Oxidase and periplasmic cytochrome assembly in *Escherichia coli* K-12: CydDC and CcmAB are not required for haem–membrane association

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The mechanism(s) that bacteria use to transport haem into and across the cytoplasmic membrane to complete the assembly of periplasmic cytochromes is unknown. The authors have tested directly the role(s) of two ATP-binding cassette (ABC) transporters – the *cydDC* and *ccmAB* gene products – in *Escherichia coli* by measuring haem uptake in everted (inside-out) membrane vesicles. If haem is exported to the periplasm *in vivo*, the same process should result in active accumulation in such everted vesicles. [¹⁴C]Haemin (chloride) with bovine serum albumin (BSA) as a carrier protein was accumulated in intact everted membrane vesicles by an energy-independent mechanism. The kinetics of this process were biphasic: rapid uptake/binding was followed by a slower uptake of haem, which was inhibited by a large excess of unlabelled haemin–BSA, but not by BSA. However, accumulated haemin was not chased out of the vesicles by unlabelled haemin–BSA, suggesting specific binding of haemin with the membrane or transport into the lumen of the vesicle. Neither ATP nor a protonmotive force (Δp) generated by lactate oxidation was required for haemin binding or subsequent transport, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), sodium vanadate and monensin had no effect on haemin transport. The rate of haemin uptake following the initial rapid binding was proportional to the external haemin concentration, suggesting that the uptake process was driven by the haemin concentration gradient across the cell membrane. The kinetics of [¹⁴C]haemin uptake were similar in wild-type and *cydD1* or $\Delta ccmA$ mutants, suggesting that the activity of neither the CydDC nor CcmAB transporters is essential for haem export to the periplasm. Cytochrome *d* levels were unaffected by mutations in *trxB* (encoding thioredoxin reductase), *trxA* (thioredoxin), or *grx* (glutaredoxin), suggesting that the CydDC transporter does not export these components of reducing pathways for cytochrome assembly.

Keywords: cytochrome *bd*, oxidase, *Escherichia coli*, haems, membrane transport

INTRODUCTION

The quinol oxidase, cytochrome *bd*, extensively studied in *Escherichia coli* and *Azotobacter vinelandii*, comprises two polypeptide subunits encoded by *cydA* and *cydB* and three cytochrome haems, *b*₅₅₈, *b*₅₉₅ and *d* (Gennis & Stewart, 1996; Poole & Hill, 1997). In *E. coli*,

at least two other genes, *cydC* and *cydD*, which constitute an unlinked operon, are necessary for cytochrome *bd* assembly and function (Georgiou *et al.*, 1987; Poole *et al.*, 1989, 1993, 1994; Delaney *et al.*, 1993; Bebbington & Williams, 1993). The gene products constitute a membrane transporter in the ABC (ATP-binding-cassette) family (Poole *et al.*, 1993). Two separate integral membrane proteins are envisaged as being necessary for function, as in the bacterial HlyB transporter and the *Drosophila* white/brown system (Higgins, 1992). The CydDC system appears to be the first prokaryotic example of a heterodimeric ABC

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Abbreviations: Δp , protonmotive force; ABC, ATP-binding cassette; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

transport system in which each polypeptide contains both hydrophobic membrane-spanning and ATP-binding domains (Poole *et al.*, 1993).

Strains having mutations in *cydD* also lack periplasmic cytochromes *c* (Poole *et al.*, 1994; Goldman *et al.*, 1996), and do not assemble cytochrome *b*₅₆₂ (Goldman *et al.*, 1996). CydD is not required for the synthesis of haem D or the assembly of the CydA and CydB subunits into the membrane (Bebbington & Williams, 1993). We therefore hypothesized that the CydDC transporter may be required for the export of haem(s) to the periplasm for maturation of soluble cytochromes *c* and *b* and perhaps assembly into periplasmically exposed components of cytochrome *bd* (Poole *et al.*, 1993, 1994). Indeed, the haem-binding site for cytochrome *b*₅₅₈ (histidine-186) is predicted to be at the periplasmic face, with the implication that haem attachment may occur there (Spinner *et al.*, 1995). Interestingly, Osborne & Gennis (1999) have reanalysed the probable topology of subunit I of cytochrome *bd* in several bacteria and conclude that the putative ligand for haem *b*₅₉₅ is also close to the periplasmic edge of the membrane, and further that the active site of the oxidase comprising this haem and haem *d* are located near the periplasmic surface. It is not clear whether such a disposition requires haem insertion from outside. The assembly of cytochrome *bo'*, the major alternative oxidase, is unaffected by a mutation in *cydD1* (G. M. Cook & R. K. Poole, unpublished data). Some doubt has been cast on the hypothesis that CydDC is a haem transporter by experiments with haem 'reporters' (Goldman *et al.*, 1996): expression of heterologous haemproteins in the periplasm of *E. coli* results in assembly of the holoprotein (i.e. with haem) even in *cydC* mutants, suggesting that a pathway for haem export must exist in the absence of the CydDC ABC transporter. An alternative hypothesis for CydDC function is that it is required for maintenance of a redox environment in the periplasm appropriate for haem ligation (Goldman *et al.*, 1996), but again no firm role has been proposed and no substrate for the transporter has been identified.

Efforts to understand periplasmic cytochrome *c* assembly in *E. coli* have focused on the eight Ccm (cytochrome *c* maturation) proteins (Thöny-Meyer *et al.*, 1994; Grove *et al.*, 1996; Thöny-Meyer, 1997; Throne-Holst *et al.*, 1997; Schulz *et al.*, 1999). One model is that the products of the *ccmABC* genes, and perhaps *ccmD* as well, constitute an ABC transporter that exports haem to the periplasm, where it is incorporated into cytochrome *c* apoproteins (Thöny-Meyer, 1997). Similar transporters have been proposed in other bacteria: in *Paracoccus denitrificans*, the *ccmABC* genes encode the subunits of an ABC transporter with the composition (CcmA)₂-CcmB-CcmC (Page *et al.*, 1997). The *helABC* and *helD* genes (haem export for ligation) of *Rhodobacter capsulatus* (Goldman *et al.*, 1997) are also thought to encode an ABC transporter necessary for cytochrome *c* assembly. No firm evidence for the transport processes catalysed by such pathways has however been presented. Fur-

thermore, since *ccmABC* mutants of *P. denitrificans* are able to assemble periplasmic cytochromes *b*, and exogenously added haem fails to restore assembly of periplasmic cytochromes *c* (Page *et al.*, 1997), this hypothesis appears untenable. Recently, Schultz *et al.* (1999) have shown in *E. coli* that CcmA and CcmB (but not CcmC) do constitute an ABC transporter but that it is not essential for cytochrome *c* maturation. (In this paper, we embrace this finding and refer to the ABC transporter encoded by the *ccm* operon in *E. coli* as CcmAB, not CcmABC.) It is proposed that CcmC (a transmembrane protein) and CcmD serve to transfer haem to CcmE, an essential haem chaperone that passes haem to periplasmic apocytochromes. Several lines of evidence suggest that CcmAB is not a haem transporter (Schulz *et al.*, 1999) but no direct measurements of haem transport have been reported.

Movement of haem into and across lipid bilayers plays a critical role not only in the biogenesis of periplasmic haemproteins in bacteria, but also in that of eukaryotic extramitochondrial haemproteins, such as globins. However, transmembrane movement of haem is likely to be slow (Light & Olson, 1990), predicting the need for haem transport systems. Nevertheless, despite concerted efforts from several groups and claims for the existence of bacterial haem transporters, no clear candidate has emerged, although putative haem exporters have been found in several bacterial genera (Beckman *et al.*, 1992; Thöny-Meyer *et al.*, 1994; Aguilar & Soberon, 1996; Page *et al.*, 1997). This may be in part a result of technical difficulties in measuring transport kinetics of haem, which self-aggregates in aqueous solutions and interacts non-specifically with cellular components. Some workers have used haem-CO, which is monomeric in dilute aqueous solutions (Light & Olson, 1990), whilst others have used haem bound to a carrier protein (e.g. Stojiljkovic & Hantke, 1994). We are unaware of direct attempts to determine rates of haem transport across *E. coli* isolated cytoplasmic membranes and report such experiments here.

METHODS

Strains, culture media and growth conditions. *E. coli* K-12 strains and plasmids used in this study are described in Table 1. Genetic crosses were performed using bacteriophage P1-mediated transduction (Miller, 1972). Cells were grown either in Luria broth, initial pH 7.0 (Miller, 1972), or in MOPS-buffered minimal medium with either 40 mM glucose or lactose as sole carbon source (Stewart & Parales, 1988). The initial pH of MOPS medium was 7.4. All additions were at the concentrations listed in the text. Kanamycin was used at a final concentration of 50 µg ml⁻¹. Agar and other dehydrated media were from Difco or Oxoid. Other components were from Sigma or BDH-Merck.

Culture optical density was generally measured with a Pye-Unicam SP6-550 spectrophotometer at 600 nm, using culture samples diluted with medium to bring OD₆₀₀ to below 0.7 when measured in cuvettes of 1 cm pathlength. Aerated cultures were grown with shaking (200 r.p.m.). An anaerobic growth atmosphere was generated using a BBL GasPak anaerobic system in an anaerobic jar.

Table 1. *E. coli* strains used in this study

Strain	Relevant genotype	Reference or source
A179	<i>trxA14::kan</i>	Russel <i>et al.</i> (1990)
A326	<i>trxB::kan</i>	Russel & Model (1988)
A410	K38 Δ <i>trxA zbi::Tn10</i> <i>grx::kan</i>	Russel & Holmgren (1988)
AN2342	F ⁻ ; referred to as wild-type	Poole <i>et al.</i> (1989)
AN2343	<i>cydD1</i> (point mutant)	Poole <i>et al.</i> (1989)
EC21	As MC1061 but Δ <i>ccmA</i>	Throne-Holst <i>et al.</i> (1997)
MC1061	F ⁻ ; referred to as wild-type	Throne-Holst <i>et al.</i> (1997)
SMB420	<i>grxA</i> Δ (<i>gal-grx</i>)	Russel & Holmgren (1988)

Cytochrome *d* assays. For identification and quantitation of cytochrome *d*, cells were grown in Luria broth (Miller, 1972) to an OD₆₀₀ of 0.9–1.0 (measured after correction for dilution where necessary). Harvested cells were washed in 0.1 M potassium phosphate buffer (pH 7.0) and used to record reduced *minus* oxidized and CO difference (CO-reduced *minus* reduced) spectra at room temperature as previously described (Poole *et al.*, 1989). Cytochrome *d* concentrations were calculated from the former using an absorption coefficient of 18.5 mM⁻¹ cm⁻¹ from $A_{630-650}$ (Kita *et al.*, 1984). The protein content of cell suspensions was assayed by the method of Markwell *et al.* (1978).

Preparation of everted membrane vesicles. Cells were grown aerobically to the mid-exponential phase of growth, either in minimal MOPS medium supplemented with glucose or lactose or in LB. Defined medium was used only for studies of lactose transport, where variation of carbon source was required (see Results). Cells were harvested by centrifugation and the cell pellet washed with pre-cooled 100 mM potassium phosphate buffer (pH 7.0) containing 10 mM disodium EDTA, and resuspended in the same buffer (5 ml per litre of original culture). To prepare everted vesicles, the procedure of Thanassi *et al.* (1997) was used. In brief, cells were disrupted by one passage through a French pressure cell at 5000 p.s.i. (34.5 MPa). Pancreatic DNase was added to 0.1 mg ml⁻¹, and the mixture was kept on ice for 1 h or until the viscosity decreased significantly. After centrifugation at 27000 *g* for 10 min, the vesicles were sedimented from the supernatant suspension by centrifugation at 150000 *g* for 1 h. Vesicles were gently resuspended in the same buffer and collected again by centrifuging under the same conditions. They were then resuspended in 100 mM potassium phosphate buffer (pH 7.2) containing 5 mM MgCl₂ to a concentration of 15 mg protein ml⁻¹. Aliquots (200 μ l) of the vesicles were stored at -70 °C after rapid freezing in liquid nitrogen.

[¹⁴C]Haemin and [¹⁴C]lactose transport assays. We have adopted the following nomenclature: the term 'haem' is used to describe the chelate complex of Fe(II) with protoporphyrin IX, the prosthetic group of, for example, cytochrome *b* or haemoglobin; 'haemin' is the chloride of the Fe(III) form, and 'haematin' is the hydroxide of the Fe(III) form. [¹⁴C]Haemin [approx. 130 mCi mmol⁻¹ (4810 MBq mmol⁻¹)] was purchased from Professor Stan Brown, University of Leeds Innovations Ltd (Woodhouse Lane, Leeds LS2 3AR, UK). Haemin transport assays were based on previously established methods (Stojiljkovic & Hantke, 1994; Tompkins *et al.*, 1997). [¹⁴C]Haemin (1 μ Ci; 37 kBq) was solubilized in a drop of 0.1 M NaOH and diluted with 50 mM potassium phosphate buffer (pH 7.2) containing bovine serum albumin

(BSA; 10 mg ml⁻¹) as a carrier for haemin to avoid polymerization, self-aggregation, and non-specific interaction with cellular components. [¹⁴C]Lactose (57 mCi mmol⁻¹; 2109 MBq mmol⁻¹; Amersham) was added to achieve a final concentration of 400 μ M in the transport assay. Everted vesicles were thawed slowly on ice and diluted to a protein concentration of 0.5–1.0 mg ml⁻¹ in 50 mM potassium phosphate buffer (pH 7.2) containing 5 mM MgCl₂. Vesicles were added to glass tubes containing 200 μ l of the same pre-warmed phosphate buffer and pre-incubated for 15 min without shaking in a water bath at 30 °C. To initiate transport, radiolabelled [¹⁴C]haemin or [¹⁴C]lactose was added to achieve final concentrations of 5.7 μ M or 400 μ M, respectively. Vesicles were energized for 15 min prior to [¹⁴C]haemin–BSA or [¹⁴C]lactose addition with either 10 mM D-lactate or ATP (2–5 mM). Vesicles were de-energized with either carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 50 μ M) to collapse the proton gradient (Δ p), monensin (100 μ M) to collapse a sodium gradient, or sodium vanadate (100 μ M), a specific inhibitor of ATP-driven transport systems (Pressman, 1976). After 0–20 min, transport was terminated by the addition of ice-cold LiCl (2 ml, 100 mM) and rapid filtration (0.45 μ m pore size cellulose-nitrate filter). The filter was washed twice with 2.0 ml of the LiCl solution, dried for 20 min at 105 °C, and radioactivity measured by liquid scintillation counting. To minimize non-specific binding of [¹⁴C]haemin–BSA to filters, the filters were pre-soaked in 100 mM LiCl containing BSA (10 mg ml⁻¹).

RESULTS

[¹⁴C]Haemin–BSA and [¹⁴C]lactose transport in everted membrane vesicles from wild-type cells

The inwardly directed transport of [¹⁴C]haemin–BSA by everted membrane vesicles was measured. Transport under these conditions would simulate the export of haem from the cytoplasm (outside of vesicle) to the periplasm (lumen of vesicle). First, to demonstrate that the everted vesicle procedure used here was effective for the strains in this study, we tested the ability of vesicles to accumulate [¹⁴C]lactose in response to an energized membrane. Lactose can be transported by both right-side-out and everted vesicles, provided that an energy source such as sodium lactate is supplied to generate a protonmotive force (Δ p). Everted vesicles prepared from cells grown in MOPS minimal medium supplemented with glucose had a low rate of lactose uptake

Fig. 1. Comparison of uptake of [¹⁴C]lactose and [¹⁴C]haemin-BSA by everted membrane vesicles from wild-type and *cydD* mutant strains of *E. coli*. In (a), vesicles were prepared from strain AN2342 (wild-type) grown in MOPS minimal medium with lactose as sole carbon source. Sodium lactate (20 mM final concentration) was added 15 min prior to addition of [¹⁴C]lactose (400 μ M final concentration) at time zero (■), but not in the control (○). Results are typical of several experiments. In (b) and (c), everted vesicles were prepared from cells grown aerobically in LB medium. In (b), ATP (●, 5 mM) or sodium lactate (△, 20 mM) was added to vesicles from the wild-type strain (AN2342), 15 min prior to addition of [¹⁴C]haemin-BSA (5.7 μ M final haemin concentration). The control (■) contained neither ATP nor sodium lactate. In (c), [¹⁴C]haemin-BSA uptake by vesicles from strain AN2343 (*cydD1*, ○) and AN2342 (wild-type, ■) is compared; no ATP or sodium lactate was added. Values shown are means $\pm$ SD bars.

[$< 1.5 \times 10^{-3}$ nmol min⁻¹ (mg protein)⁻¹; not shown] and this rate (determined over the first 60 s) was increased 25-fold [to 0.04 nmol min⁻¹ (mg protein)⁻¹] if cells were grown with lactose as the sole carbon and energy source in minimal medium. Significant [¹⁴C]lactose uptake occurred only if sodium lactate was added as an energy source in the transport assay (Fig. 1a). Lactose-grown cells were also used in preliminary experiments to measure accumulation of [¹⁴C]haemin, with similar results to those below. These results demonstrate that the everted vesicles used are capable of energization by sodium lactate and actively accumulate [¹⁴C]lactose or [¹⁴C]haemin with time. ATP (5 mM) addition was also able to energize lactose transport (data not shown). This accumulation was abolished by the addition of CCCP (50 μ M final concentration) (data not shown), demonstrating that the vesicles are intact and that lactose transport is dependent on $\Delta\mu$.

To test the ability of everted vesicles to accumulate [¹⁴C]haemin, BSA was used as a carrier protein for [¹⁴C]haemin. BSA was included at concentrations exceeding that of total haemin to prevent non-specific binding and self aggregation of the haemin (Stojiljkovic & Hantke, 1994; Tompkins *et al.*, 1997). The haemin-albumin dissociation constant is 10^{-8} M and therefore haemin is bound with high affinity by this protein (Beaven *et al.*, 1974). When [¹⁴C]haemin-BSA (5.7 μ M final concentration) was added to vesicles from wild-type strain AN2342, even in the absence of an energy source, the kinetics of [¹⁴C]haemin-BSA uptake were biphasic (Fig. 1b). Substantial association of [¹⁴C]haemin-BSA with the vesicles occurred within 2 min of haemin addition (Fig. 1b) and similar counts were obtained after incubation for only 5 s (not shown). The rapidity of the reaction and its ATP independence strongly suggest that this phase represents binding of the

haem to the lipid bilayer as reported previously for liposomes (Rose *et al.*, 1985). After this initial rapid binding, there was further uptake of [¹⁴C]haemin-BSA, which steadily increased with further incubation. The rate of this uptake calculated between 2 and 12 min after haemin addition was 0.08 nmol min⁻¹ (mg protein)⁻¹. At 12 min, the amount of [¹⁴C]haemin-BSA that had been taken up represented less than 20% of the total radioactive concentration of [¹⁴C]haemin, indicating that haemin uptake was still occurring and that the system was not fully saturated due to all the [¹⁴C]haemin-BSA having been bound.

Because CydDC is an ABC transporter presumed to be energized by ATP, ATP (5 mM) was added to the transport assay 10 min before [¹⁴C]haemin-BSA. When either ATP or sodium lactate was added to the membrane vesicles prior to [¹⁴C]haemin-BSA addition, both the initial [¹⁴C]haemin-BSA binding and the subsequent transport occurred at rates (again determined between 2 and 12 min) that did not differ significantly from that seen where neither ATP nor lactate had been added (Fig. 1b, Table 2). It is probable that the differences between the three uptake experiments shown in Fig. 1(b) and below (Fig. 3) are due to the experimental difficulties of working with solutions of such a hydrophobic substrate, and are not due to major differences in the proportion of vesicles that are everted (see Discussion). We conclude that the uptake of [¹⁴C]haemin-BSA is not dependent on an externally provided energy source. Furthermore, none of sodium vanadate (100 μ M), monensin (100 μ M) or CCCP (50 μ M) had any significant effect on the rate of [¹⁴C]haemin-BSA uptake by wild-type cells (results not shown), suggesting that an energized membrane is not required for haemin uptake and that ATP-driven transporter activity does not play a major role in haemin transport in these vesicles.

Table 2. Effect of external energy sources and metabolic inhibitors on [14 C]haemin-BSA uptake by strains of *E. coli*

[14 C]Haemin-BSA was added to a final concentration of 5.7 μ M. ATP (5 mM final concentration) or lactate (20 mM final concentration) was added, except in the controls, 15 min prior to [14 C]haemin-BSA addition. The rates reported were determined from 2 min to 12 min. The data are means of three independent determinations; errors associated with these means are demonstrated by Figs 1 and 3.

Strain, genotype	Rate of haemin transport [nmol min ⁻¹ (mg protein) ⁻¹]		
	Control	+ ATP	+ Lactate
AN2342, wild-type	0.09	0.10	0.11
AN2343, <i>cydD1</i>	0.06	0.08	0.08
MC1061, wild-type	0.15	0.16	0.13
EC21, Δ <i>ccmA</i>	0.17	0.18	0.16

Fig. 2. Dependence of the rate of [14 C]haemin uptake by everted membrane vesicles of AN2342 on haemin-BSA concentration. The rates of haemin uptake shown were determined from 2 min to 12 min after addition of [14 C]haemin-BSA. No ATP or sodium lactate was used in these assays. Values shown are means $\pm$ SD bars.

Because [14 C]haemin-BSA uptake did not appear to be dependent on an energized membrane, the driving force for [14 C]haemin-BSA was studied. When the external concentration of [14 C]haemin-BSA was varied from 5.7 μ M to 28 μ M, the rate of [14 C]haemin-BSA uptake determined between 2 and 12 min was proportional to the external haemin concentration over the range from about 5 to 20 μ M (Fig. 2), indicative of a diffusion type transport mechanism. This mechanism does not rule out the possibility that carrier proteins embedded in the membrane facilitate transmembrane movement.

It is technically difficult to determine how much of the [14 C]haemin-BSA that was taken up by the everted membrane vesicles is inside the vesicles and how much resides at the outer leaflet of the inside-out membrane vesicle. To determine if this [14 C]haemin-BSA was specifically bound, the vesicle-bound [14 C]haemin-BSA

was chased with a large excess of unlabelled haemin-BSA. In these experiments, vesicles were pre-incubated with [14 C]haemin-BSA for 10 min and then a 150-fold excess of non-radioactive haemin-BSA was added to the transport assay. The rate of [14 C]haemin-BSA uptake was determined over a further 10 min. When unlabelled haemin-BSA was added to wild-type membrane vesicles that had been accumulating [14 C]haemin-BSA for 10 min, there was no further uptake of [14 C]haemin-BSA, presumably because the binding sites and accumulation within the vesicles were saturated (Fig. 3a). That the unlabelled haemin-BSA did not displace a significant fraction of the label suggests that [14 C]haemin-BSA was either bound specifically to the membrane, probably associated with protein, or had accumulated in the lumen of the vesicle.

[14 C]Haemin-BSA transport in everted membrane vesicles from ABC transporter mutants

The ATP-independence of the haem movements described above strongly suggests that the activities of ABC-type transporters, such as CydDC or CcmAB, do not contribute significantly. This was confirmed by experiments with vesicles prepared from the *cydD1* mutant AN2343, which revealed similar patterns of [14 C]haemin-BSA uptake as in the isogenic wild-type strain (Fig. 1c). The rate of [14 C]haemin-BSA uptake as determined between 2 and 12 min was 0.06 nmol min⁻¹ (mg protein)⁻¹ (Table 2). As observed for the wild-type strain, unlabelled haemin-BSA did not displace a significant fraction of the label from vesicles prepared from the *cydD1* mutant (Fig. 3b).

To test the role of the CcmAB proteins in haemin transport, everted vesicles were prepared from cells grown anaerobically on glycerol and nitrate, conditions required for the induction of the *ccmAB* genes. Everted vesicles from either MC1061 (wild-type) or EC21 (Δ *ccmA*) accumulated [14 C]haemin-BSA with time and there was little difference between the strains in terms of the rate of [14 C]haemin-BSA uptake as calculated between 2 and 12 min (Fig. 3c, Table 2). The uptake was biphasic, and bound [14 C]haemin-BSA could not be chased in either strain and was not dependent on an exogenous energy source (Table 2).

Effect of the reducing environment on cytochrome *bd* assembly

Goldman *et al.* (1996) reported that *cydC* mutants have a more oxidized periplasm than that of wild-type cells and *cydAB* mutants. These results are consistent with the CydDC transporter exporting a reductant to the periplasm that is required either for haem ligation and/or to ensure that the redox environment of the periplasm is appropriate for haem ligation. To test this hypothesis, a number of reducing pathways in *E. coli* were tested for their effect on cytochrome *d* levels. Wild-type cells contained approximately 0.04 nmol haem *d* (mg protein)⁻¹ as measured in reduced *minus* oxidized and CO difference spectra (not shown). In contrast,

Fig. 3. [^{14}C]Haemin transport by everted vesicles from wild-type cells and ABC transporter mutants and the effects of chasing with unlabelled haemin. For strains AN2342 (wild-type) (a) and AN2343 (*cydD1*) (b), everted vesicles were prepared from cells grown aerobically in LB medium, and transport was assayed as described in Fig. 1. An excess (150-fold) of unlabelled haemin-BSA was added at 10 min (indicated by the arrows) to chase non-specifically bound haemin ($\circ$). In controls ($\blacksquare$), no excess unlabelled haemin was added. Panel (c) shows [^{14}C]haemin-BSA uptake by everted membrane vesicles prepared from strains MC1061 (wild-type, $\blacksquare$) and EC21 (ΔccmA , $\circ$) grown anaerobically on glycerol and nitrate. In all experiments, no ATP or sodium lactate was added. Values shown are means $\pm$ SD bars.

cytochrome *d* levels in an isogenic *cydD1* mutant were undetectable. Mutations in the upstream gene *trxB* did not reduce the level of cytochrome *d* significantly compared to the wild-type, confirming previous results (Poole *et al.*, 1994). Furthermore, mutations in *grxA* (glutaredoxin), *trxA* (thioredoxin), or both alleles, (*grxA trxA*) did not cause a loss of cytochrome *d* (not shown).

DISCUSSION

It is well established that haem rapidly associates with lipid bilayers. The aromatic portion of haem is thought to intercalate between the acyl chains of neighbouring lecithins in liposome model systems and the haem propionates lie in the polar region of the bilayer (Cannon *et al.*, 1984). Nevertheless, the outer membranes of Gram-negative bacteria are considered impermeable to haem (McConville & Charles, 1979). This has led to the characterization of haem-utilization systems from other bacteria by complementation of *E. coli* *hemA* mutants (Henderson & Payne, 1994; Stojiljkovic & Hantke, 1992; Stojiljkovic *et al.*, 1996; Torres & Payne, 1997).

However, there is conflicting evidence regarding the ability of haemin to penetrate the cytoplasmic membrane and the requirement for specific haem transporters, whether for inward or outward movement (with reference to the cell) in this process. First, respiration-deficient membrane vesicles from a *hemA* mutant of *E. coli*, which cannot synthesize haem, can be reconstituted *in vitro* by adding haematin and ATP (Haddock & Schairer, 1973). If cytoplasmic membranes are intrinsically impermeable to haem, this result implies either that all haemoproteins required for respiration are exposed to the outer faces of the membrane vesicles, or that haem transport is driven by ATP. Respiration is not reconstituted, however, by adding haematin to cells during growth (Haddock & Schairer, 1973), presumably

because of the requirement for outer-membrane haem receptor and transport systems in *E. coli* (Stojiljkovic & Hantke, 1992). Stojiljkovic & Hantke (1992) expressed the *Yersinia enterocolitica* haemin receptor HemR in *E. coli* K-12, thus conferring the ability to use haemin as a porphyrin source. Additional gene products appear necessary for using haemin as an iron source, since *Y. enterocolitica* mutants defective in *hemU* and *hemV* (encoding a putative haem-specific permease and ATPase, respectively) are severely impaired in haemin utilization, yet still show weak growth stimulation by haemin (Stojiljkovic & Hantke, 1994). This suggests leakage of haemin through the cytoplasmic membrane. Furthermore, Torres & Payne (1997) have demonstrated that introduction of the gene for an outer-membrane protein is sufficient to confer haem utilization in *E. coli* K-12, and growth *in vivo* of pathogenic *E. coli* strains is stimulated by haemin or haemoglobin, perhaps again indicating the presence of a cytoplasmic membrane transporter for haem (Stojiljkovic & Hantke, 1994) or perhaps that only the outer membrane presents a significant barrier to uptake to the cytoplasm. Nevertheless, it is tacitly assumed that *E. coli* has a mechanism(s) for haem translocation from the cytoplasm to the outer face of the cytoplasmic membrane, where it is required for periplasmic cytochrome assembly (Beckman *et al.*, 1992; Goldman *et al.*, 1998; Thony-Meyer *et al.*, 1994; Thony-Meyer, 1997). Such a mechanism(s) has so far remained unidentified.

The present study has used everted vesicles prepared after cell disruption in the French pressure cell. It is widely recognized that this procedure results in a population of vesicles that is predominantly inverted with respect to the *in vivo* orientation. We consider it unlikely that the small variations seen between experiments (as in Figs 1 and 3) are due to variation in the proportion of everted vesicles, since specific transport

Fig. 4. Possible pathways for haem reaction with, and transport through, the cytoplasmic membrane of *E. coli*. Haem synthesized in the cytoplasm (IN) associates rapidly with the inner leaflet of the cytoplasmic membrane, largely through hydrophobic interactions. The anticipated, relatively slow (see text) flipping of haem to the outer leaflet is shown in A. Alternatively (B), haem may be transported through the membrane via a pore formed by a specific transporter. Finally (C), haem may interact within the bilayer with a carrier, such as an ABC-type exporter, which acts as a 'flippase', speeding up, in an ATP-dependent fashion, the movement of haem to the outer leaflet or directly into the periplasm (OUT). Dissociation of haem from the outer leaflet is proposed to be facilitated by the presence of haem-binding protein(s) such as the haem chaperone CcmE (not shown). Other proteins may be involved in initial haem capture or the haem may be involved directly in insertion into apohaemoproteins like cytochrome *c* or *b*₅₆₂, or perhaps into the periplasmically exposed regions of the cytochrome *bd* quinol oxidase (not shown). The outer membrane (OM) is portrayed as being impermeable to haem, and thus requiring haem transport proteins (not detailed).

activity has been shown by others to be insensitive to experimental variables, specifically French cell pressure, for example, between 4000 and 14000 p.s.i. The procedure of Thanassi *et al.* (1997) used here gives vesicles that exhibit negligible uptake of proline (which is transported inwards *in vivo*), which is stimulated 60-fold when vesicles are prepared by lysosyme-EDTA treatment (McMurry *et al.*, 1980). Other criteria for the everted nature of vesicles isolated after French press disruption include high rates of uptake of Ca^{2+} (which is exported by intact cells), accessibility of substrates to enzymes whose activity is normally cryptic, the direction of respiration- and ATPase-driven H^+ translocation, exposure of inaccessible antigenic determinants, and morphology (Rosen & Tsuchiya, 1979). On these bases it seems highly probable that the haem-membrane interactions described here are with everted vesicles.

We found that interaction of haem with the membrane does not require ATP, an oxidizable substrate, or $\Delta\mu$. [^{14}C]Haemin-BSA uptake appears to be a biphasic process. The first rapid phase is probably haem-lipid association; the second slower phase is driven by the concentration gradient of haemin across the cell membrane and probably represents haem movement across the bilayer, possibly resulting in ultimate dissociation from the 'inner' face of the membrane (Fig. 4). The lack

of saturation kinetics suggests either that the process is not catalysed by specific transport proteins or that higher concentrations of haemin are required for saturation. These results agree with those obtained for haem-CO and unilamellar lipid vesicles (Light & Olson, 1990): 'transmembrane movement is slow and distinct from the initial binding process'. Possible involvement of the *cydDC* and *ccmAB* gene products in haemin transport could not be demonstrated, the rates of haemin uptake being comparable in wild-type and mutant strains. Thus, the CydDC and CcmAB transporters do not seem to be necessary for haem transport, but our experiments do not rule out the possibility that such proteins accelerate haem transport *in vivo* or the existence of other haem-binding membrane components. The lack of involvement of CydDC in haem transport might seem to support the work of Goldman *et al.* (1996), who expressed various haem apoproteins in the *E. coli* periplasm and showed that mutations in *cydC* did not prevent periplasmic holoprotein assembly, even in the absence of exogenous haemin. However, the experiments of Light & Olson (1990) clearly demonstrate the possibility of haem movement into and out of the bilayer in the absence of transport proteins; dissociation from the bilayer is readily demonstrated when liposomes with embedded haem are mixed with apomyoglobin. Thus the experiments of Goldman *et al.*

(1996), which utilized artefactually high periplasmic concentrations of apoproteins, may have provided a 'trap' for haem dissociating outwards from the bilayer into the periplasm. The mechanism for such haem movement in *E. coli* is becoming clearer. CcmE is a protein N-terminally anchored to the cytoplasmic membrane, which accumulates in a haem-bound form in *ccmF* mutants defective in cytochrome *c* assembly (Reid *et al.*, 1998). The haem is bound covalently by a histidine residue and is released in the presence of apocytochrome *c* and other Ccm proteins needed for assembly (Schulz *et al.*, 1998). Schulz *et al.* (1999) have shown that only CcmC is absolutely required for the charging of CcmE with haem, at least when CcmC is highly expressed. It could not be excluded from those experiments, however, that haem translocation normally occurs via the CcmAB transporter, only that high abundance of CcmC allows haem to cross the membrane in the absence of CcmAB. Perhaps the strong haem binding of CcmC (evidenced by the persistence of haem in SDS gels) could 'pull' or extract haem from the lipid bilayer, as demonstrated for the reaction of apomyoglobin with liposomes (Light & Olson, 1990).

In this work, radioactive haemin-BSA uptake was inhibited by the addition of an excess of non-radioactive haemin-BSA, but not by BSA alone. Significantly, the bound haemin was not chased by unlabelled haemin. Like Stojiljkovic & Hantke (1994), we interpret this as indicating specific binding, presumably to haem-binding components of the membrane that have a greater affinity for haemin than does BSA. The identity of these components is presently unknown, but haem-starved *Prevotella intermedia* cells express approximately 4000 haem binding sites per cell and a haem dissociation constant of 1×10^{-9} (Tompkins *et al.*, 1997). Other haem-binding proteins also have a high affinity for binding haem. For example the haem-haemopexin complex has a dissociation constant of 10^{-12} M (Hrkal *et al.*, 1974) and haptoglobin has an extremely high affinity for binding haemoglobin (K_d of $<10^{-15}$ M) (Hwang & Greer, 1980).

Phospholipid membranes have a high affinity for haem, and the interaction of phospholipid with haemin, which can be considered 'an aromatic, soap-like molecule' (Rose *et al.*, 1985), has a major hydrophobic component. It is therefore pertinent to compare possible mechanisms of haem transport with what is known about hydrophobic drugs. These have also been shown to be taken up by a biphasic process (see Bolhuis *et al.*, 1997 for a review). Normally this is observed as a fast entry into the outer leaflet of the membrane followed by a slower movement across the bilayer to the inner leaflet of the membrane (Bolhuis *et al.*, 1997). The ability of amphiphilic substrates to partition in the inner leaflet of the membrane is a prerequisite for recognition by multidrug-resistant transporters which might function as a hydrophobic 'vacuum cleaner' to remove drugs from the inner leaflet and extrude them from the membrane. There is a direct relationship between the amount of hydrophobic drug associated with the cytoplasmic leaflet of the lipid

bilayer and the rate of transport, as observed for drug extrusion by the lactococcal multidrug transporter LmrP (Bolhuis *et al.*, 1997). According to the 'flippase' model (Higgins & Gottesman, 1992), the function of the ABC transporter might be to pump substrate (perhaps haem?) from the inner to the outer leaflet (Fig. 4), from where the haem may diffuse to the periplasm, haem chaperone or membrane-associated apoprotein. In our experiments, inability to chase labelled haemin may reflect tight binding to the CydDC subunits even though they are incapable of active translocation. The idea of flipping is not new, and this term was used by Rose *et al.* (1985) in the context of haem transport. Interestingly, though, the experiments of Light & Olson (1990) with unilamellar lipid vesicles reveal a second slow phase in both the association and dissociation directions that might equate with the transmembrane movement role envisaged for ABC transporters. It is important to note that the present results do not rule out energization-dependent haem translocation from an aqueous phase on one side of the membrane to the aqueous phase on the other. Our results show only that haemin (proffered as a BSA complex) is bound to the membrane, and rendered incapable of being chased, independently of membrane energization or the involvement of CydDC or CcmAB. It is feasible, for example that, *in vivo*, haem binds to the inner leaflet of the cytoplasmic membrane bilayer and may cross to the outer leaflet, and only then might its passage into the periplasm (or the interior of a vesicle) require the ATPase activity of the transporter.

Could haem export to the periplasm proceed without a specific transporter? Although the transmembrane movement of haem-CO in liposomes having long unsaturated acyl chains is very slow (the release phase having a rate constant of about 0.001 s^{-1} ; Light & Olson, 1990), such rate constants may underestimate the *in vivo* rates for natural haem substrates. The rate may be raised further by protein-induced discontinuities in apolar regions of the membrane similar to that seen *in vitro* at temperatures near the phase-transition temperature. Finally, perhaps even a half-time of 10 min for haem flipping as estimated for egg lecithin liposomes might be sufficient for haem assembly and function in the periplasm.

Despite results from many laboratories, the substrates for the transporters encoded by both the *cydDC* and *ccmAB* gene products are unknown. Recent work from Ferguson and co-workers has suggested that the putative CcmABC haem transporter in *P. denitrificans* does not transport either haem or *c*-type apocytochromes and suggests a low-molecular-mass thiol or oxidized thiol as substrate (Page *et al.*, 1997). However, cysteine, glutathione or 2-mercaptoethanesulfonic acid could not complement a $\Delta ccmA$ mutation in *E. coli* (Schulz *et al.*, 1998). Similar proposals for the nature of the transported substrate have been put forward by Poole *et al.* (1994) and Schulz *et al.* (1999). Essentially, the substrate is considered to be a reductant that keeps haem and/or apoprotein cysteine residues in the periplasm reduced for haem ligation, despite the oxidizing environment of

the periplasm. Disulfide bond formation plays a key role in protein folding in the periplasm of Gram-negative bacteria (Raina & Missiakis, 1997). It is notable then that: (a) the gene upstream of the *cydDC* operon is *trxB*, encoding thioredoxin reductase (Russel & Model, 1988), and (b) in many cases, genes encoding an ABC exporter are adjacent to the gene encoding the transported product (Salmond & Reeves, 1993). In the cytoplasm, a reducing balance is maintained using three glutaredoxins (products of *grxA*, *grxB* and *grxC*) as well as the thioredoxin (*trxA/trxC*)/thioredoxin reductase (*trxB*) system (for a review, see Åslund & Beckwith, 1999). Furthermore, Goldman *et al.* (1996) have reported that *cydC* mutants have a more oxidized periplasm than do wild-type and *cydAB* mutants. These results are consistent with the CydDC proteins exporting a reductant to the periplasm that is required either for haem ligation and/or to ensure a redox environment of the periplasm appropriate for haem ligation. However, the present results show that none of *trxB*, *trxA* or *grxA* is required for cytochrome *bd* assembly, although *trxA* function is needed for the transfer of electrons from the cytoplasm to periplasmic DsbC for cytochrome *c* assembly (Reid *et al.*, 1998).

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