Aerobic expression of the *cyf* gene encoding cytochrome *c*-553 from *Desulfovibrio vulgaris* Hildenborough in *Escherichia coli*

W. Brent R. Pollock† and Gerrit Voordouw

Author for correspondence: Gerrit Voordouw. Tel: +1 403 220 6388. Fax: +1 403 289 9311.
e-mail: voordouw@acs.ucalgary.ca

Division of Biochemistry,
Department of Biological
Sciences, The University of
Calgary, Alberta, Canada
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Mono-haem cytochrome *c*-553 from *Desulfovibrio vulgaris* Hildenborough is encoded by the *cyf* gene which could be expressed in *Escherichia coli* to yield the periplasmic holoform of cytochrome *c*-553. Covalent haem attachment was shown by labelling with 5-amino[4-¹⁴C]laevulinic acid, the immediate precursor for haem biosynthesis. Visible-absorption spectroscopy demonstrated that the haem environment in the recombinant protein did not differ from the native protein. Optimal expression was obtained under aerobic conditions. Furthermore, efficient insertion of haem into a cytochrome *c*-553- β -lactamase fusion protein could be demonstrated. We suggest that the varying success in heterologous expression of *c*-type cytochromes in *E. coli* may arise as a result of differences in the physical properties of the apoproteins.

Keywords: cytochrome *c*-553, *cyf* gene, *Desulfovibrio vulgaris* Hildenborough, *Escherichia coli*, heterologous expression

INTRODUCTION

All *c*-type cytochromes have thioether bonds which covalently link haem to the polypeptide. These bonds are between the vinyl groups of the haem and the cysteinyl groups in the canonical polypeptide sequence CX_aX_bCH, wherein X_a and X_b are variable amino acids. It has been demonstrated (Dumont *et al.*, 1987; Drygas *et al.*, 1989) that thioether bond formation in mitochondrial cytochrome *c* requires a nuclear encoded protein which has been named cytochrome *c* haem lyase. Peculiarly, the existence of a similar protein in prokaryotes active in covalent haem insertion remains to be proven. Mutants have been isolated which are deficient in *c*-type cytochrome biosynthesis (Biel & Biel, 1990; Page & Ferguson, 1990; Ramseier *et al.*, 1991; Beckman *et al.*, 1992) but sequence analysis of the mutated loci has indicated that the encoded polypeptides may facilitate transport of *c*-type cytochromes (Ramseier *et al.*, 1991).

We have previously reported that *Escherichia coli* cannot synthesize the holoform of the tetrahaemic cytochrome *c*₃ from *Desulfovibrio vulgaris* Hildenborough (Pollock *et al.*, 1989), although *Rhodobacter sphaeroides* is able to synthesize low levels of the holoform (Cannac *et al.*, 1991). *D. vulgaris* has at least two other *c*-type cytochromes: cytochrome *c*-553, a 9 kDa, monohaemic cytochrome, and Hmc, a 70 kDa hexadeca-haemic cytochrome (Pollock *et al.*, 1991). Several attempts at expressing foreign, monohaemic *c*-type cytochromes in *E. coli* have been made with an as yet unexplained varying degree of success (McEwan *et al.*, 1989; Self *et al.*, 1990; von Wachenfeldt & Hederstedt, 1990b; Grisshammer *et al.*, 1991; Sanbongi *et al.*, 1991; Ubbink *et al.*, 1992). Therefore, it is of interest to determine whether *E. coli* can synthesize the holocytochrome *c*-553, which could be simpler than the tetrahaem cytochrome *c*₃.

The structural gene for cytochrome *c*-553, referred to from here on as *cyf*, has been cloned and sequenced (van Rooijen *et al.*, 1989). The *cyf* gene encodes a polypeptide of 103 residues (pro-Cyf). An N-terminal signal peptide of 24 residues directs the export of this polypeptide to the periplasm; cleavage of the signal peptide and haem insertion renders the mature holoprotein (Cyf). The structure of Cyf is known from X-ray crystallographic data (Nakagawa *et al.*, 1990) and 2D-NMR (Marion & Guerlesquin, 1992). Herein we report the heterologous expression of Cyf in *E. coli*.

†Present address: Department of Biochemistry, University of British Columbia, Vancouver, Canada.

Abbreviations: Amp, ampicillin; Bla, β -lactamase; *bla*, structural gene encoding Bla; Cyf, cytochrome *c*-553; *cyf*, structural gene encoding Cyf; IPTG: isopropylindolyl β -D-thiogalactopyranoside; Km, kanamycin; TMAO: trimethylamine-N-oxide; TMBZ, 3,3',5,5'-tetramethylbenzidine; TY, tryptone-yeast extract medium; X-P, 5-bromo-4-chloro-3-indolyl phosphate.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype, comments and reference
<i>D. vulgaris</i> Hildenborough	NCIMB 8303; isolated from clay soil near Hildenborough, Kent, UK (Postgate, 1984); source of the <i>cyf</i> gene encoding cytochrome <i>c</i> -553 (van Rooijen <i>et al.</i> , 1989)
<i>D. desulfuricans</i> G200	Spontaneous Nal ^r derivative of <i>D. desulfuricans</i> G100A (Weimar <i>et al.</i> , 1988)
<i>E. coli</i> TG2	$\Delta(lac-pro) supE thi hsdM hsdR recA$ [F' <i>traD36 proAB⁺ lacZ</i> ΔM15] ^q]; from T. J. Gibson*
pKUL6	Contains the levansucrase signal peptide gene fused in-frame to the gene of mature β -lactamase (Nivière <i>et al.</i> , 1992)
pKUL7	Derivative of plasmid pKUL6 with the signal peptide gene deleted (Nivière <i>et al.</i> , 1992)
pUC9	Amp ^r (Vieira & Messing, 1982)
pUC13	Amp ^r (Messing, 1983)
pUC18	Amp ^r (Yanisch-Perron <i>et al.</i> , 1985)
pJRD215	IncQ group, broad-host-range cloning vector; Km ^r Sm ^r (Davison <i>et al.</i> , 1987)†
pC41	Contains the <i>cyf</i> gene on a 736 bp fragment in pUC9
pJRC41	Contains the 736 bp <i>EcoRI</i> – <i>HindIII</i> insert of pC41 in pJRD215†
pDUL44	Contains the <i>cyf</i> gene fused in-frame to the gene of mature β -lactamase in pJRD215; this work†
pDUL45	As pDUL44 but with the 3' end of the β -lactamase gene deleted; this work†
pVIP18, pPP18, pKUL43, pKUL44 and pUC45	Intermediates in the construction of pDUL44 and pDUL45

* Constructed from *E. coli* JM101 by T. J. Gibson and M. D. Biggin at the Laboratory of Molecular Biology, MRC Centre, Cambridge, UK.

† The broad-host-range properties of this plasmid are not important for the present study, since expression was only studied in *E. coli*. Transformants were selected by their kanamycin resistance.

METHODS

Biochemical reagents. All enzymes were obtained from either Pharmacia or Boehringer Mannheim. The radiochemical 5-amino[4-¹⁴C]laevulinic acid hydrochloride [56 mCi mmol⁻¹; diluted with sterile distilled water to 83.3 μ Ci ml⁻¹ (3.082 MBq ml⁻¹)] was purchased from Amersham. Nitrocellulose was from Schleicher & Schuell. Reagents for colour development in Western blotting, alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin G, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (X-P) substrates, were purchased from Promega. All other reagent-grade chemicals were from either Sigma or Fisher.

Growth of bacteria. Liquid cultures of *E. coli* transformants were grown at 37 °C, either aerobically with shaking at 250 r.p.m., or anaerobically without agitation. *E. coli* TG2 was kept on M9 minimal glucose plates and transformants were grown on tryptone-yeast extract (TY) agar plates to which the appropriate antibiotic (100 μ g ml⁻¹ for ampicillin and 10 μ g ml⁻¹ for kanamycin), IPTG (16 μ g ml⁻¹) and X-Gal (32 μ g ml⁻¹) were added when required. Liquid cultures were in 5.0 ml of TY medium (Voordouw *et al.*, 1989) with the same concentration of antibiotics when required. M9 minimal medium (Miller, 1972) was used for radiolabelling studies. Anaerobic cultures were in TMAO medium (Bragg & Hackett, 1983). Bottles (100 ml) were filled with TMAO medium, flushed under nitrogen, sealed and autoclaved.

Strains, vectors and plasmids. The bacterial strains, vectors and plasmids used are described in Table 1. DNA manipulations were carried out essentially according to Sambrook *et al.* (1989).

Plasmid pJRC41 was obtained by transferring the 736 bp *HindIII*–*EcoRI* insert from an M13 clone (van Rooijen *et al.*, 1989) to plasmid pJRD215 (Fig. 1). In addition to pJRC41, two plasmids encoding fusions of *Cyf* and β -lactamase, pDUL44 and pDUL45, were constructed. The presence of plasmids pJRD215, pJRC41, pDUL44 or pDUL45 in *E. coli* was indicated by the kanamycin resistance of the resulting transformants.

Plasmid pDUL44 was derived from plasmid pKUL44 which was constructed as follows. The 736 bp insert of pC41 was first digested with *MaeI* (Fig. 1: a *MaeI* site is situated exactly at the 3'-end of the *cyf* gene) then blunt-ended with mung-bean nuclease. Next it was digested with *PstI* and finally ligated with pUC18 digested with *PstI* and *SmaI*. Plasmid pVIP18, isolated from the ligation mixture, was digested with *KpnI* and *EcoRI*, blunt-ended with T4 DNA polymerase in the presence of dNTPs, and self-ligated to give plasmid pPP18. Two fragments (a 2.4 kb *BamHI*–*EcoRI* fragment and a 1.8 kb *EcoRI* fragment containing the truncated *bla* gene) were isolated from a digest of pKUL7 by agarose gel electrophoresis. Ligation of the *cyf*-containing, 0.4 kb *BamHI*–*EcoRI* fragment of pPP18 and the 2.4 kb *BamHI*–*EcoRI* fragment of pKUL7 gave pKUL43. Insertion of the 1.8 kb *EcoRI* fragment of pKUL7 in the correct orientation gave pKUL44. The 2.2 kb insert of pKUL44 was transferred to vector pJRD215 by digestion with *HindIII* and

Fig. 1. A, Inserts of plasmids pJRC41, pDUL44 or pDUL45 containing the *cyf* gene or a *cyf*-*bla* fusion. Only relevant restriction enzyme sites have been illustrated. The putative *cyf* promoter (Pollock, 1992) and direction of transcription are indicated by arrows. The *bla* gene of pDUL45 is truncated at the 3' end (*bla'*). B, Features of proteins encoded by the plasmids in (A). All have the Cyf signal peptide (residues -24 to -1, shaded) followed by the complete Cyf sequence (residues 1 to 79). The haem binding site (Cys-Ile-Gly-Cys-His) starts at residue 10 (black). Residues 80 to 82 are a pUC vector derived linker peptide. Residues 83 to 344 or 83 to 240 are the Bla domain and the truncated Bla domain (*Bla'*).

SacI and ligation to give pDUL44 (Fig. 1A), which encodes the Cyf-Bla fusion protein indicated in Fig. 1 (B).

For the construction of plasmid pDUL45 the 900 bp *PstI* fragment was isolated from pKUL44 and ligated into the vector pUC13. Plasmid pUC45 with the desired orientation of the insert was selected by *Bam*HI digestion. The 900 bp *Hind*III-*Xba*I fragment was isolated from pUC45 and ligated into vector pJRD215 to give pDUL45 (Fig. 1A), which encodes a Cyf-Bla fusion protein that is truncated at the C-terminus (Fig. 1B). Both *cyf*-*bla* fusions are expected to encode proteins in which an N-terminal Cyf domain is linked to a C-terminal Bla domain through three bridging residues (-G-I-P-) encoded by DNA from the pUC vector. In both constructions the Bla domain starts at residue 2 of mature β -lactamase.

Labelling, SDS-PAGE, blotting and visible spectroscopy.

Periplasmic and membrane/cytoplasmic fractions of *E. coli* transformants were prepared as previously described (Pollock *et al.*, 1989). For *in vivo* labelling of *E. coli* TG2 transformants a single colony was used to inoculate 5.0 ml of M9 medium containing kanamycin and 1.6 μ Ci (59.2 kBq) of 5-amino[4-¹⁴C]laevulinic acid. After overnight growth, the cells were collected and periplasmic and membrane/cytoplasmic fractions were prepared. The periplasmic fractions were analysed by SDS-PAGE.

SDS-PAGE was done essentially according to Laemmli (1970), as detailed by Pollock *et al.* (1989) using gradient gels of 3% (w/v) bisacrylamide and 7% (w/v) acrylamide to 5% (w/v) bisacrylamide and 20% (w/v) acrylamide. Following SDS-PAGE, proteins were transferred electrophoretically to nitrocellulose as detailed by Pollock *et al.* (1989) except that the transfer buffer contained 0.1% (w/v) SDS. The protein blots

were incubated either with polyclonal antisera raised against purified *D. vulgaris* Hildenborough holocytochrome *c*-553 or with purified polyclonal antisera raised against *E. coli* β -lactamase (Nivière *et al.*, 1992) as the primary antibody. An alkaline-phosphatase-conjugated goat anti-rabbit IgG was used as the secondary antibody and the blot was immunostained with nitroblue tetrazolium and X-P. Blots to which ^{14}C -labelled samples were transferred were autoradiographed directly.

Visible spectroscopy was carried out with a Shimadzu UV-Visible recording spectrophotometer (model UV-265), using quartz cells with a pathlength of 1.0 cm and sample volumes of 1.0 ml. Post-electrophoretic porphyrin-*c* fluorescence analysis was carried out as previously described (Pollock *et al.*, 1989). Staining for haem peroxidase activity using TMBZ as a substrate was done according to Thomas *et al.* (1976).

RESULTS

Aerobic expression of the *cyf* gene in *E. coli*

Expression of the *cyf* gene in *E. coli* TG2(pJRC41) cells produced two forms, **a** and **b**, that were immunoreactive toward the anti-Cyf antiserum (Fig. 2, lanes 2 and 3). Form **a** migrated with the same mobility as purified *D.*

vulgaris Hildenborough Cyf (not shown), and was present predominantly in the periplasm. Form **b** was present in the membrane/cytoplasmic fraction. Neither was expressed in the *E. coli* TG2(pJRD215) control (Fig. 2, lanes 1 and 4) and forms **a** and **b** thus correspond to the processed and precursor forms of Cyf, respectively (Table 2).

Plasmid pJRC41 contains the *cyf* gene inserted into vector pJRD215, which does not possess a promoter capable of directing the transcription of any reading frame incorporated into the multiple-cloning site (Davison *et al.*, 1987). Therefore, synthesis of the cytochrome *c*-553 polypeptide in *E. coli* TG2(pJRC41) cells indicates that *E. coli* is able to direct transcription of the *cyf* gene from a *D. vulgaris* Hildenborough promoter element encoded on the insert. The -35 and -10 elements of this promoter have been tentatively identified by comparison to the upstream regions of other *D. vulgaris* Hildenborough genes (Pollock, 1992).

Both haem labelling and visible spectroscopy indicated that *E. coli* expressed form **a** as the holoprotein, as discussed in more detail below. This was not expected because *E. coli* was not able to express the holoprotein of cytochrome *c*₃, a tetrahaemic *c*-type cytochrome of the same organism, even though it could accurately process and export the apopolypeptide (Pollock *et al.*, 1989).

Covalent attachment of haem to *c*-type cytochromes can be conveniently demonstrated by labelling cells with 5-amino[4- ^{14}C]laevulinic acid (von Wachenfeldt & Hederstedt, 1990a), the first dedicated precursor in haem biosynthesis. Labelling of *E. coli* TG2(pJRC41) cells under aerobic conditions indicated that Cyf form **a** was present in the holoprotein (Fig. 3B, lane 2). Significantly, no other periplasmic *c*-type cytochromes were detected, which is in agreement with the notion that *E. coli* only expresses *c*-type cytochromes under anaerobic conditions (Bragg & Hackett, 1983; Kajie & Anraku, 1986).

Expression of Cyf in the holoprotein could also be demonstrated by direct spectroscopic analysis of stationary-phase cultures grown aerobically in TY medium, the results of which are summarized in Table 3. An air-oxidized sample of either *D. vulgaris* Hildenborough cytochrome *c*-553 or the periplasmic fraction obtained from *E. coli* TG2(pJRC41) cells was divided equally between two cuvettes. Sodium dithionite was then added to the sample cuvette. The resulting reduced minus oxidized difference spectra displayed identical absorption maxima and similar values for the ratio $\Delta\epsilon_{417}:\Delta\epsilon_{553}$. Spectra obtained for the periplasmic fraction of *E. coli* TG2(pJRD215) cells showed no discernible difference absorbance between 400 nm and 600 nm (not shown). These results indicated that the yield of recombinant holocytochrome *c*-553 was approximately 2 mg per litre of *E. coli* TG2(pJRC41) culture.

The presence of holocytochrome *c*-553 could not be detected either by post-electrophoretic porphyrin-*c* fluorescence analysis or by staining for haem peroxidase activity using TMBZ as a substrate.

Fig. 2. Expression of the *cyf* gene in *E. coli*. Forms **a** and **b** are explained in Table 2. Samples were analysed by SDS-PAGE using a 7–20% gradient gel and blotting. The protein blot was incubated with a polyclonal anti-Cyf serum. Lane 1, periplasmic fraction of *E. coli* TG2(pJRD215); lane 2, periplasmic fraction of *E. coli* TG2(pJRC41); lane 3, membrane/cytoplasmic fraction of *E. coli* TG2(pJRC41); lane 4, membrane/cytoplasmic fraction of *E. coli* TG2(pJRD215). All cultures were grown in TY medium with 10 μg kanamycin ml^{-1} .

Table 2. Properties of Cyf and Cyf–Bla fusion proteins synthesized in *E. coli* transformed with the plasmids indicated in Fig. 1

Form	Haem	Anti-Cyf*	Anti-Bla†	Mol. mass‡	Remarks
a	+	+	–	9 (9)	Periplasmic holoform of Cyf synthesized in <i>E. coli</i> TG2(pJRC41); same migration on SDS-PAGE as Cyf purified from <i>D. vulgaris</i> Hildenborough
a'	+	+	–	10	Periplasmic holoform of Cyf obtained by proteolytic degradation of forms c or e
b	ND	+	–	12 (11)	Membrane/cytoplasmic pre-Cyf; precursor of form a
c	+	+	+	45 (38)	Periplasmic Cyf–Bla fusion protein synthesized in <i>E. coli</i> TG2(pDUL44); as in Fig. 1(B), but without the signal peptide
d	–	–	+	38	Periplasmic proteolytic degradation product of form c
e	–	+	+	35 (28)	Periplasmic Cyf–Bla' fusion protein synthesized in <i>E. coli</i> TG2(pDUL45); as in Fig. 1(B), but without the signal peptide

* Reaction of protein with antiserum directed against Cyf.

† Reaction of protein with antiserum directed against Bla.

‡ Molecular mass in kDa determined by SDS-PAGE relative to pre-stained markers as well as Cyf purified from *D. vulgaris* Hildenborough. Theoretical values are in brackets. Mature Bla (theoretical molecular mass 29 kDa) ran as a 36 kDa polypeptide in SDS-PAGE.

ND, Not determined.

Table 3. Visible absorption spectroscopic data for *D. vulgaris* Hildenborough cytochrome *c*-553 expressed in *E. coli*

Sample	[Reduced sample – oxidized sample]	
	$\lambda_{\max}$ (nm)	$\Delta\epsilon_{417} : \Delta\epsilon_{553}$
<i>Cytochrome c</i> -553	417, 523, 553	4.4 ± 0.2
<i>E. coli</i> TG2(pJRC41), aerobic periplasm	417, 523, 553	4.6 ± 0.2
<i>E. coli</i> TG2(pDUL44), aerobic periplasm	417, 523, 553	4.5 ± 0.2
<i>E. coli</i> TG2(pDUL45), aerobic periplasm	417, 523, 553	4.2 ± 0.2

Aerobic expression of the *cyf*–*bla* fusions in *E. coli*

In order to further delineate the properties of the haem insertion system in *E. coli*, two vectors expressing Cyf–Bla fusion proteins were constructed (Fig. 1). Plasmid pDUL44 was designed to encode a fusion protein with an N-terminal Cyf and a C-terminal mature Bla domain. Export of this fusion protein is directed by the Cyf signal peptide. Plasmid pDUL45 encoded a similar fusion but with a truncated Bla domain (Fig. 1B, Bla').

The periplasm of stationary-phase cultures of *E. coli* TG2(pDUL44) was shown to contain three distinct forms: **a'**, **c** and **d** (Fig. 3, lane 3; Table 2). Form **c** corresponds to the processed fusion polypeptide; it was immunoreactive towards antisera directed against either Bla (Fig. 3A) or Cyf (not shown). Also, incorporation of 5-amino[4-¹⁴C]laevulinic acid into form **c** (Fig. 3B) demonstrated the Cyf domain of the fusion to be in the holoform. Form **a'**, which was also in the holoform, is thought to arise from nearly complete degradation of the

Fig. 3. Incorporation of ^{14}C -labelled haem into Cyf and two Cyf-Bla fusion proteins expressed in *E. coli* TG2. The cells were grown overnight in M9 minimal medium to which 5-amino[4- ^{14}C]laevulinic acid was added to $0.32\ \mu\text{Ci ml}^{-1}$ ($11.84\ \text{kBq ml}^{-1}$). Periplasmic fractions were analysed by SDS-PAGE using a 7–20% gradient gel. Forms a, a', c, d and e are described in Table 2. A, Protein blot incubated with anti-Bla antibodies. Lanes 1–4, *E. coli* TG2 transformed with pJRD215, pJRC41, pDUL44 or pDUL45, respectively. B, Autoradiogram of the blot in (A).

C-terminal Bla domain of form **c** because (i) it was immunoreactive to anti-Cyf serum (not shown), but not to anti-Bla serum (Fig. 3A) and (ii) it had only a slightly larger relative molecular mass than Cyf, as judged by SDS-PAGE (Fig. 3B). The haem environment in forms (a', c) was indistinguishable from that in the native cytochrome *c*-553 from *D. vulgaris* Hildenborough by visible absorption spectroscopy (Table 3). The third fusion protein, form **d**, observed in the periplasm of *E. coli* TG2(pDUL44) cells, is also likely to be a degradation product of form **c**. Form **d** may have arisen by partial degradation of the Cyf domain because (i) it demonstrated anti-Bla (Fig. 3A, lane 3) but not anti-Cyf immunoreactivity (not shown) and (ii) its molecular mass, as estimated by SDS-PAGE, was higher than that of mature Bla. Form **d** did not incorporate haem (Fig. 3B, lane 3).

A simpler, two-component pattern was seen in the periplasmic fraction prepared from *E. coli* TG2(pDUL45) cells. This fraction contained the mature, full length fusion polypeptide, form **e** (Table 2), which was immunoreactive to anti-Bla (Fig. 3A, lane 4) and anti-Cyf sera (not shown). However, unlike the corresponding form **c** encoded by pDUL44, form **e** was only seen in the apoform (Fig. 3B, lane 4). The periplasm from *E. coli* TG2(pDUL45) cells also contained a protein which was identical to form **a'** by immunoreactivity (not shown), 5-amino[4- ^{14}C]laevulinic acid incorporation (Fig. 3B, lane 4) and visible absorption spectroscopy (Table 3).

E. coli TG2(pDUL44) cells could grow in the presence of ampicillin indicating that the Bla domain of forms **c** and **d** was folded and active. *E. coli* TG2(pDUL45) could not grow in the presence of ampicillin. Form **e** cannot have β -

lactamase activity, since the truncated Bla domain lacks the β -lactam binding site (Herzberg & Moulton, 1987).

Expression of *cyf* under anaerobic conditions

Since *E. coli* expresses its *c*-type cytochromes only under anaerobic conditions, particularly when TMAO is used as the electron acceptor, the possibility of increased holocytochrome *c*-553 expression under these conditions was investigated. Haem incorporation could be demonstrated by ^{14}C -labelling (not shown), but spectroscopic analysis using *E. coli* TG2(pJRD215) controls indicated expression of only 0.03 mg of Cyf per litre of TMAO medium, approximately 70-fold less than under aerobic conditions.

E. coli TG2(pDUL44) cells grown anaerobically demonstrated ampicillin resistance comparable to cells grown aerobically. This indicated that export of the Cyf-Bla fusion protein to the periplasm was not impaired during anaerobic growth.

DISCUSSION

In the present study we have shown that holocytochrome *c*-553 from *D. vulgaris* Hildenborough can be readily expressed in the *E. coli* periplasm. This was not expected because we had shown earlier that cytochrome *c*₃ from the same organism cannot be expressed in the holoform in *E. coli* under either aerobic or anaerobic growth conditions (Pollock *et al.*, 1989). Also, in the first report of expression of a *c*-type cytochrome in *E. coli*, expression of holocytochrome *c*₂ from *Rhodobacter sphaeroides* (McEwan *et al.*, 1989), it was reported that holoprotein synthesis could only be obtained under anoxic conditions, when using

TMAO as the electron acceptor. These are the same conditions under which *E. coli* expresses its native *c*-type cytochromes (Bragg & Hackett, 1983). Others have since found that these conditions are not required for heterologous *c*-type cytochrome expression: holoprotein synthesis has been observed in aerobically grown *E. coli* for *Bacillus subtilis* cytochrome *c*-550 (von Wachenfeldt & Hederstedt, 1990b), *Hydrogenobacter thermophilus* cytochrome *c*-552 (Sanbongi *et al.*, 1991) and *Thiobacillus versutus* cytochrome *c*-550 (Ubbink *et al.*, 1992). The latter was expressed most efficiently under semi-anaerobic conditions, yielding 1–2 mg holocytochrome l⁻¹, whereas under strictly aerobic or anaerobic conditions only ca. 0.1 mg l⁻¹ was obtained. Our observation of a significant level of expression (2 mg l⁻¹) of holocytochrome *c*-553 from *D. vulgaris* Hildenborough in *E. coli* under aerobic conditions is inconsistent with the hypothesis that holo-*c*-type cytochrome expression is absolutely dependent on a host-specific factor, which is expressed in *E. coli* only under a limited set of conditions (e.g. absence of oxygen, presence of TMAO; McEwan *et al.*, 1989).

In contrast there is evidence that the success or failure of expressing a heterologous gene for a *c*-type cytochrome in *E. coli* in the holoform may depend primarily on the ability of the apoprotein to assume a three-dimensional conformation which is capable of binding haem in an orientation which facilitates covalent bond formation. Evidence for partial folding of apocytochrome *c*-550 from *Paracoccus denitrificans* was obtained by Page & Ferguson (1990) by size-exclusion chromatography experiments and indicated that, although somewhat expanded, the apocytochrome retained the dimeric quaternary structure of the holoprotein. The *T. versutus* cytochrome *c*-550 expressed in *E. coli* as the holoprotein by Ubbink *et al.* (1992) is highly homologous to the *P. denitrificans* protein. This success may thus originate from formation of a similarly folded apocytochrome in the *E. coli* periplasm. The results obtained by Sanbongi *et al.* (1991), who expressed the holoform of cytochrome *c*-552 from the thermophilic *Hydrogenobacter thermophilus* in the *E. coli* cytoplasm from a cloned gene lacking the coding region for the N-terminal signal peptide, are at first glance bizarre but can be understood if we assume that the apocytochrome *c*-552 formed in the *E. coli* cytoplasm is highly structured and, as a result, readily binds haem.

In order to understand the nature of the factors necessary for holocytochrome formation in *E. coli* it is important to note that the *in vitro* prepared apoform of mitochondrial cytochrome *c* has no recognizable tertiary (or secondary) structure and cannot react spontaneously with free haem (Sano & Tanaka, 1964; Stellwagen *et al.*, 1972; Fisher *et al.*, 1973; Cohen *et al.*, 1974). This is in direct contrast to cytochrome *b₅*, which adopts a conformation resembling that of a relaxed holoform (Huntley & Strittmatter, 1972; Moore *et al.*, 1991). Significantly, it has recently been shown that when Asn-57 of cytochrome *b₅*, which is in close proximity to the vinyl group at haem position 4, is replaced by a cysteine an α -thioether bond is formed (Barker *et al.*, 1993). This may indicate that a haem-*c* linkage can occur spontaneously as long as the apopoly-

peptide has sufficient secondary and tertiary structure to enable haem binding. A similar α -thioether bond can be spontaneously formed between a cysteinyl residue of the phytochrome apopolyptide and the linear tetrapyrrole prosthetic group phytychromobilin (Li & Lagarias, 1992). In view of these results we suggest that if a *c*-type cytochrome apopolyptide, expressed in *E. coli* under aerobic conditions, has a tertiary structure resembling that of the holoprotein its cysteinyl residues may spontaneously form covalent bonds with the haem vinyls. Our suggestion does not preclude the possibility that protein factors may facilitate covalent binding of haem to apocytochromes in other prokaryotes or even in *E. coli* under anaerobic conditions. If proof for the existence of such factors can be obtained then an important question is whether they should be considered to be true enzymes or merely chaperonins with the function to hold the apo-protein in a conformation that allows covalent haem insertion. The factor that aids in covalent haem insertion into mitochondrial cytochrome *c* has been named cytochrome *c* haem lyase (Dumont *et al.*, 1987; Drygas *et al.*, 1989), but so far no mechanism for its enzymic action has been put forward, and the possibility that this protein is in fact a cytochrome *c*-chaperonin cannot be ruled out.

It is interesting that, in this work, labelling with 5-amino[4-¹⁴C]laevulinic acid indicated haem incorporation in fusion protein form **c**, synthesized by *E. coli* TG2(pDUL44) cells, but not in form **e**, synthesized by *E. coli* TG2(pDUL45) cells. We envisage form **c** as consisting initially of a folded apo-Cyf domain connected to a folded Bla domain. The relative position of the two domains is such that spontaneous haem insertion into the apo-Cyf domain is not prevented. This can be rationalized from the three-dimensional structure, which indicates the C-terminal Leu-79 to be distant from the haem (Nakagawa *et al.*, 1990). Proteolysis of form **c** can lead to form **a'**, lacking the Bla domain, or form **d**, lacking the Cyf domain. Form **e** consists initially of a folded apo-Cyf domain and part of the Bla polypeptide, which is unlikely to be folded because it is truncated. The presence of this unfolded polypeptide may reduce access of haem to the apo-Cyf domain in the fusion protein. However, its proteolytic degradation yields form **a'**, which readily incorporates haem. These results are not inconsistent with those obtained by von Wachenfeldt & Hederstedt (1990b) for a CccA-PhoA fusion. These authors observed both the mature fusion polypeptide and a degradation product analogous to our form **a'** in the apoform, despite the fact that the holocytochrome was formed when the unfused *cccA* gene was expressed in aerobic *E. coli*. The method by which the CccA-PhoA fusion was generated truncated part of the CccA C-terminus and we suggest that this interfered with folding of the apo-CccA domain in a form competent to bind haem.

In conclusion, it appears that the varying degrees of success obtained during heterologous expression of *c*-type cytochromes in *E. coli* may possibly be explained by differences in folding of the expressed apoproteins. Progress in our understanding of the process of covalent haem insertion may accelerate upon more extensive

biophysical investigation of the conformation of these apoproteins, and upon devising strategies for finding genes, whose products modulate this conformation.

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