Synthesis of *Rhodobacter sphaeroides* cytochrome *c*₂ in *Escherichia coli*

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1. SUMMARY

The cytochrome *c*₂ structural gene, *cycA*, from *Rhodobacter sphaeroides* was expressed in *Escherichia coli*. *CycA*-specific mRNA was detected in *E. coli* both under aerobic and anaerobic conditions with trimethylamine-N-oxide as electron acceptor. However mature holocytochrome *c*₂ was only detected in anaerobically-grown cells. The mature form of cytochrome *c*₂ (Mᵣ = 12,500) was secreted into the periplasm of *E. coli* suggesting that the signal polypeptide was processed. The cytochrome *c*₂ synthesized in *E. coli* exhibited absorbance maxima in the reduced form at 550 nm (α-band) and 521 nm (β-band) and contained covalently attached haem *c*. The results indicate that a foreign *c*₂-type cytochrome can be secreted and assembled in *E. coli* under anaerobic conditions.

2. INTRODUCTION

In Gram-negative bacteria most species of *c*-type cytochrome are located in the periplasmic space where they have a variety of roles in electron transport [1]. Haem *c* is unique, compared to other haem prosthetic groups, in that it is covalently attached to the apocytochrome polypeptide [2]. The synthesis of a *c*-type cytochrome raises an additional problem in protein assembly since at some stage *c*-type haem must be attached to the apocytochrome. Many studies of protein secretion use *Escherichia coli* [3] however, the *c*-type cytochrome(s) of this bacterium are poorly defined [4] and only the membrane-bound nitrite reductase, cytochrome *c*₅₅₄ has been characterized [5]. The situation is different in photosynthetic bacteria where a number of periplasmic *c*-type cytochromes have been described in detail [6]. The most abundant cytochrome in the photosynthetic bacterium *Rhodobacter sphaeroides* is cytochrome *c*₂ [7] and its role in photosynthetic electron transport is well documented [8,9]. Recently we re-
ported the cloning and DNA sequence of the cytochrome c₂ (cyt c₂) structural gene, cycA, from *R. sphaeroides* [10] and this has allowed us to begin to address questions concerning the secretion and assembly of cyt c₂. In this paper we demonstrate that the cycA gene of *R. sphaeroides* can be expressed in *E. coli* and we describe growth conditions in which a mature form of cyt c₂ containing haem is exported into the periplasm.

3. MATERIALS AND METHODS

3.1. Bacterial growth and cell fractionation

*E. coli* strain JM83 [11] was grown in medium containing 1% glucose as carbon source either aerobically by vigorous shaking or anaerobically in completely filled screw capped bottles containing 0.3% trimethylamine-N-oxide (TMAO) [12]. Fifty μg/ml ampicillin was included during growth of *E. coli* cells harbouring plasmid pUC19 or its derivatives pC2P2.71 and pC2P2.72. The latter two plasmids contain a 2.7 kb *PstI* restriction endonuclease fragment from *R. sphaeroides* which includes the cycA gene cloned in both orientations with respect to the lac promoter present in pUC19 [10].

*E. coli* cells (20 ml) were grown to a density of about 10⁹ per ml, harvested, washed once in 100 mM sodium phosphate (pH 7.6), 5 mM EDTA (ICM buffer), and then resuspended in 5 ml of the same buffer. Cell free extracts were prepared by sonication followed by centrifugation (30,000 × g, 10 min) to pellet unbroken cells. Periplasmic fractions were prepared from 500 ml of *E. coli* cells as described by Tai and Kaplan [13].

3.2. Analysis of proteins produced in *E. coli*

Cell free extracts were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis at room temperature using a gradient of 11.5–18% acrylamide/bis-acrylamide (39: 1) [14]. Electrophoretically separated polypeptides were stained for haem-dependent peroxidase activity [15]. Cyt c₂, purified from soluble fractions of phototrophically-grown *R. sphaeroides* strain R26 [10] was used as a standard.

For immunoblotting, polypeptides were transferred, after SDS polyacrylamide gel electrophoresis, to nitrocellulose (0.2 μm pore size) as in Chory et al. [16]. Cyt c₂ was identified by its reaction with rabbit antibody raised against cyt c₂ purified from *R. sphaeroides* [9]. Immunoblots were developed using goat anti-rabbit antibody conjugated to biotin, strepavidin-β-galactosidase and the chromogenic substrate Bluogal (Bethesda Research Laboratories). Reduced minus oxidized difference spectra of periplasmic fractions were recorded using a Cary 2300 spectrophotometer with 1 mM sodium ascorbate as reductant and 400 μM potassium ferricyanide as oxidant. Cyt c₂ contents of fractions were calculated using $E_{\text{mm}} = 20$ at 550–540 nm [17]. The protein content of samples was determined as in Markwell et al. [18]. Samples from cells grown in the presence of TMAO were dialyzed against ICM buffer to remove trimethylamine before the assay. Malate dehydrogenase activity was determined as described by Markwell and Lascelles [19].

3.3. Recombinant DNA techniques

Bulk RNA was prepared from *E. coli* as described [20] and orcinol assays were used to quantitate RNA. Purification and analysis of DNA fragments has been described [21] as have methods for nick translation of DNA fragments for use as probes [22] and stringency conditions for DNA-RNA hybridization [20]. Restriction endonucleases and nick translation kits were obtained from either Bethesda Research Labs or New England Biolabs and were used according to manufacturers instructions. Nytran transfer membranes for RNA dot blots were purchased from Schleicher and Schuell. [α-³²P]-dCTP (3000 Ci/mmol) was obtained from New England Nuclear.

4. RESULTS

4.1. Synthesis of cyt c₂ in *E. coli*

The cycA gene of *R. sphaeroides* is located on a 2.7 kb *PstI* DNA restriction endonuclease fragment [10]. This *PstI* fragment was cloned into the lac multiple cloning site of plasmid pUC19 to generate pC2P2.71 in which the directions of tran-
that in cell free extracts from cells containing
plasmid pC2P2.71 an additional haem-staining polypeptide of low molecular weight was synthe-
sized. This polypeptide had the same electro-
phoretic mobility as R. sphaeroides cyt c$_2$ (Fig. 1,
lane a). This c-type cytochrome was not detectable
in soluble extracts of E. coli cells which did not
contain the cycA gene (Fig. 1, lanes d, e) or in
cells containing plasmid pC2P2.72 (Fig. 1, lane b).
This result suggested that cyt c$_2$ synthesis in E.
coli was dependent on transcription from the
plasmid lac promoter. No haem-staining poly-
peptides were detected in cell free extracts from
aerobically grown cells; including those harboring
the cycA gene on either plasmid pC2P2.71 or
pC2P2.72 (data not shown).

4.2. Localization of cyt c$_2$ in the periplasm of E.
coli

Fig. 2 shows an ascorbate-reduced minus ferri-
cyanide oxidized spectrum of the periplasmic frac-
tion from E. coli JM83 containing plasmid
pC2P2.71, grown anaerobically with TMAO. Ab-
sorption maxima were observed at 550 nm and

Fig. 1. Haem-staining profile of polypeptides after SDS-PAGE.
Lane a, 0.4 µg pure cyt c$_2$ from phototrophically grown R.
sphaeroides. Thirty µg cell-free extract from E. coli JM83
grown anaerobically with TMAO and harboring the following
plasmids: lane b, pC2P2.72; lane c, pC2P2.71; lane d, pUC19;
lane e, no plasmid.

The expression of the cycA gene in E. coli was monitored in cells grown
anaerobically with TMAO as electron acceptor. Cell-free extracts were prepared, the polypeptides
were separated by SDS-PAGE and then stained
for haem-dependent peroxidase activity. Since only
haem c is retained following electrophoresis under
denaturing conditions at room temperature [23],
the results in Fig. 1 indicate the presence of c-type
cytochromes. Haem-staining polypeptides of high
molecular weight were observed in all E. coli
extracts (Fig. 1 lanes b, c, d, e). These poly-
peptides probably represent endogenous c-type
cytochromes of E. coli since their synthesis was
dependent on the use of TMAO as an electron
acceptor (data not shown) but independent of the
cycA gene (Fig. 1 lanes d, e). Fig. 1 (lane c) shows
256 nm and this spectrum was identical to the spectrum of cyt c₂ purified from *R. sphaeroides* [1]. No cyt c₂ was observed in ascorbate reduced minus ferricyanide oxidized difference spectra of either the membrane or cytoplasmic fractions (data not shown). Malate dehydrogenase was used as a marker for cytoplasmic enzymes [19]; the cellular distribution of this activity was periplasmic fraction 5%; cytoplasmic and membrane fraction 95%. These data show that no uncontrolled lysis of cells occurred during cell fractionation and they support the conclusion that cyt c₂ was located in the periplasm of *E. coli*. No ascorbate-reducible c-type cytochrome was observed in periplasmic fractions of *E. coli* cells which did not contain the cycA gene (Fig. 2). The endogenous c-type cytochrome(s) of *E. coli* synthesized when cells are grown anaerobically with TMAO were observed in the membrane fraction (data not shown). These cytochromes were found to be reducible with dithionite but not ascorbate (data not shown), in agreement with Bragg and Hackett [12].

4.3. Immunochernical identification of cyt c₂

We have shown in an earlier report that the cycA gene encodes a putative signal polypeptide and directs the synthesis of a cyt c₂ precursor polypeptide (*Mₚ* = 15,500) in an *R. sphaeroides* in vitro transcription-translation system [10]. The data in Fig. 1 showed that the cyt c₂ synthesized in *E. coli* had the same electrophoretic mobility as the mature form of cyt c₂ from *R. sphaeroides*. This suggested that the putative signal polypeptide of cyt c₂ was cleaved during its secretion into the *E. coli* periplasm. Periplasmic fractions from *E. coli* were analyzed by immunoblotting using anti-cyt c₂ antibody. Fig. 3 shows that no cross-reacting material corresponding to cyt c₂ could be detected in the periplasm of cells grown in the absence of cycA (lane 2) but that cyt c₂ was detected in cells containing cycA which were grown anaerobically with TMAO (lane 3). No cyt c₂ was immunologically detectable in cells containing cycA grown aerobically lane (1). The cyt c₂ synthesized in *E. coli* was estimated to have a molecular weight of 12,500. This indicates that a mature form of cyt c₂ is synthesized in *E. coli*.

4.4. Levels of cycA-specific mRNA in *E. coli*

Since no mature cyt c₂ was found in aerobically grown cells, the levels of cycA-specific mRNA were monitored by dot blots to confirm that the synthesis of cyt c₂ was not limited by cycA transcription. An internal cycA stuI restriction endonuclease fragment [9] was used to measure cycA-specific transcripts in bulk mRNA prepared from cells grown aerobically and under anaerobic conditions in the presence of TMAO (Table 1). There was a low degree of hybridization of the cycA

Fig. 3. Immunoblot using anti-cyt c₂ antibody against SDS-PAGE separated periplasmic fractions from *E. coli*. Lane 1, JM83 (pC2P2.72), aerobically grown; Lane 2, JM83 (pUC19), anaerobic plus TMAO; Lane 3, JM83 (pC2P2.71), anaerobic plus TMAO. Thirty µg of protein was electrophoresed in each lane.
Table 1
Levels of cycA-specific transcripts in E. coli

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Electron acceptor</th>
<th>CPM over background</th>
<th>Relative amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pC2P2.71</td>
<td>O2</td>
<td>1143</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>TMAO</td>
<td>567</td>
<td>47</td>
</tr>
<tr>
<td>pC2P2.72</td>
<td>O2</td>
<td>570</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>TMAO</td>
<td>223</td>
<td>14</td>
</tr>
</tbody>
</table>

a Cells were grown as described in MATERIALS AND METHODS.
b Transcript levels were measured from dot blots containing 5 μg of RNA per sample. Background hybridization of this probe to 5 μg of RNA from JM83 cells containing plasmid pUC19 was approximately 50 cpm.

probe to RNA from cells which did not contain cycA and this was subtracted from all the data presented in Table 1. CycA-specific transcripts were observed in all RNA preparations from cells containing the cycA gene. However the level of cycA-specific mRNA was highest in aerobic cells containing plasmid pC2P2.71 and it was reduced by about 50% in the same cells grown anaerobically with TMAO (Table 1). The level of cycA-specific transcripts was lower under either growth conditions in cells containing plasmid pC2P2.72 in which the cycA gene would be transcribed in a direction opposite to the lac promoter of plasmid pUC19.

5. DISCUSSION

The biogenesis of mature cyt c2 requires covalent attachment of haem to the apocytochrome polypeptide, cleavage of the signal sequence from the putative cyt c2 precursor polypeptide and secretion of the protein into the periplasmic space. This paper shows that under certain physiological conditions the R. sphaeroides cyt c2 structural gene can direct synthesis of a mature form of cyt c2 in E. coli. There are several examples of genes encoding b-type cytochromes from other organisms that have been expressed and assembled into functional redox proteins in E. coli [24,25], but this paper reports the first successful processing and export of a foreign c-type cytochrome in E. coli.

The level of c-type cytochrome(s) in aerobically grown E. coli is extremely low [4], and similarly no mature cyt c2 was detected by haem-staining or by immunoblotting when the cycA gene was expressed in aerobically-grown cells. In contrast c-type cytochromes are thought to be involved in respiratory electron flow to TMAO [12] and they were detected in the membrane of cells grown anaerobically with this electron acceptor. The observation that cyt c2 is synthesized only under anaerobic conditions suggests that its assembly in E. coli may be limited by the availability of the cell to either synthesize haem c or covalently attach it to the cyt c2 apoprotein under aerobic conditions. Cytochrome c-haem lyase, which covalently attaches haem c to apocyt c, has only been studied in detail in mitochondria [26], and it is possible that the equivalent enzyme activity in E. coli may only be present or active in significant amounts in anaerobically grown cells where there is a physiological requirement for c-type cytochromes. The low levels of cyt c2 synthesized in E. coli may also reflect an inefficient attachment of haem to the cyt c2 apoprotein by the cytochrome c-haem lyase of this bacterium. This would be particularly true if the E. coli enzyme(s) had a relatively narrow substrate specificity as is found for a yeast haem lyase which is apparently specific for mitochondrial cytochrome c [27].

The failure to detect mature cyt c2 in E. coli grown aerobically was not due to a lack of transcription since cycA-specific transcripts, presumably driven from the vector lac promoter, were the highest under aerobic growth conditions. We also know that cycA-phoA protein fusions constructed either upstream (Varga and Kaplan, unpublished) or downstream (Brandner et al., unpublished) of the site of covalent haem attachment are processed and enzymatically active in E. coli under aerobic conditions when these fusions are downstream of the same plasmid lac promoter. This suggests that the cyt c2 precursor protein is synthesized and processed to the apocyt c2 under aerobic conditions in E. coli, but that the apoprotein is rapidly turned over in either the cytoplasm or periplasm in the absence of haem attachment. Efforts to test this hypothesis by pulse-chase experiments have so far been unsuccessful; however this may be
because antibodies raised against mature holiest c$_2$ appear to have only very weak reactivity towards the cyt c$_2$ precursor polypeptide [10].

The dependence of cyt c$_2$ accumulation on the orientation of the cycA gene in E. coli requires comment. We have consistently observed that transcription from R. sphaeroides promoters is low or undetectable in E. coli [28]. However, insertion of the cycA gene downstream of the lac promoter of pUC19 allows detection of mature cyt c$_2$ under anaerobic growth conditions in plasmid pC2P2.71. This observation suggests that high level transcription driven by the lac promoter is required for accumulation of detectable cyt c$_2$ in E. coli, although we have not addressed whether transcription also initiates from within the R. sphaeroides sequences on either pC2P2.71 or pC2P2.72 in E. coli. The cycA-specific mRNA levels from both plasmids were higher in aerobic cells compared with anaerobic cells and this supports the view that a post-transcriptional activity, necessary for synthesis of mature cyt c$_2$ in E. coli is absent or limiting in aerobically grown E. coli cells. The use of well-characterized protein export mutants of E. coli in future studies may aid in identifying regions of cyt c$_2$ which are important for processing, secretion, or possibly haem attachment as well as aid in the identification of equivalent gene products involved in the biogenesis of cyt c$_2$ in R. sphaeroides. Such information may well be of value in understanding the assembly and secretion of other c-type cytochromes in Gram-negative bacteria.

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