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Expression of prokaryotic and eukaryotic cytochromes *c* in *Escherichia coli*

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Abstract

C-type cytochromes from various sources show substantial structural conservation. For the covalent attachment of heme groups to apocytochromes, however, three different enzyme systems have been described so far. We have examined the ability of the heme ligation systems of *Escherichia coli* and of *Saccharomyces cerevisiae* to process cytochromes from *S. cerevisiae, Paracoccus denitrificans*, and *Synechocystis* sp. PCC 6803. *E. coli*'s maturation system with at least eight different proteins accepted all these cytochromes for heme ligation. The single subunit heme lyase from *S. cerevisiae* mitochondria, on the other hand, failed to attach heme groups to cytochromes of prokaryotic origin.

Keywords: Cytochrome *c*; Heme attachment; Heme lyase; *ccm* system; Heterologous expression; *Escherichia coli*

1. Introduction

In respiratory energy conversion, redox-driven electron transport chains generate the driving force for chemiosmotic ATP synthesis [1]. Despite the large variety of electron sources and acceptors utilized by such respiratory chains, *c*-type cytochromes are ubiquitously involved as electron carriers [2]. Throughout the prokaryotic and eukaryotic kingdoms, most *c*-type cytochromes comprise a conserved structure of at least three helices, which are connected by more variable regions. A heme group acting as a redox cofactor is covalently attached to the sulfhydryls of a CxxCH signature motif via two α-thioether bonds. In contrast to this level of conservation, attachment of heme to apocytochrome *c* is catalyzed by at least three different enzyme systems in different organisms [3]. Proteobacteria like *Escherichia coli,* archeae, and plant and protozoal mitochondria employ a set of proteins (system I), which are mainly located in the cytoplasmic membrane [2,4]. Maturation of cytochromes is performed after separate transport of apoproteins and heme groups into the periplasmic space. In *E. coli*, several genes encoding proteins involved in this process (*ccm*A–H) are organized in the tightly regulated *aeg*46.5 operon and are only expressed under anaerobic conditions and in the presence of nitrate [5,6]. A second, at least partially homologous multi-enzyme complex (system II) has been found in some Gram-positive bacteria, cyanobacteria, and chloroplasts [7,8]. In fungi, invertebrates and vertebrates, cytochrome *c* heme lyase (CCHL, system III) appears to be responsible for

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cytochrome *c* maturation in the mitochondrial intermembrane space [9].

Wild-type and mutated versions of cytochrome *c* are required in large amounts for numerous different lines of investigation, examining e.g. interactions with other complexes of respiratory chains [10] intra- and intermolecular electron transfer [11] or folding dynamics [12]. So far, both homologous [13] and heterologous [14–16] expression of eukaryotic cytochromes *c* have been achieved in yeast. The employed yeast strains should be devoid of cytochromes, only allowing for the expression of physiologically functional variants. Moreover, protein yields are notoriously low with yeast expression systems. One possible circumvention of these restrictions would be heterologous expression in bacteria. Whereas cytochromes with non-covalently attached heme groups have been successfully expressed in *Escherichia coli*, production of *c*-type cytochromes with covalently attached heme groups mostly failed (see [17] and references therein). Major obstacles are the periplasmic location of the *E. coli* cytochrome *c* maturation system and its tight regulation. A potential solution to this problem has recently been developed by the transfer of the *ccm*A–H genes from their chromosomal locus to a plasmid, thereby allowing constitutive expression. This system was shown to sustain maturation of a variety of heterologously expressed prokaryotic cytochromes [10,18], but its efficiency still suffers from the necessity to transfer the expressed apocytochromes into the periplasmic space for heme ligation. A different approach for the maturation of yeast cytochromes in *E. coli* involved cytoplasmic coexpression of yeast cytochrome *c* heme lyase. Although hampered by its low solubility in *E. coli*, this enzyme forms eukaryotic holocytochrome *c* with yields up to one order of magnitude higher than upon expression in yeast [17].

As pointed out by Kranz et al. [3], so far little was known about the cross-compatibility of various cytochromes and maturation systems. We have examined the potential of *E. coli* as a host for overexpression of *c*-type cytochromes from different sources with either the prokaryotic or eukaryotic maturation enzymes present. We found that upon export into the periplasmic space, all cytochromes tested were matured by the prokaryotic *ccm* system. Coexpression of a yeast cytochrome *c* heme lyase (CCHL) variant with yeast apocytochromes in *E. coli* yielded high amounts of holocytochrome *c*, but CCHL completely failed to mature the prokaryotic proteins, thereby indicating a much higher specificity as compared to the more complex *E. coli* maturation system.

2. Materials and methods

2.1. Plasmids

Cytochrome *c* genes from *Saccharomyces cerevisiae* (*cyc*1, *cyc*7, provided by F. Sherman, Rochester), *Paracoccus denitrificans* (*cyc*A, provided by M. Lübben, Bochum), and *Synechocystis* sp. PCC 6803 (*pet*J) have been amplified by polymerase chain reaction. Recognition sequences for restriction nucleases *Nde*I and *Bam*HI were introduced during the amplification and used to clone the coding sequences into expression vector pET-3a (Novagen, Madison). By means of an oligonucleotide cassette with *Nde*I-compatible ends, a streptavidin affinity tag (StrepTag II; [19]) was introduced at the 5′ ends of the cytochrome genes. In another set of plasmids, a sequence encoding the OmpA signal peptide for *sec* mediated transport into the periplasmic space was additionally introduced. All constructs were verified by DNA sequencing prior to expression. Genes encoding the *ccm* maturation apparatus have been provided by L. Thöny-Meyer on plasmid pEH86 [18]. The *S. cerevisiae* cytochrome *c* heme lyase gene (*cyc*3, provided by F. Sherman, Rochester) was amplified by PCR and cloned into plasmid pLysS, replacing the sequence encoding T7 lysozyme (Novagen, Madison). A pair of oligonucleotides encoding a N-terminal polyhistidine sequence (HisTag) was introduced in order to allow subsequent purification of the enzyme. All molecular genetics procedures were done as published [20]. Plasmids used in this work have been compiled into Table 1.

2.2. Bacterial strains and growth conditions

Expression of cytochromes was carried out in *E. coli* BL21 (DE3). When appropriate, plates and media were supplemented with 100 μg/ml ampicillin and/or 34 μg/ml chloramphenicol. After transformation and overnight incubation on plates, single colo-

Table 1
Plasmids used in this work

Plasmid	Genes	OmpA signal	Affinity Tag	Source/reference
pET-3a	–	–	–	Novagen
pLysS	*lys*S	–	–	Novagen
pEH86	*ccm*A–H	–	–	Thöny-Meyer
pOScyc1	*cyc*1	+	Strep	this work
pOScyc7	*cyc*7	+	Strep	this work
pOScycA	*cyc*A	+	Strep	this work
pOSpetJ	*pet*J	+	Strep	this work
pScyc1	*cyc*1	–	Strep	this work
pScyc7	*cyc*7	–	Strep	this work
pScycA	*cyc*A	–	Strep	this work
pSpetJ	*pet*J	–	Strep	this work
pACcyc3	*cyc*3	–	His	this work

*cyc*1, *Saccharomyces cerevisiae iso*-1-cytochrome *c*; *cyc7*, *S. cerevisiae iso*-2-cytochrome *c*; *cyc*A, *Paracoccus denitrificans* cytochrome *c*; *pet*J, *Synechocystis* sp. PCC 6803 cytochrome *c*553; *cyc*3, *S. cerevisiae* cytochrome *c* heme lyase; *ccm*, *E. coli* cytochrome *c* maturation system; pLysS, plasmid carrying the gene for T7 lysozyme (Novagen).

nies were transferred into 20 ml YT medium and grown at 37°C overnight. This culture was used to inoculate 500 ml of fresh medium and grown further at 30°C for 8 h, reaching an approximate OD_{600} of 2.0. The volume was then increased to 1 l by the addition of fresh medium, supplemented with 0.1 mM isopropylthiogalactoside (IPTG), and incubated 16 h at 30°C under gentle shaking. It was crucial to maintain a low oxygen concentration during expression. Cells were harvested by centrifugation (10 min, $5000 \times g$), washed in 20 mM Tris–HCl, pH 8.0, and pelleted again.

2.3. Purification of expressed proteins

Cells were resuspended in 10 ml of buffer, containing 100 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.2% (v/v) Tween 20, 100 μg/ml lysozyme, and 100 μM Pefabloc SC (Biomol, Hamburg), chilled on ice for 20 min and then incubated 10 min at room temperature. The cells were subjected to four 30-s cycles of sonication (Branson Sonifier B15, microtip, 70% power, 50% pulse) and then frozen in liquid nitrogen. The solution was allowed to thaw for 30 min at room temperature and again sonicated for two 30-s cycles. Insoluble material was removed by 1 h of centrifugation at $100\,000 \times g$, 4°C, and 5 ml of the supernatant was applied to a column, containing 2 ml of StrepTactin Sepharose resin (IBA, Göttingen). The column was then washed and eluted following the manufacturer's instructions.

2.4. Gel electrophoresis and staining

Protein concentrations were measured according to Sedmak and Grossberg [21], with bovine serum albumin as a standard. Polyacrylamide gel electrophoresis (SDS–PAGE) was done on 15% acrylamide gels in a Bio-Rad MiniProtean apparatus, and gels were stained for proteins with Coomassie brilliant blue. When appropriate, proteins were blotted to nitrocellulose membranes (Schleicher and Schüll, Dassel). The membranes were either used for heme staining according to Vargas et al. [22] or for detection of StrepTag II by means of a streptavidin–peroxidase conjugate following the manufacturer's recommendations (IBA, Göttingen).

2.5. Spectroscopy

An Aminco DW2000 spectrophotometer was used for absorption spectroscopy. Cytochromes were diluted to 10 μM protein in 50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, and 100 μM $K_3Fe(CN)_6$, and spectra were recorded between 350 and 700 nm. $Na_2S_2O_4$ was added to a final concentration of 1 mM and spectra were recorded again. From the difference spectrum (reduced minus oxidized), we de-

termined the heme concentration by means of a decadic absorption coefficient at 553–556 nm of 19 mM^{-1} cm^{-1} [11].

3. Results

In a first series of experiments, we examined the maturation of cytochromes from various sources by *E. coli*'s *ccm* system. Apocytochromes as indicated in the uppermost row of Table 2 were fused to the OmpA transfer signal and the Streptavidin affinity tag (StrepTag II). Upon coexpression of these cytochromes and *E. coli*'s *ccm*A–H genes from pEH86 (Table 2, last four columns), already visual inspection of cells pelleted from 1 ml of culture clearly revealed the development of red color after induction, indicative of successful cytochrome *c* maturation. In the experiment shown in the first column, there was no expression plasmid for any cytochrome *c* maturation system present in the cells. In the experiment documented in the second column, cells expressed *cyc*3 in the cytoplasm, whereas in the experiment shown in the third column, the employed cytochrome lacked the OmpA leader sequence.

The left part of Fig. 1 (lanes a–g) documents the maturation of cytochromes by means of peroxidase activity of covalently bound heme [22]. All four cytochromes generated positive heme stains, whereas no heme was detectable in the absence of either the *ccm* genes or a heterologously expressed cytochrome. Lanes g and h in Fig. 1 exemplify the purification of yeast *iso*-2-cytochrome *c* by affinity chromatography on StrepTactin resin. We tentatively attribute the occurrence of a double band in the purified sample to the presence of uncleaved proteins with their signal sequences still attached. We obtained 0.9 mg of cytochrome *c* from 1 g (wet weight) of cells in this purification; the other cytochromes have been purified with comparable yields (not shown).

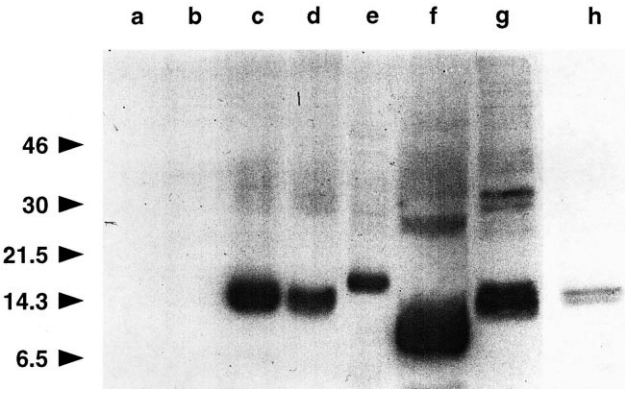

Fig. 1. Heme-containing proteins in the periplasmic space of cytochrome *c* producing cells. Various *c*-type cytochromes have been coexpressed in BL21 (DE3) together with *E. coli ccm* genes. Periplasmic fractions comprising 20 μg protein were run on SDS–PAGE and stained for the presence of heme (lanes a–g). Expressions: Lane a, pET-3a without insert; b, CcmA–H alone; c, CcmA–H and Cyc7; d, CcmA–H and Cyc1; e, CcmA–H and CycA; f, CcmA–H and PetJ; and g, yeast *iso*-2-cytochrome *c* from the periplasmic fraction after chromatography on StrepTactin resin. This purified cytochrome is shown in lane h as well, but now Coomassie stained.

In a second series of experiments, the same set of cytochromes was expressed without signal sequence for *sec*-mediated export. Upon coexpression of yeast cytochrome *c* heme lyase, however, color development was observed only with those cells expressing yeast cytochromes (Table 3, rows 4 and 5). Notably,

Table 2
Coexpression of CcmA–H and various cytochromes *c*

Genes	Plasmids						
	pOScyc7/ pLysS	pOScyc7/ pACcyc3	pScyc7/ pEH86	pOScyc1/ pEH86	pOScyc7/ pEH86	pOScycA/ pEH86	pOSpetJ/ pEH86
*lys*S	+	−	−	−	−	−	−
*cyc*3	−	+	−	−	−	−	−
*ccm*A–H	−	−	+	+	+	+	+
*omp*A signal	+	+	−	+	+	+	+
Color development	−	−	−	+	+	+	+

E. coli BL21 (DE3) cells were used to express combinations of proteins as indicated. Pelleted cells from 1 ml cultures were examined for color development after 4 h of induction.

Table 3
Coexpression of cyc3 and various cytochromes *c*

Genes	Plasmids						
	pScyc7/pLysS	pScyc7/pEH86	pOScyc7/ pACcyc3	pScyc1/ pACcyc3	pScyc7/ pACcyc3	pScycA/ pACcyc3	pSpetJ/ pACcyc3
*lys*S	+	−	−	−	−	−	−
*cyc*3	−	−	+	+	+	+	+
*ccm*A–H	−	+	−	−	−	−	−
*omp*A signal	−	−	+	−	−	−	−
Color development	−	−	−	+	+	−	−

E. coli BL21 (DE3) cells were used to express combinations of proteins as indicated. Pelleted cells from 1-ml cultures were examined for color development after 4 h of induction.

we observed no color development in either cells expressing the *ccm* system instead of *cyc*3 (row 2), or in cells expressing *cyc*3 together with yeast cytochrome genes fused to the OmpA leader peptide (row 3). As shown in Fig. 2, this finding was corroborated by heme staining, showing peroxidase activity only with cells that produced yeast cytochromes together with heme lyase. Yeast cytochromes produced in the cytoplasm could also be easily purified by affinity chromatography on StrepTactin. We obtained up to 8 mg of protein per g cells (wet weight), which, in contrast to the periplasmic expression, appeared as a homogenous band on SDS–PAGE (Fig. 2, lane h).

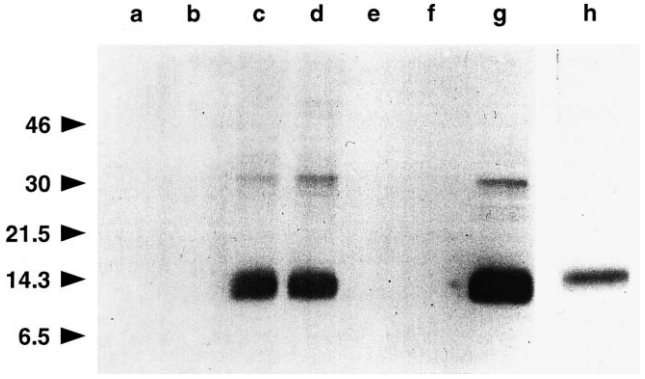

Fig. 2. Heme-containing proteins in the cytoplasm of cytochrome *c* producing cells in *E. coli*. Various *c*-type cytochromes have been coexpressed in BL21 (DE3) together with cytochrome *c* heme lyase. Sonicated cells comprising 20 μg protein were run on SDS–PAGE and stained for the presence of heme (lanes a–g) as in Fig. 1. Expressions: Lane a, pET-3a without insert; b, Cyc3 alone; c, Cyc3 and Cyc7; d, Cyc3 and Cyc1; e, Cyc3 and CycA; f, Cyc3 and PetJ; and g, yeast *iso*-2-cytochrome *c* after chromatography on StrepTactin resin. This purified cytochrome is shown in lane h as well, but now Coomassie stained.

Several attempts under various conditions to achieve maturation of prokaryotic cytochromes with yeast heme lyase failed. Therefore, we had to test whether those apocytochromes were actually produced in *E. coli*. Purification of the apocytochromes by affinity chromatography yielded proteins with the expected electrophoretic mobilities, but attempts for N-terminal sequencing failed for unknown reasons. Therefore, we monitored the expression of apocytochromes by detection of StrepTags. Fig. 3 shows the respective experiment with a streptavidin–peroxidase conjugate, revealing that all four apocytochromes were present in the cytoplasmic extracts after expression. This finding suggests that the

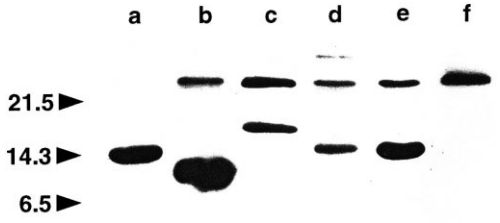

Fig. 3. Strep-tagged proteins in the cytoplasm of cytochrome *c*-producing cells. *E. coli* BL21 (DE3) cells were used to coexpress various combinations of cytochromes and yeast cytochrome *c* heme lyase. Bacterial lysate comprising approximately 20 μg of protein were run on SDS–PAGE and blotted onto nitrocellulose membranes. Membranes were then decorated with streptavidin–peroxidase conjugate and StrepTags were visualized according to the manufacturer's recommendations (IBA, Göttingen. The strongly staining band at 22 kDa which shows up in all cell lysates probably represents the *E. coli* biotin carboxyl carrier protein, which was decorated despite preincubation of the lysates with avidine). Lane a, purified *iso*-2-cytochrome *c* (Cyc7) as in Fig. 2, lanes g and h; b, PetJ; c, CycA; d, Cyc1; e, Cyc7; and f, plasmid pET-3a without insert.

failure to mature prokaryotic cytochromes was due to a much higher specificity of yeast heme lyase as compared to the *E. coli ccm* system.

Yeast *iso*-2-cytochrome *c* samples purified from the two different expression systems (cf. Figs. 1 and 2, lanes h) were compared by absorption spectroscopy. As depicted in Fig. 4, redox difference spectra recorded from both proteins were qualitatively identical, indicating the production of mature and correctly folded holocytochrome *c* by both expression methods, but with a significantly lower heme content in the sample obtained from periplasmic expression (here approximately 30% holocytochrome after cytoplasmic and 15% after periplasmic expression). Obviously, in both experiments, maturation was outpaced by the high rates of protein production mediated by the T7 expression system.

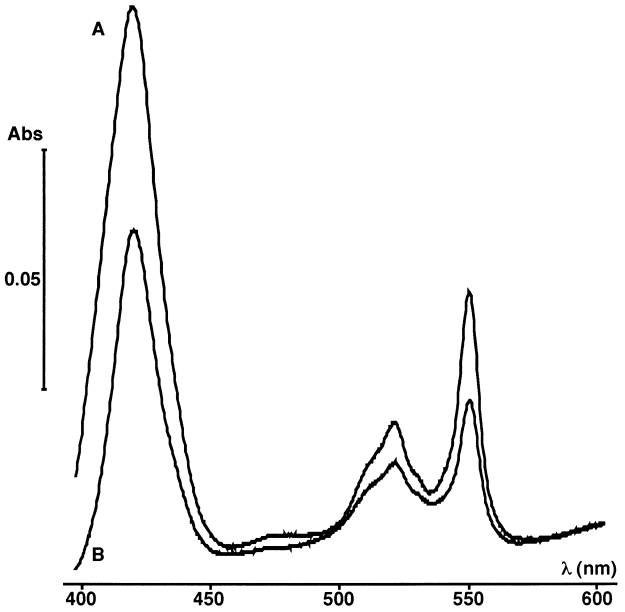

Fig. 4. Comparison of yeast *iso*-2-cytochrome *c* purified from the periplasmic space and from the cytoplasm. *Saccharomyces cerevisiae iso*-2-cytochrome *c* has been coexpressed either with *S. cerevisiae* CCHL in the cytoplasmic space (trace A) or with the *E. coli ccm* system in the periplasmic space (trace B). Redox difference spectra have then been generated from spectra obtained with the purified cytochromes at a protein concentration of 10 μM in the presence of 100 μM $K_3Fe(CN)_6$ or 1 mM $Na_2S_2O_4$.

4. Discussion

Up to now, three different systems have been described for the covalent attachment of heme cofactors to *c*-type cytochromes [3]. Here, we examine heme attachment in *Escherichia coli* by its own *ccm* system (maturation system of type I) and the heterologously expressed yeast cytochrome *c* heme lyase (type III) to cytochromes that are originally matured by systems of type I (CycA), II (PetJ), and III (Cyc1, Cyc7).

In both series of experiments, without constitutive expression of either maturation system from plasmids, no heme attachment was observed at all (cf. Tables 2 and 3, first columns). This finding corroborates earlier observations that *E. coli*'s *ccm* genes are tightly regulated and only expressed under anaerobiosis and with nitrate present in the medium [5,6]. From other control experiments (cf. rows 2 and 3 of Tables 2 and 3), we conclude that heme attachment is also spatially tightly controlled: apoproteins had to be translocated across the cytoplasmic membrane in order to be matured by the *ccm* system, and on the other hand cytochromes exported to the periplasmic space entirely escaped heme attachment mediated by heme lyase, which was located in the cytoplasm. This finding also saved us the tedious and sometimes unreliable work of localization of expressed proteins in the various compartments.

In this study, protein yields achieved by heterologous cytoplasmic expression of yeast cytochromes in *E. coli* were considerably higher than those reported by Pollock et al., who first described the CCHL coexpression system [17]. This improvement may be due to increased solubility of CCHL in the cytoplasm which seems to be a side effect of the polyhistidine sequence initially introduced for purification purposes. Whereas Pollock et al. reported precipitation of CCHL into inclusion bodies, we have not encountered such effects with the His-tagged enzyme.

We found that yeast CCHL exhibited a high specificity for yeast cytochromes and failed to attach heme groups to cytochromes of prokaryotic origin. The prokaryotic maturation apparatus, on the other hand, accepted all four apocytochromes for heme attachment and did not differentiate between prokaryotic and eukaryotic proteins. Several of the proteins encoded by *ccm* genes appear to be involved in auxiliary processes, such as transmembrane heme transport (CcmAB [4]), intermediate covalent heme

binding [23], or apocytochrome reduction (CcmG, [24]). As with CCHL, the active site actually responsible for the attachment of heme groups to apocytochrome appears to be located on one distinct protein (CcmF, [2]). Future work, aiming at the alteration of CCHL for use in the maturation of prokaryotic cytochromes, will therefore concentrate on the interactions of these two proteins with apocytochromes and heme groups.

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