. However, our voltammetric data (Figure 5) provide convincing evidence that in our buffer system (100 mM Mops/KOH/5 mM EDTA, pH 7.0), the LPC $E_{m,7}$ is indeed -310 mV. In determining the LPC $E_{m,7}$, we chose to use the same buffer as that used to generate our steady-state kinetic data. The relatively large difference in $E_{m,7}$ between PB and LPC of 270 mV is reasonable considering reported $E_{m,7}$ and E_0 (E_m at pH 0) values for hydroxy- and alkyl-functionalized 1,4-naphthoquinones [41]. The $E_{m,7}$ value for 2-hydroxy-1,4-naphthoquinone (lawsone, $E_{m,7} = -139$ mV) is 172 mV more negative than that of 5-hydroxy-1,4-naphthoquinone (juglone, $E_{m,7} = +33$ mV) as determined by redox titration. The combined effect of adding an alkyl group to the 3- position of lawsone relative to adding a methyl group to the 2- position of juglone is expected to lower $E_{m,7}$ by an additional ~ 65 mV [41]. Thus, from these values, we expect the $E_{m,7}$ for LPC to be ~ 237 mV more negative than that of PB. Considering differences in solution composition and methodology, this agrees well with our observed difference of 270 mV.

The interaction of LPC with electron-transfer chains was originally studied in 1947 by Ball et al. [46] using a bovine heart preparation and the malarial parasite *Plasmodium knowlesi*, and it was shown to be an inhibitor of respiratory electron transfer. Respiratory chains exposed to PB under aerobic conditions have been shown to generate superoxide [47], and as a result it has been found to be both toxic and mutagenic [48]. The interaction of PB with the respiratory chain of *E. coli* has been studied in detail by Imlay and Fridovich [49], who showed that, in addition to toxicity arising from superoxide production, PB is able to divert electron flow from primary dehydrogenases to such an extent that the loss of electron flux through the electron-transport chain to PB is in itself toxic to *E. coli*. The proposed role of PB in superoxide generation suggests that it is unlikely to be suitable for quinol assays carried out under aerobic conditions. In any case, we have observed that both PBH_2 and $LPCH_2$ become rapidly oxidized on exposure to air (results not shown).

The kinetic parameters determined here are consistent with those observed for other quinol and naphthoquinol substrates. For example, the K_m of $DMNH_2$ oxidation by fumarate reductase from *Vibrio succinogenes* has been determined to be 120 μM [24], in reasonable agreement with our K_m values for $LPCH_2$ and PBH_2 oxidation by *E. coli* FrdABCD of 85 and 155 μM respectively. Few kinetic data are available for quinol oxidation by *E. coli* FrdABCD beyond reports of turnover numbers for various site-directed mutants reported by Cecchini and co-workers (for example, see [12]). These workers report turnover numbers for MQ_6H_2 and $DMNH_2$ oxidation by FrdABCD of 321 s^{-1} and 68 s^{-1} respectively. These numbers are of the same order of magnitude as those reported by us for PBH_2 and $LPCH_2$ oxidation (Table 2). The availability of the methodologies and substrates reported here should make more thorough studies of the enzymology of MQH_2 oxidation by the three anaerobic reductases significantly easier to carry out.

Kinetic parameters determined using membrane vesicles or detergent-solubilized enzyme are representative of the relative affinities of the enzymes studied, but must be interpreted with some caution because of the partitioning of lipophilic quinols into the membrane bilayer or micelle at higher concentrations than those calculated for the bulk milieu [50]. This results in a significant underestimation of the K_m for quinol substrates in studies of the mitochondrial cytochrome bc_1 complex. The more hydrophobic a quinol is, the greater the concentration in the membrane vesicles compared with the calculated concentration in the assay cuvette. Also, increased hydrophobicity reduces aqueous solubility such that it may not be possible to have quinol concentrations in the cuvette that are in excess of the apparent K_m. PBH_2 and $LPCH_2$ appear to have an appropriate balance of hydrophobicity and aqueous solubility, so the K_m values of the three enzymes studied are accessible. However, using our methodology, we are unable to report K_m values based on the actual concentration of quinol within the lipid bilayer. In this sense, the parameters reported in Table 2 are apparent rather than actual values.

In none of the studies reported here did we observe deviations from simple Michaelis–Menten kinetics that would be consistent with the presence of multiple MQH_2-binding sites within the enzymes studies. The results are consistent with quinol binding and oxidation occurring at a single dissociable site. It has been suggested that, at least in the case of FrdABCD, there are two binding sites for MQH_2 binding within FrdCD, similar to those observed in the the bacterial photoreaction centre [11,12]. In the model for MQH_2 binding and oxidation for FrdCD, it has been proposed that one of the sites is a high-affinity Q_A site, and the other is a lower-affinity Q_B site. The latter would be the site at

which MQH_2 would bind, be oxidized and then dissociate from the enzyme as MQ. Our data are consistent with a single dissociable Q_B-type site being present in NarGHI, FrdABCD and DmsABC.

Overall, the data reported here demonstrate that PBH_2 and $LPCH_2$ are effective and convenient substrates for *E. coli* anaerobic reductases. PBH_2 and $LPCH_2$ oxidations are sensitive to HOQNO, indicating that their site of binding and oxidation is identical with the site of MQH_2 binding and oxidation. A quick and convenient method of quinol reduction has been developed which eliminates excess reductant from the reaction cuvettes. The methods developed should constitute useful tools in the study of the enzymology of quinol oxidation by anaerobic reductases in *E. coli* and other organisms.

Note added in proof (received 19th March 1998)

We have recently become aware of a cyclic voltammetry study of 4 mM lapachol in 100 mM Hepes buffer (pH 7.0)/5 mM EDTA in which a midpoint potential of −157 mV was measured at a stationary, rather than at a rotating, electrode (K. Heffron and F. A. Armstrong, personal communication).

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