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# Heterologous expression of soluble fragments of cytochrome $c_{552}$ acting as electron donor to the *Paracoccus denitrificans* cytochrome *c* oxidase

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**Abstract**

A membrane-bound *c*-type cytochrome, $c_{552}$, acts as the electron mediator between the cytochrome $bc_1$ complex and cytochrome *c* oxidase in the branched respiratory chain of the bacterium *Paracoccus denitrificans*. Unlike in mitochondria where a soluble cytochrome *c* interacts with both complexes, the bacterial $c_{552}$, the product of the *cyc*M gene, shows a tripartite structure, with an N-terminal membrane anchor separated from a typical class I cytochrome domain by a highly charged region. Two derivative fragments, lacking either only the membrane spanning region or both N-terminal domains, were constructed on the genetic level, and expressed in *Escherichia coli* cotransformed with the *ccm* gene cluster encoding host-specific cytochrome *c* maturation factors. High levels of cytochromes *c* were expressed and located in the periplasm as holo-proteins; both these purified $c_{552}$ fragments are functional in electron transport to oxidase, as ascertained by kinetic measurements, and will prove useful for future structural studies of complex formation by NMR and X-ray diffraction. 

*Keywords:* Membrane anchor; Cytochrome *c* biogenesis; Electron transfer; Supercomplex; *ccm* gene; Cytochrome *c* maturation

## 1. Introduction

The interaction and subsequent electron transfer (ET) between redox-active enzymes and their mediators has long been of considerable interest (e.g. [1–3]), but at the same time it has been hampered for most cases by the fact that respiratory ET complexes are integral membrane proteins not easily amenable to structural studies. Only in a few selected model systems has the structural basis for ET been understood in molecular detail (e.g. [4–6]).

Cytochrome *c* is a well-established electron mediator between the last two complexes of the mitochondrial redox chain, the cytochrome $bc_1$ complex and the $aa_3$-type cytochrome *c* oxidase, and it has long been appreciated that its interaction with either complex is primarily governed by electrostatic forces. Despite recent progress in the elucidation of the structure of both multi-subunit enzymes [7–13], no

*Abbreviations:* ET, electron transfer; TXRF, total reflection X-ray fluorescence spectroscopy; SDS, sodium dodecyl sulfate; PAG(E), polyacrylamide gel (electrophoresis); IPTG, isopropyl β-D-thiogalactoside; PCR, polymerase chain reaction

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information on the actual complex formation with their electron donor/acceptor, cytochrome *c*, is available due to the lack of co-crystals.

Functional and mutagenesis studies have defined individual residues interacting at the docking site on either complex [14–17]. For oxidase, a two-stage binding model has been suggested [18,19], in which (i) the primary orientation is achieved by long-range electrostatic forces, followed by (ii) a fine-tuning of the interaction exerted by hydrophobic patches at or around the docking site. In conjunction with results from other systems (e.g. [20,21]) this has led to the notion of pseudospecificity in the primary recognition process governed by the overall electrostatic potential, thereby granting sufficient conformational freedom to both partners to attain an optimal ET conformation in the subsequent step.

Bacterial ET chains often display a much higher degree of complexity when compared to the mitochondrial counterpart, due to their greater nutritional adaptability and flexibility to environmental demands. For a bacterium such as *Paracoccus denitrificans*, considered here, a large number of cytochromes *c* have been described, both soluble and membrane-integrated (see [22] for a recent review). A membrane-bound 18 kDa cytochrome $c_{552}$, product of the *cyc*M gene [23], was identified as the genuine electron mediator in this region of the ET chain of *Paracoccus*: it copurifies under certain experimental conditions with a supercomplex of both respiratory complexes [24], and its deletion, or inhibition by a specific antibody, abolishes ET between complexes III and IV in membranes [23]. However, using conventional purification strategies, this protein has not been available in sufficient amounts for in vitro experiments, and as in many other cases, its heterologous expression in *Escherichia coli* resulted in rather poor yield [23,25]. Recently, our understanding of cytochrome *c* assembly has been boosted by the elucidation of the individual steps involved in this organism, and by recognizing the requirement of several cytochrome *c* maturation factors encoded in the *ccm* operon [26–31].

Here we report the synthesis of two soluble fragments of the *P. denitrificans* cytochrome $c_{552}$ in an *E. coli* host cell overexpressing the *ccm* genes from a plasmid *in trans*. The purified cytochrome fragments interact with isolated *Paracoccus* oxidase and sustain ET activity. Unlike earlier attempts to express *c* cytochromes in *E. coli*, this approach yields holo-protein at levels suitable for 3-D and solution structure studies.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*E. coli* DH5α [32] was used for subcloning, and BL21(DE3) (Novagen) for expression of the *P. denitrificans* $c_{552}$ fragments (see below); where appropriate, antibiotics were added to a concentration of 50 μg/ml (ampicillin) or 30 μg/ml (chloramphenicol) and cells were grown aerobically at 37°C in Luria-Bertani medium. For expression, IPTG was added to a concentration of 1 mM at a cell density of $OD_{600\,nm}$ of 0.5, and cells were harvested after 3–4 h; induction of cells additionally containing plasmid pEC86 (carrying the *ccm* gene cluster, see below) was initiated typically at a cell density of 3, and the medium supplemented with 0.1% glycerol at this point.

### 2.2. Cloning of gene fragments for heterologous expression

All DNA manipulations were performed essentially according to [32]. Using the cloned *cyc*M gene on plasmid pAT3 [23], restriction sites were introduced by PCR to generate gene fragments encoding both shortened $c_{552}$ versions (see Fig. 1). A 300 bp fragment was obtained by the *Nco*I/*Bam*HI pair (primers BR 1 (5′-GTC CTG GCC ATG GCC GAT-3′) and BR 2 (5′-GGA CGC CGG ATC CTT GCG CG-3′)), cloned into the respective sites of pET-22b(+) (Novagen), resulting in pBR2 which encodes fragment A (see Fig. 1). Correspondingly, fragment B is encoded by a 420 bp *Nco*I/*Not*I PCR product (primer MA1 (5′-CGT CGG CAC CAT GGG CCA CGG-3′) and MA2 (5′-GGC ATG GGG GGG CGG CCG CTA CTG CTG-3′)), resulting in pMA1. Both plasmids were introduced individually into strain BL21(DE3) and cotransformed with plasmid pEC86, yielding expression strains B2E (fragment A) and M1E (fragment B). pEC86 encodes the complete *ccm*A–H gene cluster originat-

ing from *E. coli* (see Section 1) under the control of the *tet* promoter from pACY184, which allows for their aerobic expression [33].

### 2.3. Cell fractionation and protein purification

*E. coli* cells were harvested by centrifugation and periplasmic fractions prepared essentially as described by Witholt et al. [34]. Both soluble fragments of cytochrome $c_{552}$ were purified by column chromatography in three steps (see Table 1). (i) Ion exchange chromatography on Q-Sepharose fast flow (Pharmacia) in 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 (for fragment B, pH 7.0), and eluted by a salt gradient of 0–350 mM NaCl in the same buffer (for B, 100–400 mM). (ii) Fractions with the highest specific heme content were pooled and separated further by gel filtration on Sephacryl S-200 (Pharmacia) in the initial Tris-EDTA buffer (for B, at pH 7 and 100 mM salt). (iii) Pooled fractions were rechromatographed as in (i), using gradients of 0–300 mM NaCl (for B, 100–300 mM).

Previously published methods were used for recording redox difference spectra of *c*-type cytochromes employing an extinction coefficient $\Delta\varepsilon_{(red-ox)} = 19.4\ mM^{-1}\ cm^{-1}$ [23], for SDS-PAGE [36], heme staining and Western blotting using antisera directed against the $c_{552}$ [23], for element determinations by TXRF [35], and for the cytochrome *c* oxidase assay [18].

## 3. Results and discussion

### 3.1. Heterologous expression of c-type cytochromes in E. coli

Using two different fragments of the cloned *cyc*M gene encoding the *P. denitrificans* cytochrome $c_{552}$ located on pET-22b(+) expression vectors, the two *E. coli* strains synthesize the two truncated forms of the cytochrome (see Figs. 1 and 2). Whereas the native gene encodes a membrane-bound protein ([23], and see below), both fragments were devised to encode soluble forms, differing from the parent protein by approximately 4 and 8 kDa in size. Employing the vector-encoded *pel*B leader sequence, their expression is targeted to the periplasm of the bacterium, and both forms are indeed found predominantly in this cellular fraction (details not shown). By necessity, this location is required for covalent heme attach-

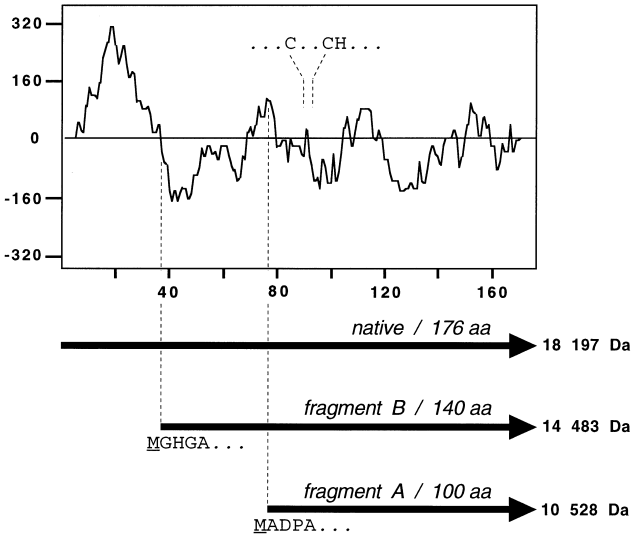


Fig. 1. Domain structure of the *P. denitrificans* cytochrome $c_{552}$ and derived fragments expressed in *E. coli*. Hydropathy plot of the nucleotide sequence-based amino acid sequence of the native cytochrome and site of covalent heme attachment; below, size representation and molecular mass of the native protein and of fragments A and B. For both fragments, the N-terminal methionine (underlined) is vector sequence-derived. The hydropathy plot was generated using the program Geneworks (Oxford Molecular Group) and a window size of 11 residues.

Table 1
Purification and yield of heterologously expressed *P. denitrificans* cytochrome $c_{552}$ fragment A isolated from strain B2E

| Fraction | Heme *c* (nmol) | Protein (mg) | Heme:protein (nmol/mg) | Yield (%) |
|---|---|---|---|---|
| Periplasm[a] | 1330 | 429 | 3.1 | (100) |
| First ion exchange chromatography | 1277 | 97.5 | 13.1 | 96 |
| Gel filtration | 1143 | 31.8 | 35.9 | 86 |
| Second ion exchange chromatography | 1073 | 11.5 | 93.3 | 81 |

[a]Corresponding to 1 l of growth medium, containing 14 mg of fragment A.

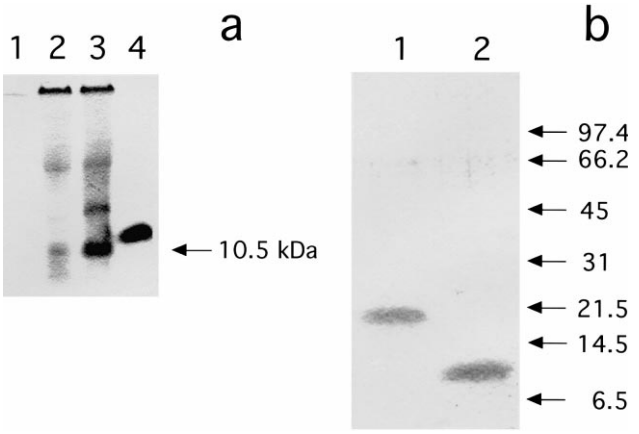


Fig. 2. SDS-PAGE and heme stain of periplasmic fractions and purified fragments A and B isolated from different strains of *E. coli*. (a) 50 μg each of the isolated periplasmic fraction was separated on SDS-PAGE and stained for covalently attached heme (see Section 2); host strain BL21 without plasmid (lane 1); carrying plasmid pBR2 alone (lane 2); carrying in addition pEC86 plasmid (lane 3); for comparison, lane 4, horse heart cytochrome *c*, 5 μg. (b) Coomassie-stained gel of isolated fragment A (lane 2) and fragment B (lane 1); 3 μg each. Arrows and numbers denote position and mass of standard proteins given in kDa.

ment (see below), but it also provides an advantage for the purification of the protein of interest.

We obtained the best yield for the recombinant cytochrome(s) with an induction schedule that starts at rather high cell density, since we observed poor growth progress after IPTG addition. After 3 h of induction in the presence of 0.1% glycerol as an additional carbon source, the expression level reached values of around 8–14 mg cytochrome *c* per liter of growth medium, as determined spectrally in the periplasmic fraction (see Table 1).

### 3.2. Expression levels are critically dependent on cotransformation of the ccm gene cluster

As exemplified by heme staining (Fig. 2a) and spectral analysis (Fig. 3, traces 1 and 2) of the periplasm, the untransformed host strain shows virtually no *c* cytochromes under aerobic growth conditions, both in the presence and absence of plasmid pEC86. Small amounts (below 1 mg/l) show up in the periplasmic fraction in cells transformed with pBR2 alone (Fig. 3, trace 3), encoding fragment A. Only when cells contain the plasmid pEC86 in addition to the plasmid-encoded *cyc*M gene fragments, the above mentioned high level of periplasmic $c_{552}$ is reached.

Under anaerobic conditions, few endogenous *c*-type cytochromes are known to be expressed in *E. coli*, confirming the presence of at least basic levels of enzymatic activities present for cofactor activation and insertion into the apoprotein. Nevertheless, *E. coli* has not gained much attention as a foreign host for heterologous expression of cytochromes *c*, due to generally low yields under both aerobic and anaerobic conditions (for a recent exception, and a comprehensive overview of the relevant literature, see [37]). Within the past 2 years our understanding of the individual steps of cytochrome *c* biogenesis in various systems has grown considerably, and a cluster of eight *E. coli* cytochrome *c* maturation genes has already been shown qualitatively to enhance the synthesis of a *Bradyrhizobium japonicum* cytochrome *c* initially under anaerobic conditions in *E. coli* [38]. A derivative plasmid, pEC86 [33], allowing aerobic expression of the *ccm* genes, has been used here to greatly enhance the synthesis of the target gene, clearly surpassing the yield of a *Thermus thermophilus* cytochrome *c* expressed in *E. coli* from a plasmid of comparable properties [37]. We assume that in those studies, and to an even larger extent in our strain only containing the $c_{552}$ structural gene *in trans* (see above), the expression of maturation factors is

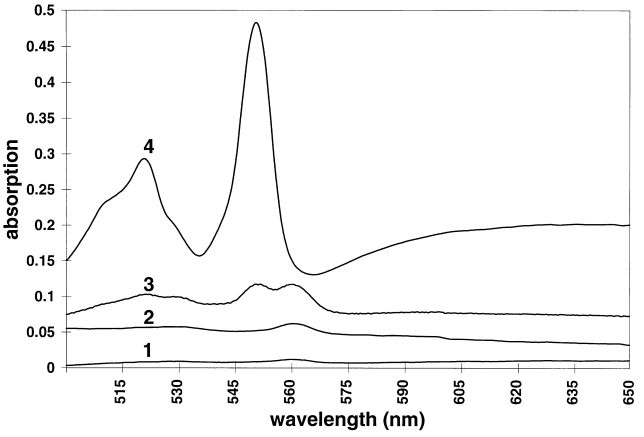


Fig. 3. Redox difference spectra of various *E. coli* strains expressing cytochrome $c_{552}$ fragments. Periplasmic fractions (isolated according to [34]) from strain BL21 carrying no plasmid (lane 1), BL21 transformed with pEC86 (2), pBR2 (3), or both plasmids (4) were recorded as difference spectra, dithionite-reduced minus ferricyanide-oxidized.

the limiting parameter for reaching maximal holoprotein levels, and this situation may even lead to synthesis of non-homogeneous material [37] when using high copy plasmids encoding *c* cytochromes.

### 3.3. Homologous expression of the soluble functional domain of P. denitrificans cytochrome $c_{552}$

A previous approach to express a soluble form of the cytochrome $c_{552}$ in *P. denitrificans* led to correct targeting within the cell and to heme association with the fragment, but levels were completely unsatisfactory for biophysical studies (not detailed here): in short, both the promoter and the region encoding the signal sequence of the *P. denitrificans* cytochrome $c_{550}$ (CycA [39]) were fused to that part of the coding region of *cyc*M representing fragment A (see Fig. 1). The chimeric pre-protein was translocated across the cytoplasmic membrane, its signal sequence cleaved, and heme attached to yield small amounts of the correctly assembled heme domain matching fragment A. However, this expression strategy could not be pursued further as presumably either the promoter activity under single carbon compound growth was insufficient [39] or the gene product degraded at an elevated rate.

### 3.4. Biochemical characterization of engineered cytochrome fragments expressed in E. coli

When cytochrome $c_{552}$ fragments A or B are isolated with the periplasmic fraction, fragment A exhibits the expected size when the SDS-PAG is stained for heme (Fig. 2), while fragment B migrates at a position close to 20 kDa, considerably higher than its calculated mass (see Fig. 1). A 4 kDa difference between calculated and experimentally determined gel-derived mass has been noted for the native cytochrome before [23], and may be explained by the high charge density present in the N-terminal region of fragment B; as confirmed by peptide sequencing (see Fig. 1), this fragment is correctly processed to its mature size. For both proteins, spectral redox characteristics (Fig. 3) are indistinguishable from published data of the parent protein, and both fragments react with an antiserum directed against the native protein in a Western blot (not shown). We conclude from these facts that the hybrid pre-protein (carrying the PelB leader sequence, see Section 2) underwent correct membrane translocation, signal sequence cleavage, and assembly pathways specific for *c* cytochromes in the *ccm* plasmid background of the host strain.

Both proteins were purified to homogeneity in excellent yield, using a conventional column chromatography protocol (see Table 1 for fragment A, and Section 2) by combining ion exchange and gel filtration separation. Purified cytochrome fragments were again checked for their spectral characteristics and protein composition (Figs. 2 and 3), and their correct N-termini (see Fig. 1) confirmed by peptide sequencing. To establish protein homogeneity in terms of complete heme cofactor attachment, we analyzed fragment A for its heme-iron and sulfur stoichiometry (from its five sulfur-containing amino acid residues) by TXRF; we conclude that an experimental ratio of Fe:protein of 0.88, within the error margin of the method, indicates quantitative covalent heme attachment in the purified cytochrome. This conclusion is also supported by a specific heme:protein ratio of 93 nmol/mg of protein, close to the theoretical value of 95 nmol/mg. While any apo-protein lacking the heme moiety should most likely not copurify with the holo-protein under the chromatographic conditions used here, we have no evidence that the apocytochrome accumulates in any of the other cellular fractions to any large extent.

As a final proof of functionality in terms of both protein interaction and ET competence, the efficiency of each of the two fragments as electron donors in a standard cytochrome *c* oxidase assay was tested, using the purified *P. denitrificans* enzyme. When prereduced by dithionite for use in the spectroscopic enzyme assay, we note a quite high stability towards autoxidation at 4°C, explaining also to some extent the fact that purified fragments are predominantly in the reduced form, as isolated. Both fragments are active as donors to oxidase, and preliminary kinetic data suggest that for fragment A similar values for $k_{cat}$ are obtained, when compared to the standard assay electron donor, horse heart cytochrome *c* [18]. Once the native, full-size $c_{552}$ is available in sufficient amounts, exact kinetic data for all three electron donor proteins will make it possible to draw conclusions on the specific role of individual domains.

### 3.5. Domain structure of the P. denitrificans cytochrome $c_{552}$

Within the large number of soluble and membrane-bound *c*-type cytochromes of this bacterium [22], this protein is the most likely candidate for the direct electron mediator between complexes III and IV of the respiratory chain (see Section 1). As noted previously [23], its amino acid sequence suggests a tripartite domain structure (see also Fig. 1): an N-terminal domain of approximately 40 residues shares large homology with a typical signal sequence which, however, is not cleaved, providing a membrane anchor function for the protein; the next 40 residues are characterized by pronounced hydrophilicity with many negative charges in excess, which led to the speculative view that this region might endow the protein with some flexibility between the membrane anchor and its *c*-heme domain; the remaining almost 100 residues represent a typical small class I *c* domain, with many of the characteristic residues conserved in many other cytochromes *c* [23].

A functional necessity for such a domain structure is not immediately evident, in particular since the mitochondrial ET between reductase and oxidase does not rely on such a complex structure: its cytochrome $c_{550}$ is a soluble protein of around 100 amino acids. If, however, both respiratory redox complexes form a supercomplex comprising also the mediator cytochrome $c_{552}$, as initially isolated from *P. denitrificans* [24], a membrane (and supercomplex) association could yield an advantage in terms of its kinetic interaction when it is fixed in place. At the same time such a structural constraint poses a problem not encountered for the soluble mediator in mitochondrial ET: does the $c_{552}$ interact, with its same interface, with both the cytochrome $c_1$ of complex III and with subunit II of oxidase, or does it lack any rotational flexibility, requiring different entry and exit routes when locked in a supercomplex? To eventually address such issues, a distinct interaction analysis between these components is required.

Our present approach makes available two differently truncated forms of the bacterial cytochrome *c*, designed according to its domain character. The minimal structure is that of a classical *c* domain of 100 amino acids with a calculated p*K* of 6.32 (fragment A, see Fig. 1), while fragment B (140 residues with a calculated p*K* of 4.53) carries the additional domain, now at its N-terminus, of high negative excess charge, providing a closer homology tribute to the genuine bacterial situation. Whereas the native protein is membrane-bound, both engineered fragments are soluble proteins, available in amounts sufficient for biophysical studies both by X-ray diffraction (A. Harrenga, H. Michel et al., unpublished data) and NMR spectroscopy in solution. Their high expression rates in the heterologous system also make isotopic labelling economically feasible, which will be required even more when studies of complex formation of each of these fragments with soluble domains of their partner proteins in solution are envisaged.

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