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Specific thiol compounds complement deficiency in *c*-type cytochrome biogenesis in *Escherichia coli* carrying a mutation in a membrane-bound disulphide isomerase-like protein

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Abstract *Escherichia coli* JCB606 carries a mutation in the *dipZ* gene, known to code for a disulphide isomerase-like protein, with the consequence that holo forms of neither exogenous nor endogenous *c*-type cytochromes are synthesised. This failure has been overcome by adding compounds containing thiol groups to the growth medium. Only L-cysteine and 2-mercaptoethane sulphonic acid were effective, suggesting a (stereo)specific binding site that could be occupied by these compounds in the absence of the catalytic domain of DipZ.

Key words: Bacterial *c*-type cytochrome biogenesis; Protein disulphide isomerase; L-Cysteine

1. Introduction

Escherichia coli strain JCB606 carries a mutation that affects a locus which is essential for biogenesis of not only endogenous *c*-type cytochromes but also the exogenous *Paracoccus denitrificans* cytochrome c_{550} [1]. The mutation responsible for the phenotype of JCB606 has been shown to be in a gene coding for a membrane-bound protein disulphide isomerase, or thioredoxin homologous protein, named as DipZ (H. Crooke and J.A. Cole, personal communication; GenBank accession no. X77707). The current hypothesis that haem attachment occurs in the periplasm during bacterial *c*-type cytochrome biogenesis [2], and that general function of protein disulphide isomerase is related to the interconversion of cysteine and cystine, together suggest that DipZ could be essential for maintaining apo-proteins of *c*-type cytochromes in the competent state for the covalent attachment of haem to the correct pairs of cysteine residues at the periplasmic side of the cytoplasmic membrane.

In the present paper, the effects on the *c*-type cytochrome biogenesis in *E. coli* JCB606 were examined when thiol or disulphide compounds were added to the growth media. In addition the alkaline phosphatase (AP) activity, which requires disulphide bond formation in the periplasm, was measured in JCB606 cells carrying a plasmid containing a *P. denitrificans* cytochrome c_{550}-AP fusion gene.

2. Materials and methods

2.1. E. coli *strains and growth conditions*

The *E. coli* K-12 strain JCB606, which is deficient in both endogenous and exogenous periplasmic and membrane *c*-type cytochromes [1], and its parental strain JCB387 [3], which can produce *c*-type cytochromes normally, were used in this study. These strains were transformed by the plasmid pKPD1 which carries the complete gene for *P. denitrificans* cytochrome c_{550} [4]. These strains were also transformed by the plasmids carrying the cytochrome c_{550} and *E. coli* alkaline phosphatase (AP) translational fusion gene described below. The cells were grown at 37°C anaerobically in minimal media [3] in the presence of glycerol, fumarate and nitrite supplemented with various chemical compounds described in section 3. The antibiotics ampicillin (50 μg/ml) and kanamycin (25 μg/ml) were added to the media.

2.2. *Construction of translational fusion of* P. denitrificans *cytochrome* c_{550} *with alkaline phosphatase*

Two oligonucleotides shown in Fig. 1 were synthesised to amplify a DNA fragment including the AP gene in the plasmid pRT733 [5]. The primer XF09 was designed to be annealed with the mature region of AP DNA sequence corresponding to the 6th to 10th amino acid in the sequence and the 5′ end was flanked by restriction site for *Xho*I (5′-CTCGAG-3′). The primer XF10 was designed to be annealed with the sequence of the 3′ AP non-coding region. Both primers were 5′ phosphorylated and PCR amplification was done between these primers using Taq polymerase under the condition of 30 cycles of denaturation at 93°C (1 min), annealing at 54°C (1 min) and extension at 72°C (1 min). The resulting PCR fragment (approximately 1.4 kb) was treated with T4 DNA polymerase to make blunt ends and inserted into the pUC119 cloning vector which had been restricted by *Hinc*II and dephosphorylated by shrimp alkaline phosphatase. A recombinant clone in which 1.4 kb AP gene could be recovered by *Xho*I (in the insertion) and *Pst*I (in the vector) was selected. The 1.4 kb *Xho*I–*Pst*I AP gene fragment was substituted for the 0.5 kb *Xho*I–*Pst*I DNA fragment of the plasmid pKPD1 to make a new plasmid pKPD60 which contains the N-terminal 61 amino acid residues of *P. denitrificans* cytochrome c_{550} gene plus the 20 amino acid residues of its signal sequence and the mature region of AP except for the N-terminal 5 amino acid residues.

2.3. *Analysis of cytochrome* c *formation*

Fractionation of periplasm from *E. coli* cells carrying pKPD1 or pKPD60 was performed by the cold osmotic shock procedure described previously [4]. The spheroplast protein fraction was obtained from the cold osmotic shock precipitate followed by sonication and removal of unbroken cells by low-speed centrifugation. The haem staining method [6] was used to detect holo *c*-type cytochromes after SDS-PAGE (12.5% acrylamide).

2.4. *Enzyme assay*

AP enzyme activity was measured essentially according to Brickmen and Beckwith [7]. Briefly, 1 ml of the fractionated and diluted protein solutions in 50 mM Tris-HCl (pH 8.0) were added to 0.1 ml of phosphatase substrate Sigma 104 (0.4% w/v) in 37°C and the increase in absorbance at 420 nm was measured. Activity of a cytoplasmic marker enzyme, glutamate dehydrogenase, was measured as described previously [1]. Protein concentration was determined by Bio-Rad protein assay kit using bovine serum albumin as a standard.

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Abbreviations: AP, alkaline phosphatase; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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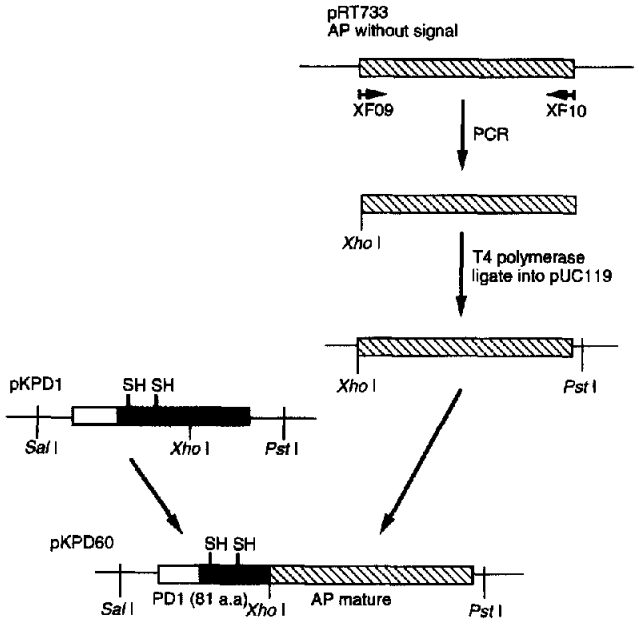

Fig. 1. Strategy for construction of *P. denitrificans* cytochrome c_{550}-alkaline phosphatase (AP) translational fusion gene. The relevant parts of the plasmids, pRT733, pKPD1 and pKPD60 are shown (size is not to scale). The mature part of the AP gene is shown by the striped box. The coding regions for the cytochrome c_{550} signal sequence and mature protein are shown by the open box and the black box, respectively. PCR primers are shown by the arrows with the direction from 5′ to 3′. The sequences are: XF09 (5′-CTCGAGCCTGTTCTGGAAAAC-3′), XF10 (5′-TCTAGAAAATTCACTGCCGGG-3′).

3. Results

3.1. Chemical complementation of the c-type cytochrome biogenesis deficiency of JCB606

The chemical compounds tested in this study are listed in Table 1. Among these compounds, two thiol compounds, L-cysteine and 2-mercaptoethane sulphonic acid (coenzyme M of methanogenic bacteria) restored the formation of endogenous and exogenous holo cytochromes *c* in JCB606. Fig. 2 shows haem stains following separation by SDS-PAGE of the periplasmic proteins from JCB606 cells with the pKPD1 plasmid following growth in the presence of L-cysteine or 2-mercaptoethane sulphonic acid. Haem stained bands for *E. coli* native cytochrome c_{552} (nitrite reductase) and exogenous *P. denitrificans* cytochrome c_{550} can be seen in the presence of over 1.0 mM L-cysteine. A higher concentration of 2-mercaptoethane sulphonic acid, than of L-cysteine, was required for formation of holo *c*-type cytochromes in JCB606; at least 5.0 mM of this compound was needed. On the other hand, none of disulphide compounds tested (Table 1) nor amino acids structurally similar to L-cysteine, L-serine which only differs from L-cysteine having a hydroxyl group instead of thiol, and L-alanine, in which L-cysteine thiol is missing, had an effect on the formation of the *c*-type cytochrome in JCB606, indicating that a thiol group is necessary for complementation of *dipZ* mutation. Moreover, D-cysteine, cysteamine, reduced DTT and reduced glutathione, even though having thiol(s), were ineffective over a range of concentrations. Any physiological role for glutathione in *c*-type cytochrome biogenesis is further rendered unlikely by our finding that a glutathione-deficient mutant of *E. coli* (kindly provided by Professor I.R. Booth, University of Aberdeen) synthesises *c*-type cytochromes (data not shown).

The consequence of addition of L- or D-cysteine to growth media containing cells at the late exponential phase of growth was also examined. Just as when either isomer of this amino acid was added at the start of growth, only L-cysteine, after three hours incubation, caused the appearance of *c*-type cytochromes (data not shown). The concentrations of the thiol groups of either isomer, measured by the method of Ellman [8], fell equally during this three hour incubation, but this consumption of cysteine was clearly not directly related to the role in restoring *c*-type cytochrome biogenesis.

3.2. Expression of cytochrome c_{550}-AP fusion and cytochrome b_{562} in JCB387 and JCB606

While *E. coli* strains JCB387 and JCB606 gave weak and pale blue colonies on AP indicating plates supplemented with 5-bromo-4-chloro-3-indolyl phosphate (100 μg/ml), cells of both strains carrying pKPD60, which contains the cytochrome c_{550} gene fused with mature part of AP gene, gave dark blue colonies on such plates. AP activities in both of the fusion-containing strains were mainly detected in the periplasmic fractions and their levels of activities were much greater than the control value for *E. coli* cells carrying pKPD1 which contains the wild type intact cytochrome c_{550} gene (Table 2). In addition, the preferential distribution of glutamate dehydrogenase activity in the spheroplast fractions confirms that significant amounts of cell lysis did not occur during the cold shock fluid preparation (Table 2). These findings, plus the fact that AP is only active in the periplasm [9], indicate that the fusion protein is being efficiently exported to the periplasm of JCB606 as well as JCB387. Thus the absence of DipZ is presumed not to affect the translocation of apo *c*-type cytochromes across the cytoplasmic membrane. A lack of effect of DipZ on the provision of periplasmic haem could be deduced from the synthesis of the

Table 1
Compounds tested for ability to restore holo cytochrome *c* formation in JCB606

Compound	Minimum concentration for complementation (mM)	Maximum concentration tested (mM)
Thiol		
L-cysteine	1	5[a]
2-MESA	5	20
D-cysteine	–	20
cysteamine	–	20
DTT(red)	–	5[a]
glutathione(red)	–	20
Disulphide		
L-cystine	–	5
D-cystine	–	5
cystamine	–	15
DTT(ox)	–	5
glutathione(ox)	–	15
Amino acid		
L-serine	–	15
L-alanine	–	15

[a]Toxic for cell growth; 2-MESA, 2-mercaptoethane sulphonic acid; –, no concentration tested restored *c*-type cytochrome biogenesis; red, reduced; ox, oxidised.

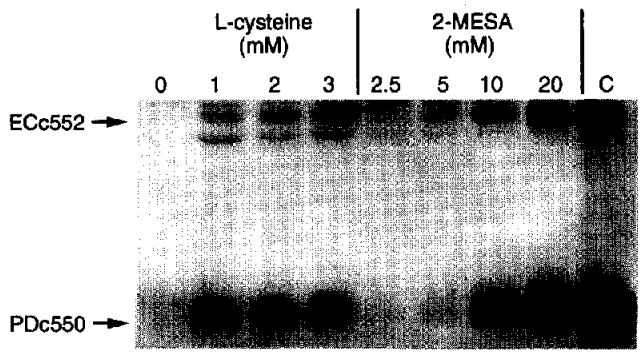

Fig. 2. The restoration of holo-cytochromes *c* formation in JCB606 carrying pKPD1 grown in the presence of thiol compounds. The thiol compounds and their added concentrations are shown above the panel. Periplasmic protein fractions from approximately 10^9 cells were subjected to SDS-PAGE and stained for haem. The periplasmic fration from parental strain JCB387 carrying pKPD1 grown without the thiol compounds was used as the control shown at the right end of the gel. The positions of the *E. coli* native cytochrome c_{552} (ECc552) and exogenous *P. denitrificans* cytochrome c_{550} (PDc550) are shown. 2-MESA, 2-mercaptoethane sulphonic acid; C, control.

periplasmic cytochrome b_{562}, which has non-covalent haem, in JCB606 (not shown).

4. Discussion

On the reasonable assumption that haem is attached to apo-cytochromes *c* in the periplasm, it is important whether the apo-protein arrives in the periplasm possessing thiol or disulphide groups on cysteine residues in the characteristic haem binding motif Cys-X-X-Cys-His, and how such cysteine thiol-disulphide redox state is subsequently controlled before the haem attachment. The evidence that the deficiency in *c*-type cytochrome biogenesis in *E. coli* JCB606 carrying the *dipZ* mutation is overcome by the addition of specific thiol compounds, L-cysteine and 2-mercaptoethane sulphonic acid, indicates that reducing power is required to form holo-cytochromes *c* and that DipZ may relate to an as yet undefined stereospecific thiol-disulphide redox potential balancing system, providing reducing power to apo-cytochromes *c* to keep the cysteine residues of their haem binding motif reduced in the periplasm. The membrane-impermeable sulphonic acid group should ensure that 2-mercaptoethane sulphonic acid acts in the periplasm.

The function of DipZ is distinguished from that of DsbA [10,11], DsbB [12] and DsbC [13,14] proteins, which also have a thioredoxin motif, but function to provide oxidising power to periplasmic and outer membrane proteins for synthesis of disulphide bonds in the periplasm. Thus mutations in these Dsb proteins in *E. coli* result in inability to assemble F pilus [10], flagellae [15] and active forms of periplasmic proteins such as alkaline phosphatase [11], in all of which disulphide bonds are required for their functions. However, results from the cytochrome c_{550}-AP fusion protein expression in JCB606 show that *dipZ* mutation does not affect the disulphide bond formation in the AP domain of the cytochrome c_{550}-AP fusion protein.

It is possible that the apo-cytochromes *c* are acted upon by the Dsb proteins with consequential disulphide bond formation. Subsequently, this bond would be reduced in apo-cytochromes *c*, but not in proteins such as alkaline phosphatase which require disulphide bonds, and then haem attached. Although it has not yet been shown whether DipZ relates to thiol groups of other proteins, it seems likely that Dsb proteins and the reductive system involving DipZ may act synergistically to fold proteins correctly, the former promoting oxidative protein folding and the latter reducing non-native disulphides formed spontaneously or by the former.

Our present work does not show whether the DipZ is a direct reductant for apo-cytochromes *c*. It is possible that small reductants such as L-cysteine directly interact with apo-cytochromes *c*. Also such a reducing potential transfer system to apo-cytochromes *c* may be mediated by the periplasmic HelX-like, or other, thioredoxin-type protein, whose mutation results in the deficiency of *c*-type cytochrome biogenesis in *Rhodobacter capsulatus* [16] and *Rhizobium leguminosarum* [17]. HelX-like genes are found in *E. coli* at *aeg-46.5* [18] and in *Bradyrhizobium japonicum* [19] but it is unclear whether such HelX-like proteins function as a reductant or an oxidant in these organisms. If we assume that a HelX-like protein acts as a reductant, it is conceivable that, just as DsbB oxidises DsbA in a reaction that can also be achieved by cystine and oxidised glutathione [12], DipZ is responsible for regeneration of a reduced HelX-like protein in a reaction where L-cysteine or 2-mercaptoethane sulphonic acid can act as surrogates for DipZ.

Table 2
Alkaline phosphatase (AP) activity in the cellular fractions from *E. coli* expressing the cytochrome c_{550}-AP fusion gene

Strain/plasmid	Enzyme activity			
	AP[a]		GDH[b]	
	Periplasm	Spheroplast	Periplasm	Spheroplast
JCB387/pKPD60	29.9	0.8	9.2	69.2
JCB387/pKPD1	0.02	0.005	12.2	33.7
JCB606/pKPD60	32.2	0.8	5.6	60.7
JCB606/pKPD1	0.01	0.004	7.6	34.6

[a]AP, alkaline phosphatase activities are given in the decrease on the absorbance at 420 nm · min^{-1} · mg $protein^{-1}$ (see section 2).
[b]GDH, glutamate dehydrogenase activities are given in nmoles NADPH oxidised · min^{-1} · mg $protein^{-1}$.

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