The *Escherichia coli* CcmG protein fulfils a specific role in cytochrome *c* assembly

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In *Escherichia coli* K-12, *c*-type cytochromes are synthesized only during anaerobic growth with trimethylamine-*N*-oxide, nitrite or low concentrations of nitrate as the terminal electron acceptor. A thioredoxin-like protein, CcmG, is one of 12 proteins required for their assembly in the periplasm. Its postulated function is to reduce disulphide bonds formed between correctly paired cysteine residues in the cytochrome *c* apoproteins prior to haem attachment by CcmF and CcmH. We report that loss of CcmG synthesis by mutation was not compensated by a second mutation in disulphide-bond-forming proteins, DsbA or DsbB, or by the chemical reductant, 2-mercaptoethanesulphonic acid. An anti-CcmG polyclonal antibody was used in Western-blot analysis to probe the redox state of CcmG in mutants defective in the synthesis of other proteins essential for cytochrome *c* assembly. The oxidized form of CcmG accumulated not only in *trxA* or *dipZ* mutants defective in the transfer of electrons from the cytoplasm for disulphide isomerization and reduction reactions in the periplasm, but also in *ccmF* and *ccmH* mutants. The requirement of both CcmF and CcmH for the reduction of the disulphide bond in CcmG indicates that CcmG functions later than CcmF and CcmH in cytochrome *c* assembly, rather than in electron transfer from the membrane-associated DipZ (also known as DsbD) to CcmH. The data support a model proposed by others in which CcmG catalyses one of the last reactions specific to cytochrome *c* assembly.

Key words: cytochrome *c* maturation, disulphide reduction, periplasmic nitrite reduction, redox state of CcmG, thioredoxin-like proteins.

INTRODUCTION

Unlike mammals and many aerobic micro-organisms, *c*-type cytochromes are not essential for the aerobic growth of enteric bacteria. During anaerobic growth, five *c*-type cytochromes are synthesized by *Escherichia coli* in response to the availability of trimethylamine-*N*-oxide, nitrite or growth-limiting concentrations of nitrate [1]. The structural genes for the five *c*-type cytochromes, all of which contain multiple haem groups, are located in the *nap* (for nitrate reductase in the periplasm), *nrf* (nitrite reduction by formate) and *tor* (trimethylamine-*N*-oxide reduction) operons [2–4].

Synthesis of *c*-type cytochromes in *E. coli* is a complex process, involving a series of sequential or co-ordinated steps culminating in the attachment of haem to form the holo-cytochrome in the bacterial periplasm [5–7]. In addition to the structural genes, 12 other genes are essential for the assembly of all holo-cytochromes *c* in *E. coli*: these are the eight *ccm* genes located downstream of the *napF* operon (where 'Ccm' is derived from 'cytochrome *c* maturation') [2,8], *dsbA* (where 'Dsb' is derived from 'disulphide-bond formation') [9], *dsbB* [10], *trxA* [11] (where 'Trx' is derived from thioredoxin) and *dipZ* [12] (also known as *dsbD*) (where 'Dip' is derived from 'disulphide-isomerase-like protein'). Three additional genes, *nrfE*, *nrfF* and *nrfG* (where 'Nrf' is derived from 'nitrite reduction by formate'), have been shown to be required (although *nrfF* has not been shown to be essential) for the attachment of a haem group to a Cys-Trp-Ser-Cys-Lys motif at the active site of the terminal nitrite reductase, NrfA [13]. The unfolded apoprotein is first secreted into the periplasm [14], where pairs of reduced cysteine residues, to which haem is finally attached, are oxidized into disulphide bonds by DsbA [9]. The reduced DsbA is re-oxidized by its membrane-bound partner protein, DsbB [15,16], which in turn is regenerated by transferring electrons via the quinone pool to terminal reductases [17–19]. Haem is transferred from its site of synthesis in the cytoplasm to the membrane, where it binds covalently to a histidine residue in CcmE prior to transfer to the cytochrome *c* apoprotein [11,20]. Whether CcmA, CcmB, CcmC and CcmD are all required for haem transfer remains controversial (compare refs [21] and [22]). When overexpressed from a multi-copy plasmid, CcmC alone is essential for the accumulation of haem-loaded CcmE, which is apparently stabilized by CcmD [21]. However, under more natural conditions, CcmA and CcmB are also required for transfer of haem from CcmC to CcmE [21]. The disulphide bonds formed by DsbA must be reduced before haem can be attached: two key players in the reductive step are the cytoplasmic TrxA and the membrane-bound DipZ proteins, which together transfer two electrons from the cytoplasm to the periplasm [10–12,22–29].

The final three components, CcmF, CcmG and CcmH, are believed to catalyse the reduction of the disulphide bonds in the apoproteins and haem attachment [5–7,8,10,28–34]. CcmG and its homologues in other bacteria are thioredoxin-like proteins implicated in the reduction of disulphide bonds of the apocytochrome *c* prior to haem ligation [10,11,28,33,35–40]. The final two components, CcmF and CcmH, are postulated to form a haem lyase that includes a second Cys-Xaa-Xaa-Cys thioredoxin-like motif in the essential N-terminal domain of CcmH [2,7,32,41]. However, it is still uncertain whether the two steps occur sequentially or in a co-ordinated reaction (compare the models discussed in [28] and [32]). A specific point requiring clarification is whether DipZ transfers electrons via CcmG to the N-terminus of CcmH, or vice versa. If the former is correct, it can be predicted that CcmG will be predominantly reduced in a mutant

Abbreviations used: AMS, acetamido-4′-maleimidyl-stilbene-2,2′-disulphonate; DTT, dithiothreitol; LB, Luria–Bertani broth; 2-MESA, 2-mercaptoethanesulphonic acid.

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defective in the synthesis of CcmH. Furthermore, CcmG would function in partnership with DipZ as a pair of redox proteins, similar to the coupling of DsbB with DsbA. An alternative possibility is that electrons from DipZ are transferred first to the cysteine motif at the N-terminal domain of CcmH, and then to CcmG. This latter model predicts that CcmG will remain predominantly oxidized in a mutant defective in CcmH. Experiments were therefore designed to determine whether CcmG plays a general role in transferring electrons from the cytoplasm to the periplasm, or a role specific to cytochrome *c* assembly. To this end, we have investigated whether a mutation in *ccmG* can be compensated by a mutation in the periplasmic oxidative proteins, DsbA and DsbB, or by the chemical reducing agent, 2-mercaptoethanesulphonic acid (2-MESA; [42]). Also reported are experiments to determine the redox state of CcmG in mutants defective in the synthesis of other proteins known to be essential for the assembly of bacterial *c*-type cytochromes.

MATERIALS AND METHODS

Bacterial strains and plasmids used in this project

The *E. coli* K-12 strains used, their relevant genotypes and their method of construction or source are listed in Table 1. Plasmids used in the project are listed in Table 2.

Strains RI536 (*grxA*), RI342 (*trxA*), RI393 (*dsbC*), RI318 (*dsbB*,*dipZ*) and the parental strain, RI89 [22], were transduced to ampicillin resistance using P1 from strain JCB712Δg. JCB712Δg contains an ampicillin-resistance cassette within the *cysG* gene, which confers a Nir$^-$ (defective in NADH-dependent nitrite reduction) phenotype to the strain. The resulting strains are JCB70536 (*grxA*), JCB70342 (*trxA*), JCB70393 (*dsbC*), JCB70318 (*dsbB*,*dipZ*) and the parent strain, JCB70089.

Table 1 *E. coli* K-12 strains used in this work

Strain	Genotype	Source
JCB387	RV Δ*nir*	[3]
JCB3510	JCB387 Nrf$^-$ *dsbA*	[9]
JCB3871	Δ*ccmG* derivative of JCB387	[11]
JCB3872	*trxB*::*kan* derivative of JCB387	[11]
JCB3873	*trxA*::*kan* derivative of JCB387	[11]
JCB3874	JCB387 *dsbAccmG*	Transduce JCB3510 to erythromycin resistance with JCB3871-P1
JCB3875	JCB387 *dsbBccmG*	Transduce JCB3512 to erythromycin resistance with JCB3871-P1
JCB38712	JCB387 *dsbB*	[10]
JCB603	JCB387 *ccmH* (Δ*nirB nrf*-601)	[48]
JCB606	JCB387 *dipZ* (Δ*nirB nrf*-606)	[48]
JCB60611	JCB387 *dsbAdipZ*	Transduce JCB3510 to kanamycin resistance with JCB606-P1
JCB60612	JCB387 *ccmGdipZ*	Transduce JCB606 to erythromycin resistance with JCB3871-P1
JCB712Δg	Δ *cysG* derivative of JCB712	[49]
JCB70536	Δnir derivative of RI536	This work
JCB71202	JCB7120Δ*ccm* (A'-'H)::Ω	[2]
JCB70089	Δ*nir* derivative of RI89	This work
JCB70393	Δ*nir* derivative of RI393I89	This work
JCB70318	Δ*nir* derivative of RI318	This work
M15	Nal^S, Str^S, Rif^S, RecA$^+$, Uvr$^+$, Lon$^+$ *lac, ara, gal, mtl, f*	Qiagen
RI89	MC1000 *phoR* Δ*ara714 leu*$^+$	[22]
RI318	RI89 *dsbB*::Kan^r *dsbD*::mini-Tn*10* Cam^r	[22]
RI342	RI89 Δ*trxA*::*kanR*	[22]
RI393	RI89 *dsbC*::*mini-Tn10 kan*	[22]
RI536	RI89 *grxA*::*Tn10-kan*	[22]

Table 2 Plasmids used in this work

Plasmid	Description	Reference
pJG70	*ccmA-H* cloned into pBR322	[2]
pJG77	pJG70 with an in-phase deletion in *ccmF*	[2]
pJG87	Derivative of pJG70, which lacks *ccmH*	[2]
pJG97	Deletion in the downstream half of *ccmH* in pJG70	[2]
pQE-60	T5 promoter, C-terminal His_6 tag	Qiagen
pREP4	*lac* repressor plasmid	Qiagen
pER1	Coding sequence of the *ccmG* gene in the overexpression vector pQE-60	This work

Construction and use of plasmid pER1 for overproduction of CcmG

The *ccmG* gene was amplified by PCR using pJG70 [2] as a template. The upstream primer, 5′-GGC-CTC-ATG-AAG-CGC-AAA-GTA-TTG-TTA-ATT-3′, included an *Rca*I site engineered at the 5′ end. The downstream primer, 5′-AAA-AGA-TCT-TTG-TGC-GGC-CTC-CTT-ACT-3′, contained an engineered *Bgl*II site. The resulting 564 bp PCR fragment, which included the ATG start codon for *ccmG*, was cloned into pQE-60 (Qiagen, Crawley, West Sussex, U.K.). The resulting plasmid, pER1, was transformed into *E. coli* strain M15[pREP4] for overexpression of the CcmG polypeptide.

Strain M15[pREP4]pER1 was grown aerobically, and overproduction of CcmG was induced by the addition of isopropyl β-D-thiogalactoside ('IPTG'). The N-terminal amino acid sequence of the 20 kDa polypeptide was determined to be MKRKVL (single-letter amino acid code), identical with the sequence of unprocessed CcmG.

Purification of recombinant CcmG, and production of anti-CcmG antibodies in guinea-pigs

Bacteria were harvested, washed and broken by passage through a French pressure cell at 70 MPa. Unbroken cells were removed by centrifugation. The membranes were isolated by centrifugation at 150000 ***g*** at 4 °C, and washed with 50 mM Tris/HCl, pH 7.0/2 M NaCl to remove peripheral membrane proteins. The recombinant CcmG protein was recovered from the membrane fraction by washing with 50 mM Tris/HCl, pH 8.0/1 % (w/v) sodium deoxycholate.

The partially purified CcmG protein solubilized in 1 % (w/v) sodium deoxycholate was separated from other proteins by electrophoresis under denaturing conditions on a 15 % (w/v) polyacrylamide gel. The CcmG protein band was excised from the gel, and eluted for antibody production. Anti-CcmG was recovered from the antiserum by ammonium sulphate precipitation. Cross-reactive antibodies were then removed by incubating the antiserum with membranes prepared from strain JCB71202 (a deletion mutant that lacks all of the *ccm* genes), followed by removal of these membranes by centrifugation at 150000 ***g***. Further purification of the supernatant was achieved by incubating the antiserum with the soluble cytoplasmic proteins, prepared from the *ccm* deletion strain JCB71202, which had been attached to CNBr-activated Sepharose 4B (Sigma Chemical Co., Poole, Dorset, U.K.).

Derivative-formation of CcmG

For analysis of the oxidation state of CcmG *in vitro*, 600 μg of purified CcmG protein was reduced with 17 mM dithiothreitol (DTT) in a final volume of 500 μl. Excess DTT was removed by gel filtration through a Nap 5 column (Pharmacia Biotech, St Albans, Herts., U.K.). Either the oxidized or the reduced CcmG was then reacted with 5.6 mM 4-acetamido-4′-maleimidyl-stilbene-2,2′-disulphonate (AMS; Molecular Probes Inc., Leiden, The Netherlands) [43]. The reaction was stopped by the addition of one-tenth volume of 3 M HCl.

For analysis of the oxidation state of CcmG *in vivo*, the total cell protein from 750 μl of culture was precipitated with 750 μl of 10% (w/v) trichloroacetic acid (TCA), and collected by centrifugation in a Microfuge. The protein pellet was then washed in cold acetone. The protein precipitate was air-dried and resuspended in AMS reaction buffer [5.6 mM AMS/50 mM Tris/HCl (pH 8.0)/1% (w/v) SDS/1 mM EDTA] for 30 min. [43]. Proteins were re-precipitated with acetone and resuspended in Laemmli sample buffer for electrophoresis. All steps sensitive to air oxidation were prepared under a nitrogen atmosphere.

Western-blot analysis and other biochemical procedures

Proteins were resolved by Tris/Tricine SDS/PAGE using a 16.5% (w/v) polyacrylamide gel, and subsequently transferred on to a Hibond transfer membrane (Amersham Pharmacia Biotech., Little Chalfont, Bucks., U.K.). The membrane was incubated with purified antiserum followed by anti-(guinea-pig IgG) alkaline phosphatase conjugate (Sigma). The resultant antibody precipitate was detected using Problot detection system (Promega, Chilworth, Southampton, U.K.).

Previously published procedures were used to determine the Nrf phenotype [3], to determine glycerol- and formate-dependent rates of nitrite reduction by suspensions of washed bacteria [44,45] and for SDS/PAGE [46]. Concentrations of *c*-type cytochrome in the soluble protein fraction (which includes both periplasmic and cytoplasmic proteins) of bacteria broken in the French press were determined spectroscopically, as described previously [45], except that the oxidant was potassium ferricyanide, rather than nitrite. The major cytochrome in these fractions is cytochrome c_{552}, the NrfA product of the *nrfA* gene [47].

RESULTS

Specificity of compensation for defects in thiol oxidation in the periplasm by a second mutation in *dip*Z or *ccm*G

We and others have previously reported that the defect in cytochrome *c* assembly resulting from a *dsbA* mutation can at least in part be compensated by a second mutation in *dipZ* (*dsbD*) in the reductive branch of electron transfer to the periplasm. It is assumed that compensation occurs due to the achievement of a balanced redox potential in the periplasm that allows both random chemical oxidation of cysteines to form essential disulphide bonds in the cytochrome *c* apoproteins, followed by their reduction prior to haem attachment. If this assumption is correct, a *dipZ* mutation should also be compensated by a second mutation in *dsbB*. If CcmG also plays a general role in the transfer of electrons from DipZ to the periplasmic haem lyase, a mutation in *ccmG*, like the *dipZ* mutation, should compensate for loss of DsbA or DsbB.

To test these proposals, an isogenic series of double mutants was constructed by bacteriophage P1-mediated transduction. The resulting strains were grown under conditions optimal for synthesis of NrfA and NrfB, which are *c*-type cytochromes

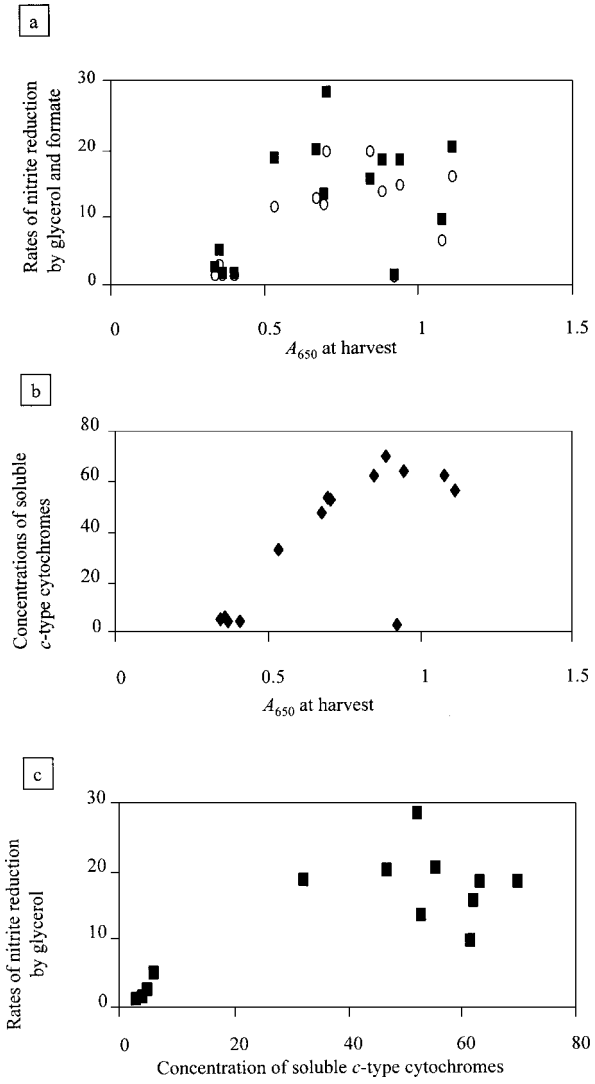


Figure 1 Rates of nitrite reduction by glycerol and formate and concentration of soluble *c*-type cytochromes in the *dsbA,dipZ* strain JCB60611 harvested at different absorbances

Bacteria were grown anaerobically in minimal medium supplemented with 10% Lennox broth, 0.4% (v/v) glycerol, 40 mM fumarate and 2.5 mM nitrite, harvested, washed and the rates of nitrite reduction in the presence of glycerol (■) or formate (○) were determined. Rates are in nmol of nitrite reduced/min per mg bacterial dry mass. Cytochrome concentrations (◆) are pmol/mg of soluble protein released by the French press.

required for formate-dependent nitrite reduction. However, in initial experiments with the previously described control strain that is defective in both *dsbA* and *dipZ*, the growth rate and yield were extremely variable. No nitrite reduction was detected with washed bacteria from these cultures, and no *c*-type cytochromes had accumulated during growth. No similar growth problems were encountered with the *dsbB,dipZ* strain or other double mutants.

The most likely explanation for the failure to reproduce earlier results appeared to lie in the variability of the growth of the culture, which probably reflected variations in the redox state of the periplasm caused by the defects in disulphide-bond management. The effect of growth phase on the ability of a *dipZ* mutation to compensate for loss of *dsbA* was therefore investigated (Figure 1). Results from many independent cultures

Table 3 Rates of nitrite reduction by glycerol and formate and concentrations of soluble cytochrome *c* produced by *ccmG,dsbA*, *ccmG,dsbB* and *ccmG,dipZ* double mutants and *ccmG*, *dsbA*, *dsbB* and *dipZ* single mutants, compared with the parent strain

Bacteria were grown anaerobically in minimal salts medium supplemented with 10% (v/v) Lennox broth, 0.4% (v/v) glycerol, 40 mM fumarate and 2.5 mM nitrite, harvested, washed, and the rate of nitrite reduction by washed bacteria was determined. Rates are nmol of nitrite reduced/min per mg bacterial dry mass. Cytochrome concentrations are pmol/mg of soluble protein released by the French press (periplasmic and cytoplasmic proteins, including both cytochrome c_{550} and cytochrome c_{552}). Results are the average values for two or more independent cultures.

Strain	Relevant genotype	Nitrite reduction by glycerol	Nitrite reduction by formate	Soluble cytochrome *c*
JCB387	Wild-type	62.0 ± 13.7	35.0 ± 9.1	218 ± 61
JCB60611	*dsbA,dipZ*	16.7 ± 7.3	12.1 ± 5.3	51 ± 19
JCB3874	*ccmG,dsbA*	2.1 ± 0.4	1.1 ± 0.2	< 10
JCB3875	*ccmG,dsbB*	3.4 ± 2.4	2.2 ± 1.0	< 10
JCB60612	*ccmG,dipZ*	1.3 ± 0.5	0.9 ± 0.4	< 10
JCB3510	*dsbA*	4.0 ± 0.9	2.3 ± 0.1	< 10
JCB38712	*dsbB*	2.8 ± 0.6	1.1 ± 0.1	< 10
JCB606	*dipZ*	1.7 ± 0.6	1.3 ± 0.1	< 10
JCB3871	*ccmG*	1.7 ± 0.4	1.3 ± 0.2	< 10
JCB70089	Wild-type	19.1 ± 3.9	13.2 ± 4.9	273 ± 10
JCB70318	*dsbB,dipZ*	16.7 ± 8.3	13.0 ± 7.6	60 ± 24

revealed that significant rates of nitrite reduction and the accumulation of periplasmic *c*-type cytochromes were detected only with cultures harvested during the active phase of growth (which was not always exponential). Neither Nrf activity nor cytochrome *c* accumulation was detected in cultures that failed to reach a moderately high bacterial density.

In subsequent experiments, standard procedures were used to prepare inocula that were sequentially transferred from 10-ml Luria–Bertani (LB) cultures via 200 ml of anaerobic cultures to the final 1 litre cultures harvested for biochemical assay. Using this procedure, highly reproducible data for all double mutants, except for the *dsbA,dipZ* strain, were obtained (Table 3). In typical experiments, the *dsbA,dipZ* double mutant gave glycerol-dependent rates of nitrite reduction that were 30–40% of those of the parental strain. Significantly higher rates, typically about 85% of the parental strain, were also detected with a double mutant defective in both *dsbB* and *dipZ*. We conclude that loss of ability to re-oxidize DsbA by DsbB can be compensated by a secondary mutation in the reducing branch of periplasmic dithiol-disulphide redox reactions.

Double mutants defective in both *ccmG* and *dsbA,dsbB* or, as a negative control, *dipZ*, were also tested in the same series of experiments. In contrast with the compensation of the *dsbA* and *dsbB* mutations by a second mutation in *dipZ*, rates of nitrite reduction by the *ccmG* double mutants were insignificant compared with those of the parental strain, but were comparable with those of strains carrying single mutations in the genes tested. This was the first indication that CcmG plays a specific role in the reduction of cysteine residues of apocytochromes *c*, rather than a general role in disulphide–thiol redox reactions in the periplasm.

Effect of 2-MESA on cytochrome *c* assembly during growth of strains defective in the synthesis of TrxA, DipZ and various Ccm polypeptides

Several laboratories have reported that loss of disulphide reduction proteins, TrxA and DipZ, can be compensated for chemically by the addition of reducing agents to the growth medium [10,42]. To explore whether a chemical reducing agent can compensate for loss of CcmG in the same way that it can compensate for loss of DipZ or TrxA, both single and double mutants were grown anaerobically in the presence of nitrite; 20 mM 2-MESA was added to some of the cultures. All of the strains tested grew well in the presence of 2-MESA; the unpredictable growth defect of the *dsbA,dipZ* double mutant was also eliminated.

As expected [11], the presence of 2-MESA during the growth of *trxA* and *dipZ* single mutants restored about half of the rate of glycerol-dependent nitrite reduction found for the parental strain that had been grown in the absence of 2-MESA (Figure 2). Particularly striking was that, although the presence of 2-MESA during growth stimulated the rate of nitrite reduction by the *dsbA,dipZ* double mutant, it was strongly inhibitory for both the parental strain and the *dsbA,dipZ* double mutant. Although the reason for this is unknown, it seems possible that 2-MESA prevents the formation of essential disulphide bonds in apocytochrome *c* by spontaneous chemical oxidation in the periplasm. Following growth in the presence of 2-MESA, the

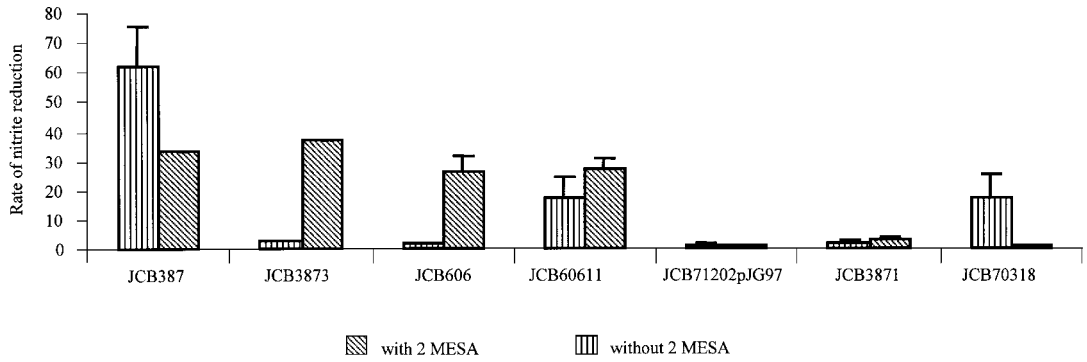


Figure 2 Complementation of strains defective in *trxA* (JCB3873), *dipZ* (JCB606) and *dsbA,dipZ* (JCB60611), but not *ccmG* (JCB3871), *ccmH*^{N} (JCB71202pJG97) or *dsb,BdipZ* (JCB70318), for formate-dependent nitrite reduction by 2-MESA

Bacteria were grown as shown in Figure 1, supplemented where appropriate with 20 mM 2-MESA, harvested, washed, and the rates of nitrite reduction in the presence of glycerol were determined. Rates are in nmol of nitrite reduced/min per mg bacterial dry mass. Error bars show the S.D. from the mean.

Table 4 Complementation of *trxA* and *dipZ* single mutants and the *dsbA,dipZ* double mutant, but not the *ccmG* or *ccmH^N* single mutants or the *dsbB,dipZ* double mutant, for cytochrome *c* synthesis and formate-dependent nitrite reduction by 2-MESA

Strain	Relevant genotype	Nitrite reduction by formate		Soluble cytochrome *c*	
		−	+	−	+
JCB387	Wild-type	35.0 ± 9.1	23.9 ± 2.4	218 ± 61	97 ± 8
JCB3871	*ccmG*	1.3 ± 0.1	1.0 ± 0.1	< 10	< 10
JCB71202pJG87*	*ccmH^N*	0.9 ± 0.1	0.7 ± 0.1	< 10	< 10
JCB71202pJG97*	*ccmH^C*	56.1 ± 5.3	31.5 ± 4.6	568 ± 50	255 ± 22
JCB71202pJG77*	*ccmF*	1.0 ± 0.1	0.8 ± 0.1	< 10	< 10
JCB71202	*ccmA-H*	0.9 ± 0.1	0.8 ± 0.1	< 10	< 10
JCB3873	*trxA*	0.8 ± 0.1	26.5 ± 7.8	< 10	109 ± 7
JCB606	*dipZ*	1.3 ± 0.1	15.0 ± 2.8	< 10	211 ± 24
JCB603	*ccmH*	1.1 ± 0.1	1.3 ± 0.1	< 10	< 10
JCB60611	*dsbA,dipZ*	12.3 ± 5.3	25.5 ± 4.4	55 ± 11	84 ± 18
JCB70089	Wild-type	13.2 ± 4.9	n/d	273 ± 10	n/d
JCB70318	*dsbB,dipZ*	13.0 ± 7.6	0.8 ± 0.1	60 ± 24	< 10

* Strains JCB71202pJG87, JCB71202pJG97 and JCB71202pJG77 are strain JCB71202 transformed with plasmids pJG87, pJG97 and pJG77 respectively.

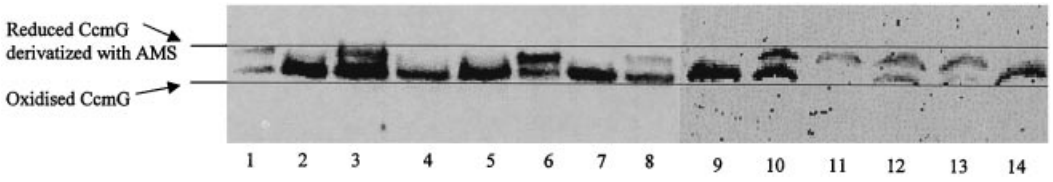


Figure 3 Redox state of the putative active site Cys-Xaa-Xaa-Cys motif of CcmG in various mutant backgrounds

Bacteria were grown as for Figure 1. Total cellular proteins were denatured with TCA, reacted with AMS under nitrogen, and separated by Tris/Tricine SDS/PAGE (16.5% gels). CcmG was detected by Western blotting with anti-CcmG antibody. Lane 1, JCB387; lane 2, JCB606 (*dipZ*); lane 3, JCB606 + 2-MESA; lanes 4 and 5, JCB71202pJG77 (*ccmF*); lane 6, JCB71202pJG77 + 2-MESA; lane 7, JCB3873 (*trxA*); lane 8, JCB3873 + 2MESA; lane 9, JCB3873 (*trxA*); lane 10, JCB3872 (*trxB*); lane 11, JCB603 ($ccmH_c$) + 2-MESA; lane 12, JCB603; lane 13, JCB71202pJG97 ($ccmH_N$) + 2-MESA; lane 14, JCB71202pJG97.

stimulated rates of nitrite reduction by *trxA* and *dipZ* mutants were similar to the inhibited rate for the parental strain (Figure 2).

Rates of nitrite reduction by formate were slightly lower than the corresponding glycerol-dependent rates, and the effects of 2-MESA were similar to those for the glycerol-dependent rates (Table 4). The concentration of soluble cytochromes decreased in parallel with the decreased rates of nitrite reduction in the parental strain after growth with 2-MESA. On the other hand, the cytochrome concentrations increased in parallel with the rates of nitrite reduction in the *trxA* and *dipZ* single mutants and in the *dsbA*,*dipZ* double mutant. The rates of nitrite reduction appeared to be proportional to the concentration of cytochromes (predominantly the terminal nitrite reductase, cytochrome c_{552}) accumulated. Under these conditions, the concentration of cytochrome *c* therefore appears to exert most control over the rate of electron transfer from the primary dehydrogenases via the quinone pool to nitrite.

Rates of nitrite reduction in the presence of either glycerol or formate by the *ccmF*, *ccmG* and *ccmH* mutants, as well as the concentrations of *c*-type cytochromes, were as low after growth in the presence of 2-MESA as in its absence. Therefore 2-MESA was unable to compensate for mutations in the *ccm* genes, including *ccmG*, implicated in the final steps of cytochrome *c* assembly (Table 4 and Figure 2) [11].

Determination of the redox state of CcmG by Western-blot analysis

The parental strain, JCB387, and a series of single and double mutants defective in proteins required for cytochrome *c* assembly were grown anaerobically in test tubes containing 10 ml of minimal medium supplemented with 10% (v/v) LB, glycerol, fumarate and nitrite. To determine whether the active-site cysteines of CcmG were present in the oxidized, disulphide form or as reduced dithiols, total bacterial protein was denatured with TCA and reacted with AMS. Proteins were then separated by 16.5% (w/v) Tris/Tricine SDS/PAGE, transferred on to Amersham Hibond transfer membrane and visualized by Western blotting with anti-CcmG antiserum. The addition of two molecules of AMS to each pair of reduced cysteines increases the molecular mass, and hence decreases the mobility, by about 1 kDa.

Cellular proteins from the parental strain, JCB387, produced two bands on the immunoblot after staining for alkaline phosphatase activity (Figure 3, track 1). Therefore the thioredoxin-like motif, Cys-Xaa-Xaa-Cys, of CcmG exists as a mixture of oxidized and reduced forms *in vivo*. Only the oxidized form of CcmG was detected in mutants defective in TrxA (Figure 3, tracks 2 and 9) or DipZ (track 7), but addition of 2-MESA during growth restored both oxidized and reduced forms of

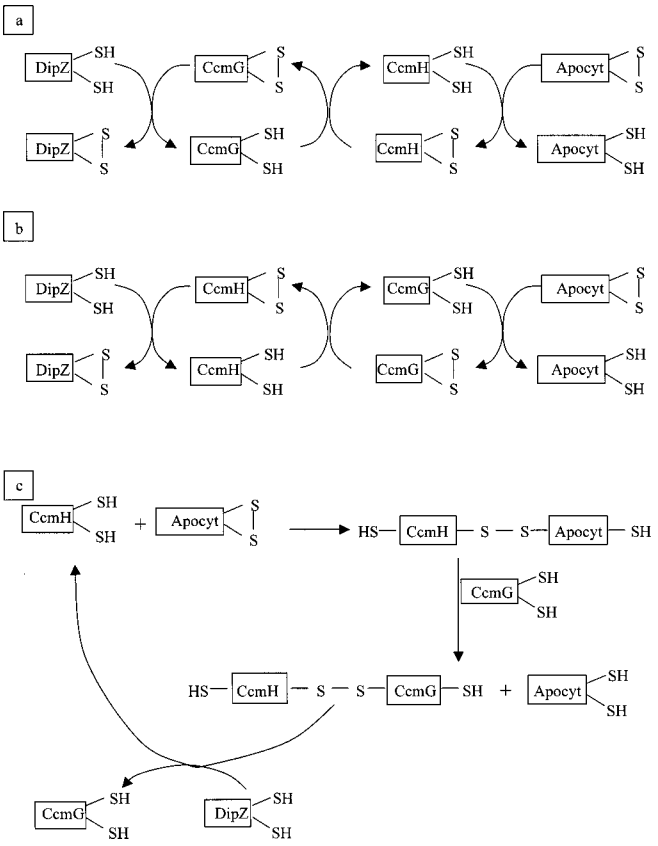
CcmG (Figure 3, tracks 3 and 8). Significantly, in the absence of 2-MESA, only the oxidized form of CcmG was detectable in mutants defective in *ccmF* (Figure 3, tracks 4 and 5) or the essential 5′-end of *ccmH* (track 14). On the other hand, both oxidized and reduced forms of CcmG were detected in strains defective in the synthesis of the non-essential TrxB (Figure 3, track 10), or in the 3′-end of *ccmH* (track 12). Addition of 2-MESA during growth resulted in substantial reduction of CcmG in strains defective in CcmF (Figure 3, track 6), and complete reduction in strains defective in CcmH (tracks 11 and 13). These results were confirmed in a further series of experiments that also revealed the presence of a mixture of oxidized and reduced forms of CcmG in mutants defective in GrxA (glutaredoxin A) and the thiol-disulphide isomerase, DsbC. The demonstration (i) that 2-MESA reduces CcmG, but cannot compensate for loss of CcmF and CcmH, (ii) that only the oxidized form of CcmG accumulates in mutants defective in CcmF or CcmH, and (iii) that 2-MESA is unable to compensate for the loss of CcmG provides strong evidence that CcmF and CcmH are essential for CcmG reduction. Consequently, the function of CcmG cannot simply be to transfer electrons from DipZ to a putative haem lyase complex that includes CcmF and CcmH.

DISCUSSION

We have previously reported that mutations in *dsbA*, *dsbB*, *trxA* and *dipZ*, as well as in the eight Ccm proteins encoded by the *nap–ccm* region of the *E. coli* chromosome, all result in loss of cytochrome *c*-dependent nitrite reduction due to their inability to assemble *c*-type cytochromes in the periplasm [2,9–12]. Loss of DsbA apparently results in a redox potential in the periplasm that is too low for the formation of one or more disulphide bonds that are essential for cytochrome *c* assembly. On the other hand, loss of DipZ or TrxA results in a periplasmic redox potential too high for the reduction of a critical disulphide bond prior to haem attachment. Both cytochrome *c* accumulation and functional nitrite reductase activity were partially restored to a *dsbA* mutant by a second mutation in *dipZ*, a protein essential for the transfer of electrons from TrxA in the cytoplasm to the periplasm in the reducing branch of the cytochrome *c* assembly pathway. This restoration of function by a mutation in a second essential gene was assumed to be a general effect due to partial restoration of a balanced redox environment in the periplasm. If this interpretation is correct, a mutation in dipZ should also restore function to a mutant defective in *dsbB*, a gene that encodes the partner to DsbA that is essential to re-oxidize DsbA by transferring electrons via the quinone pool to a terminal electron acceptor. This prediction was readily confirmed (Table 2 and Figure 1). Furthermore, the chemical reducing agent, 2-MESA, could also compensate non-specifically for loss of either DipZ or TrxA (Figure 2 and Table 4). We previously reported that loss of DsbC has little effect on cytochrome *c* synthesis or cytochrome-*c*-dependent electron-transfer pathways [10]. It would be interesting to extend our current experiments to triple mutants defective in *dipZ*, *dsbB* (or *dsbA*) and *dsbC* to determine whether restoration of cytochrome *c* maturation in the double mutants is more dependent upon the protein-disulphide isomerase activity of DsbC.

CcmG and its homologues in other bacteria are small proteins with a thioredoxin-like motif facing the periplasm, but tethered to the cytoplasmic membrane by a hydrophobic N-terminal domain [28,31]. By analogy to the way that DsbA is the periplasmic partner of the integral membrane protein, DsbB, in the oxidative branch of disulphide-bond formation in periplasmic proteins, it was possible that CcmG is the periplasmic partner of DipZ in the transfer of electrons across the membrane for disulphide-bond reduction in the periplasm, as shown in Scheme 1(a). If this were correct, it was likely that loss of CcmG by mutation might restore cytochrome *c* synthesis and nitrite reduction to mutants defective in *dsbA* and *dsbB*, and that 2-MESA could also restore loss of function to strains carrying a *ccmG* mutation. Neither of these proposals was correct, implying that CcmG plays a more specific role, or multiple roles, in cytochrome *c* assembly. Furthermore, in the absence of the essential CcmH, CcmG should still be reduced by DipZ and hence accumulate in the reduced form in a *ccmH* mutant. The opposite result to this prediction was obtained, suggesting that CcmH is essential for CcmG reduction, as shown in Scheme 1(b). This conclusion is entirely consistent with results from other laboratories [28,29,32,33]. However, this model predicts that chemical reduction of CcmG by 2-MESA should be sufficient to restore cytochrome *c* assembly to a *ccmH* mutant. Although we have demonstrated chemical reduction of CcmG by 2-MESA (Figure 3, lanes 11 and 13), in contrast with the prediction from Scheme 1(b), cytochrome *c* assembly was not restored.

Evidence is accumulating that CcmF, CcmG and CcmH might function together as a haem lyase complex, and a model of how



Scheme 1 Three alternative models to explain the roles of CcmG and CcmH proteins in the reduction of the apocytochrome *c*

In (**a**), it is suggested that electrons from DipZ are transferred via CcmG to CcmH; CcmH then reduces the apocytochrome. In (**b**), the order of electron transfer is reversed: electrons from DipZ are transferred first to CcmH, and then to CcmG, and it is reduced CcmG that reduces the apocytochrome. In (**c**), CcmH and the apocytochrome form a complex via a disulphide bond in their active sites. The disulphide bond is resolved by CcmG, resulting in reduced apocytochrome and a CcmH–CcmG complex, the latter being the substrate for reduction by DipZ.

such a complex might work has been suggested [32]. Our data are entirely consistent with several features of that model, which incorporates the idea that CcmG and CcmH work in close partnership as a specific part of the cytochrome *c* assembly apparatus. However, the model presented previously, if correct, still predicts that DipZ is the direct reductant for CcmG, and hence that CcmG should accumulate in a reduced form in mutants defective in CcmH. To provide a satisfactory explanation for the accumulation of oxidized CcmG in a strain defective in CcmH and the inability of chemical reduction of CcmG by 2-MESA to restore cytochrome *c* assembly to a *ccmH* mutant, we propose that the substrate for DipZ reduction is not fully oxidized CcmG, but a mixed disulphide between CcmG and CcmH, as shown in Scheme 1(c). This proposal fully accounts for all of the data available at present, and extends, rather than replaces, the model presented previously [32]. We propose further that CcmF catalyses the ligation of haem to the reduced apocytochrome, although no direct evidence relevant to this point has been presented.

In summary, we have presented four lines of evidence that CcmG catalyses a late, reductive step in the assembly of *c*-type cytochromes in the bacterial periplasm. First, CcmG was shown by two methods not to function as a non-specific mediator of electron transfer from TrxA in the cytoplasm via the membrane-bound DipZ to proteins like CcmF or CcmH, which have more specific roles. Particularly significant was the observation that the chemical reductant, 2-MESA, can bypass the requirement for loss of both TrxA and DipZ in cytochrome *c* assembly, but cannot replace CcmG. That CcmG functions after CcmF and CcmH was confirmed by the detection of only the oxidized form of CcmG in mutants defective in CcmF and CcmH.

This work was supported by Project Grant Z03923C from the U.K. Biotechnology and Biological Sciences Research Council (BBSRC), and a BBSRC Research Studentship to E.R. Excellent technical assistance from Lesley Griffiths is also gratefully acknowledged. We are also grateful to Dr A. Rietsch and Professor J. Beckwith (Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA, U.S.A.) for the gift of strains.

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Received 13 September 2000/1 December 2000; accepted 19 January 2001