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Maturation of a eukaryotic cytochrome *c* in the cytoplasm of *Escherichia coli* without the assistance by a dedicated biogenesis apparatus

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Abstract Maturation of c-type cytochromes involves the covalent and stereospecific enzymatic attachment of a heme *b* via thioether linkages to two conserved cysteines within apocytochromes. Horse cytochrome *c* is readily matured into its native holoform in the cytoplasm of *E. coli* when co-expressed with yeast cytochrome *c* heme lyase. Here we report the low yield formation of holocytochrome with covalently attached heme also in the absence of heme lyase. This is the first demonstration of in vivo maturation of a eukaryotic cytochrome *c* in a prokaryotic cytoplasm without the assistance by a dedicated enzymatic maturation system. The assembled cytochrome *c* can be oxidized by cytochrome *c* oxidase, indicating the formation of a functional protein. The absorption spectrum is typical of a low spin, six coordinated c-type heme. Nevertheless, minor spectral differences relative to the native cytochrome *c*, deviation of the midpoint reduction potential and slightly altered kinetic parameters of the interaction with cytochrome *c* oxidase emphasize the importance of cytochrome *c* heme lyase in folding cytochrome *c* into its native conformation.

Keywords Apocytochrome · Holocytochrome · Heme · Heme lyase · Spontaneous maturation

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Abbreviations

Ccm	cytochrome *c* maturation
Ccs	cytochrome *c* synthesis
COX	cytochrome *c* oxidase
CCHL	cytochrome *c* heme lyase
ECL	enhanced chemiluminescence
IPTG	isopropyl-beta-D-thiogalactopyranoside
Na-PSB	sodium phosphate buffer
TMPD	NNNN-tetramethyl-p-phenylenediamine
DTT	dithiothreitol

Introduction

C-type cytochromes are unique in the sense that their heme moiety is covalently bound via thioether linkages to two cysteines in the conserved Cys-Axx-Axx-Cys-His motif, with the histidine serving as the proximal axial ligand to the central iron atom. Only a few exceptions are known with single cysteines, with more than two amino acids between the cysteines, or with lysine serving as the proximal ligand (for recent reviews see Bowman and Bren 2008; Kranz et al. 2009; Hamel et al. 2009). No unique advantage of the covalent heme attachment has been identified, while numerous cytochromes exist with non-covalently bound heme, and perform similar tasks—most notably participate in reduction-oxidation reactions (Barker and Ferguson 1999; Kranz et al. 2009). Nevertheless, cytochromes *c* plays a vital role in the energy transduction of eukaryotes and most prokaryotes. Their maturation, which includes the stereospecific covalent heme attachment, is catalyzed by various cytochrome maturation systems. System I (Cytochrome *c* maturation, Ccm) operating e.g. in plant mitochondria and in α- and

γ-proteobacteria, as well as System II (Cytochrome *c* synthesis, Ccs) found for example in Gram positive bacteria and in chloroplasts, are complex multicomponent systems (Kranz et al. 2009). System I functions in the periplasm of *E. coli* where the prerequisite for cytochrome *c* assembly is the transport of the pre-apocytochrome with an N-terminal signal peptide through the cytoplasmic membrane by the secretory apparatus (Thony-Meyer 1997, 2002). System III operates in the mitochondria of fungi, invertebrates and vertebrates: cytochrome *c*, the mobile electron carrier between complex III and IV (cytochrome *c* oxidase, COX) and cytochrome c_1, a subunit of complex III are matured in most organisms by a single heme lyase, although in fungi and green algae they are matured by two separate enzymes, cytochrome *c* heme lyase (CCHL) and cytochrome c_1 heme lyase (CC_1HL) (Giege et al. 2008). (In fungi another protein, Cyc2p, has been implicated in the reduction of heme as a necessary prerequisite for cytochrome *c* maturation (Bernard et al. 2005; Hamel et al. 2009).) In addition to the catalysis of the covalent binding of heme to the apocytochrome, CCHL is thought to assist in the appropriate folding of apo- and holocytochrome. The interaction of CCHL with its two substrates, apocytochrome and heme, and the mechanism of the enzymatic process at the molecular level are hardly understood.

Mitochondrial holocytochrome *c* can be heterologously produced in *E. coli* when co-expressed with CCHL from yeast. Various groups, including us (Dolgikh et al. 1998; Pollock et al. 1998; Patel et al. 2001; Rumbley et al. 2002; Tenger et al. 2007) have optimized the conditions for the high-yield production of holocytochrome *c*. Spontaneous assembly of a heterologous cytochrome *c* in the cytoplasm of *E. coli* has not been observed so far, with one prokaryotic exception, the cytochrome c_{552} from *Hydrogenobacter thermophilus*, whose maturation has been explained by the appropriate folding of this thermostable protein before its encounter with the heme (Sanbongi et al. 1991; Sinha and Ferguson 1998). Here we demonstrate that when horse cytochrome *c* is expressed in the absence of CCHL, a small amount of holocytochrome is produced. The yield of this lyase-free assembly is approximately 2% of the yield obtained in the presence of the yeast mitochondrial CCHL. Our eukaryotic cytochrome *c*, which is matured in vivo without known enzymatic assistance, contains covalently bound heme in 6 coordinated, low spin configuration. Its oxidation by COX displays slightly distinct kinetics as compared to the native cytochrome *c*. Minor spectral differences and a -17 mV shift of the midpoint reduction potential also indicate that the protein conformations of the native and the non-enzymatically matured cytochromes *c* differ measurably.

Materials and methods

Constructs for the expression of cytochrome c and heme lyase The coding region of Strep affinity tag II was fused to the 3' end of the horse cytochrome *c* gene by PCR amplification, and this tag-fused gene was cloned into the *Nco*I and *Sma*I digested pBAD24 plasmid (Guzman et al. 1995), downstream of its arabinose inducible p_{BAD} promoter. This pBAD24Cyc1-STII construct was transformed into BL21-AI (Invitrogen) and EC06 (Thony-Meyer et al. 1995) *E. coli* strains. For enzymatic maturation, the cytochrome *c* was co-expressed with yeast CCHL. The *cyc3* gene of CCHL was cloned under the control of the isopropyl-beta-D-thiogalactopyranoside (IPTG) inducible tac promoter/lac operator system. This plasmid (Sanders and Lill 2000) and the EC06 strain were kind gifts from Dr. Carsten Sanders, Kutztown University of Pennsylvania.

Expression and purification of cytochrome c A starter culture was inoculated into fresh super broth medium supplemented with 100 μg/ml ampicillin and 1% of glycerol (Ausubel et al. 1996). The culture was incubated at 35°C until it reached OD_{600}=0.7-0.8. Protein overproduction was induced by 0.5% arabinose and also by 1 mM IPTG in the case of enzymatic maturation of cytochrome *c*. The medium was incubated for various periods at 35°C with rigorous shaking. Cells were harvested by centrifugation for 10 min at 10,000 × g, 4°C and they were kept frozen at -70°C until use. The harvested cells were resuspended in 50 mM sodium phosphate buffer (Na-PSB), pH 7.00, containing 100 μg/ml lysozyme and 2.5 units/ml benzonase. After 30 min incubation on ice, the cells were subjected to two cycles of sonication (MSE sonicator, 15 μm amplitude, 40 s). The soluble fraction was separated by ultracentrifugation (100,000 × g, at 4°C, 1 h). Prior to chromatography, the soluble fraction containing Strep-tag II fused cytochrome *c* was subjected to a 50% saturated ammonium sulfate purification. The precipitated proteins were removed by centrifugation for 10 min at 16,000 × g, at 4°C. Following the replacement of the buffer with 50 mM Na-PSB, pH 7.00, the cytochrome was separated on a CM-Sephadex C50 cation exchange matrix. The final purity of the protein was reached by affinity chromatography on a Strep-Tactin Sepharose (IBA) resin following the manufacturer's instructions. Absorption spectra were measured on a UNICAM UV4 spectrophotometer at micromolar cytochrome concentrations, in 50 mM Na-PSB, pH 7.00. Holocytochrome concentrations were estimated from the Soret and the visible absorption bands.

Heme peroxidase activity For the heme peroxidase activity measurements, the samples were specially treated as in (Vargas et al. 1993). Before loading on SDS gels the protein

solutions were incubated for 15 min at 43°C in 120 mM Tris buffer, pH 7.50, containing 20% glycerol and 4.6% SDS. Following the electrophoresis the gel buffer was changed to 20 mM potassium-sodium PSB, pH 7.40, with 115 mM NaCl, for the in-gel heme peroxidase activity measurements. The heme-dependent enhanced chemiluminescence (ECL) signal was developed by the two components (in 50-50%) of the SuperSignal® West Pico Chemiluminescent Substrate (Pierce) kit and recorded by a VersaDoc imaging system.

Immunodetecton of tagged holocytochrome Following the SDS-PAGE, the Strep-tag II fused cytochrome *c* was electrophoretically transferred onto a nitrocellulose membrane (0.45 μm pore size) for western blot analysis. The ECL signal related to horseradish peroxidase was measured as above.

Pyridine hemochrome spectral measurement The protocol was performed according to (Bartsch 1971). The pyridine ferrohemochrome absorption spectrum of 10 μM cytochrome *c* was measured in 30% (v/v) pyridine and 200 mM NaOH after reduction by sodium dithionite. Authentic horse-heart cytochrome *c*, purchased from Sigma-Aldrich (C-7752), was used without further purification in control experiments, following identical protocols.

Reduction potential measurement The reduction potentials of cytochromes c were determined by spectrophotometric titration with Na-ferricyanide, based on (Craig and Nichols 2006). Briefly, in ~1 ml volume 10 μM of cytochrome *c* in 10 mM ferrocyanide, 50 mM Na-PSB, pH 7.00 was titrated with 5–10 microliter aliquots from millimolar ferricyanide stock solutions, and the amounts of reduced and oxidized cytochrome *c* were determined by nonlinear least squares fit of the spectra in the 475–600 nm spectral interval.

Cytochrome c oxidase activity measurement The purification of bovine heart COX was performed according to (King 1967; Yonetani 1967). 0.2 to 38 μM of cytochrome *c* was used in the activity measurement. The solution also contained 20 μM NNNN-tetramethyl-p-phenylenediamine (TMPD), 10 mM ascorbate, 0.05% dodecylmaltoside, 100 nM COX in 50 mM Na-PSB, pH 7.00. The activities were monitored by a Model 10 Oxygraph (Rank Brothers Ltd.). Kinetic parameters (K_m and V_{max}) of the Michaelis-Menten reaction were calculated by fitting the initial rate of oxygen consumption as a function of the substrate (cytochrome *c*) concentration with a nonlinear least squares routine.

Autoxidation experiment 1 ml volumes of the three cytochrome *c* species (non-enzymatically matured, CCHL-matured recombinant, as well as authentic) at approx. 2 μM concentration were fully reduced by the addition of 3 mM dithiothreitol (DTT). DTT was removed by dialysis and the three samples were further dialysed over a time period of several days against 25 mM Na-PSB, pH 7.00 at room temperature, under aerobic conditions and vigorous stirring of the buffer solution. The UV-visible absorption spectra of the three samples were periodically measured.

Results

Heterologous expression and lyase-free maturation of horse cytochrome *c* in *E. coli*

Recently, we have developed our own expression system for producing various site-directed horse cytochrome *c* mutants in order to study the electron transfer routes in this protein (Tenger et al. 2005, 2007). Our reliable, tightly controllable system consisted of the *ara*⁻ BL21-AI *E. coli* strain (Invitrogen) as an expressing host, and a pBAD24 based plasmid. Gene expression from this plasmid was induced by arabinose. The high yield of holocytochrome *c* production was obvious already from the reddish color of the cell extract, whereas in experiments performed with cells expressing only cytochrome *c* without CCHL, no reddish color of the cell extract (and neither of the supernatant nor of the pellet after centrifugation) was recognized. However, when cytochrome was purified from this cell extract, the absorption spectrum displayed typical (although weak) features indicating the formation of holocytochrome *c* in the absence of CCHL from a small fraction of the expressed apocytochrome in *E. coli*.

The heme binds covalently to cytochrome *c* without enzymatic assistance

The expression of Strep-tag II fused, recombinant cytochrome *c* was demonstrated by its appearance in the SDS PAA gel as well as in the western blot immunoassay of the BL21-AI *E. coli* whole cell extract (Fig. 1a and c, lanes 1). The production of matured cytochrome *c* could only be proven after affinity purification of the Strep-tag II fused protein. The purity of the heterologously expressed and non-enzymatically matured cytochrome *c* is shown in Fig. 1a. The presence of heme was demonstrated by in-gel heme peroxidase activity visualized by enhanced chemiluminescence (Fig. 1b), which is only possible if the heme is covalently attached to the protein and, therefore, retained in SDS gel. The western blot immunoassay (Fig. 1c) confirms that two polypeptide variants were obtained after Strep-tag II based affinity purification, which are also visible as distinct bands on the SDS-gel (Fig. 1a, lane 2). The higher molecular

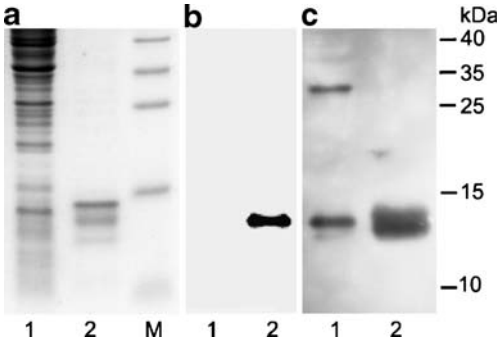

Fig. 1 Purification of non-enzymatically matured cytochrome *c* from the BL21-AI *E. coli* strain. **a** 15% SDS–PAGE, stained with Coomassie BB. **b** In-gel ECL assay for holocytochrome *c* heme peroxidase activity. **c** Immunodetection of Strep-tag II fused cytochrome *c* by StrepMAB-Classic horseradish peroxidase-conjugated antibody. The signal was detected by autoradiography. 1: whole cell extract; 2: purified cytochrome *c*, 80 pmol; M: protein ladder (Fermentas, SM0671)

weight upper band corresponds to the holocytochrome *c* with heme peroxidase activity (Fig. 1b), whereas the lower one, without heme peroxidase activity, corresponds to the tagged apocytochrome.

Control experiments were performed with the same BL21-AI strain and the same plasmid, but without the cytochrome gene. The conditions of cell growth and the method of cytochrome purification were identical to those in the cytochrome expression experiments. No detectable amount of apocytochrome or holocytochrome could be registered either by gel electrophoresis or by spectroscopy. Similarly, no detectable amount of apocytochrome was present in cells harboring the *cytC* expression cassette (with the cytochrome c and the cytochrome c heme lyase genes), without induction of the cytochrome *c* expression by arabinose. In another set of control experiments the cytochrome *c* maturation deficient EC06 strain was used, while the cell growth, the expression and the purification conditions were similar to the experiments with the BL21-AI strain. Gel electrophoresis, in-gel heme detection and Strep-tag II immunostaining all demonstrated the formation of holocytochrome *c* in the absence of CCHL (not shown). Comparison of the near-UV-visible absorption spectra of the holocytochromes expressed in these *E. coli* strains indicated that the binding of heme took place similarly in both cases (see below). Since cytochrome yields, when co-expressed with CCHL, are generally lower in the EC06 strain than in BL21-AI, and the latter strain also lacks several proteases, which may contribute to the degradation of the recombinant apoprotein, the BL21-AI based system was used for further studies of lyase-free cytochrome *c* production.

The heme attachment to both cysteines of the apoprotein via thioether bonds was demonstrated by measuring the pyridine hemochrome spectrum of the non-enzymatically matured cytochrome *c* (see Fig. 4e). The Q band maximum of the pyridine hemochrome spectrum was exactly at 550 nm, as it was also for the authentic horse heart cytochrome *c* and for the CCHL-matured recombinant protein (not shown), indicating native-like covalent heme binding.

Apocytochrome is continuously expressed and degraded in the cytoplasm of *E. coli*

The time course of apocytochrome expression and degradation in the BL21-AI strain was followed in experiments where the expression in the absence of CCHL was interrupted at various times after arabinose induction (Fig. 2). In order to examine the stability of the expressed protein, the translation was either continued overnight (for approx. 16 h), or blocked with chloramphenicol after 1 h or after 3 h following induction. Efficient cytochrome production during the whole expression process could be demonstrated by SDS-PAGE

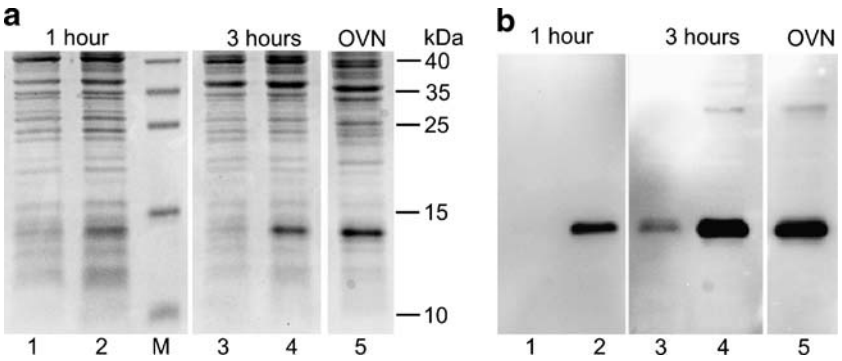

Fig. 2 Time dependence of the expression level of apocytochrome in the BL21-AI strain in the absence of CCHL. A: 15% SDS–PAGE of whole cell extracts, stained with Coomassie BB. B: Immunodetection of Strep-tag II fused cytochrome *c* by StrepMAB-Classic horseradish peroxidase-conjugated antibody. Protein expression induced by arabinose was allowed for 1 h (lanes 1,2), 3 h (lanes 3,4) or overnight (lane 5), and then blocked by the addition of chloramphenicol in the first two cases. Cells were either further incubated overnight after the chloramphenicol block (lanes 1,3) or harvested immediately (lanes 2,4). M: protein ladder (Fermentas, SM0671)

and by Strep-tag II immunodetection in samples taken from the culture after the chloramphenicol addition and frozen immediately at -70°C until further analysis (Fig. 2, lanes 2,4). The amounts of cytochrome *c* were comparable to the amount accumulated during overnight incubation without chloramphenicol addition (Fig. 2, lanes 5). Cells further incubated overnight after blocking the translation/cytochrome expression produced drastically reduced amounts of cytochrome, although still immunodetectable following 3 h of expression (Fig. 2, lanes 1, 3). These experiments indicated that—although the apocytochrome is degraded much faster than the other proteins in the cell extract—it is sufficiently stable to allow the presumably slow lyase-free formation of holocytochrome *c* in low amounts. (Note that these experiments account for the total Strep-tagged cytochrome amounts, without distinguishing between the apo- and holoforms.).

The non-enzymatically matured cytochrome *c* is redox active and physiologically functional

The midpoint reduction potential of the non-enzymatically matured cytochrome *c* was compared to that of the authentic cytochrome and the CCHL matured recombinant protein with and without the Strep-tag II. The non-enzymatically matured cytochrome *c* has a slightly lower potential, 243±4 mV, than the other proteins at 260±5 mV (pH 7.0, versus standard hydrogen electrode). This small difference indicates a heme environment or geometry that is somewhat different from the native environment or geometry.

The functionality of the non-enzymatically matured cytochrome *c* was also investigated with COX, the physiological partner of the native cytochrome *c*. A unique set of K_m and V_{max} values was sufficient to fit the initial rate of oxygen consumption with the non-enzymatically matured cytochrome *c*, but the fit yielded slightly different parameters relative to the authentic protein. The activity parameters for the non-enzymatically matured cytochrome *c* were K_m=9.1±3.6 μM and V_{max}=9.0±1.8 (μmol O_2) min^{-1} (mg of enzyme)$^{-1}$, whereas the parameters for the authentic cytochrome with the same COX preparation were K_m=2.4±0.9 μM and V_{max}=7.5±1.1 (μmol O_2) min^{-1} (mg of enzyme)$^{-1}$.

The non-enzymatically matured and the CCHL-matured cytochromes *c* autoxidized even slower than the authentic protein under the present experimental conditions. The authentic cytochrome c reached complete oxidation only within 3 days, and it took even somewhat longer to fully oxidize the two other species.

The non-enzymatically bound heme has a slightly altered molecular environment or geometry

The near-UV-visible-near-IR absorption spectrum of the non-enzymatically matured, purified, oxidized cytochrome *c* is compared to that of the authentic protein in Fig. 3. Whereas only minor differences are observed in the Soret and Q bands, the 695 nm charge transfer band—which reports the native Met80-Fe axial ligand bond—is absent in the non-enzymatically matured cytochrome *c*. This absorption band was clearly present, however, in the spectrum of the CCHL-matured recombinant protein (not shown).

The absorption spectrum of the non-enzymatically matured, reduced cytochrome *c* is compared to the spectra of the authentic horse heart cytochrome *c* and the recombinant cytochrome *c* co-expressed with CCHL in Fig. 4. Whereas the latter two are spectrally identical, the non-enzymatically matured, reduced cytochrome *c* has slightly red-shifted absorption maxima at 417 nm (Soret band) and at 521 nm and 551 nm (Q-band). The spectrum of the non-enzymatically matured cytochrome *c* from the EC06 strain is similar to the one from the BL21-AI strain, which again indicates that similar proteins are produced in the two strains, independent of the presence of the bacterial maturation apparatus. In order to further characterize these minor spectral differences, the Q-bands of the reduced proteins were resolved into Gaussian components. As shown in Fig. 5 and in Table 1, the same five components with only minor differences in amplitude, position and width fit the spectra of the three species very well. The 1 nm shift of the 550 nm peak is reflected by a similar shift of the 5th Gaussian component, and the 4 nm red-shift, increased amplitude and width of the underlying 4th component is mostly responsible for the small but characteristic differences in the shape of the Q band of the non-enzymatically matured, reduced cytochrome relative to the other two species.

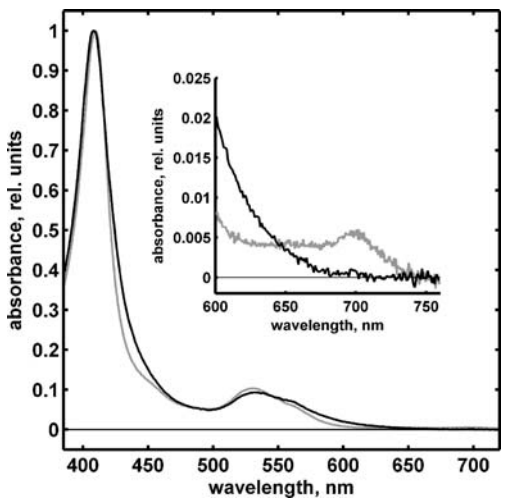

Fig. 3 Near-UV-visible-near-IR absorption spectra of oxidized horse holocytochrome *c*. Protein matured non-enzymatically in the BL21-AI strain (*black*) and the authentic protein (*gray*). Spectra are normalized at the Soret band maxima after the subtraction of a small amplitude scattering spectrum

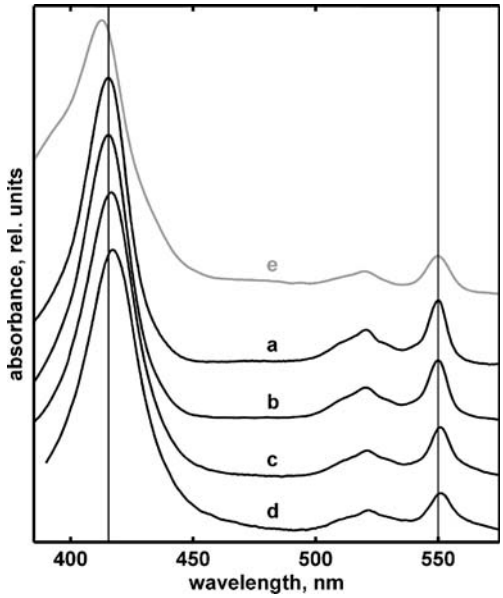

Fig. 4 Near-UV-visible absorption spectra of reduced horse holocytochrome *c*. Authentic **a**, as purified from BL21-AI *E. coli* strain when co-expressed with yeast CCHL **b**, and non-enzymatically matured in BL21-AI **c** and in EC06 **d** *E. coli* strains. Appropriate scattering spectra are subtracted from b, c, and d. Pyridine hemochrome spectrum obtained from the non-enzymatically matured cytochrome *c* **e**. All spectra are normalized at the Soret band maxima

Discussion

The mechanism of the enzymatic maturation of mitochondrial cytochrome *c* by CCHL remains to be elucidated. We have demonstrated that even in the absence of CCHL, the low-yield formation of recombinant horse holocytochrome *c* (i.e. one with appropriate covalent heme attachment) is possible in the cytoplasm of *E. coli*. No cytoplasmic formation of a eukaryotic holocytochrome *c* in an aerobic prokaryotic cell, in the absence of known maturation factor(s), has so far been reported.

The cytochrome *c* maturation machinery (Ccm) of *E. coli* cannot be responsible for the in vivo maturation because our apocytochrome lacks the N-terminal signal peptide necessary for cytoplasmic membrane transport to the periplasm, where Ccm operates (Thony-Meyer 1997). Moreover, the activation of the bacterial Ccm system requires anaerobic ambience and the presence of special substrates (Thony-Meyer 2002), and neither condition was met in our experiments. In addition, we have observed maturation also in an *E. coli* strain lacking the prokaryotic cytochrome *c* maturation genes. Although the likely explanation for the holocytochrome formation in our experiments is spontaneous maturation, we cannot rule out the operation of a so far unknown factor in the cytoplasm of *E. coli* which would catalyze the covalent heme attachment. Examples for in vitro spontaneous (non-enzymatic) maturation have been published in the case of bacterial and mitochondrial cytochromes *c* (Daltrop et al. 2002; Daltrop and Ferguson 2003). For horse cytochrome *c* this maturation was partial and rather slow, requiring 48 h of incubation of the apocytochrome with heme, whereas in our in vivo experiment the covalent heme attachment was accomplished on a much shorter timescale. The absorption spectrum of the reduced, in vitro matured horse cytochrome *c* was characteristic of a native holoprotein, but other physicochemical parameters were not available for comparison with the in vivo assembled holocytochrome in the present study.

The yield of apocytochrome production is comparable to the yield of holocytochrome production when CCHL is co-expressed with the cytochrome *c* in the BL21-AI *E. coli* strain. The SDS-PAGE analysis revealed that the non-processed apocytochrome was progressively degraded over time in the cytoplasm. The lyase-free maturation of cytochrome *c* is probably much slower than the enzymatic

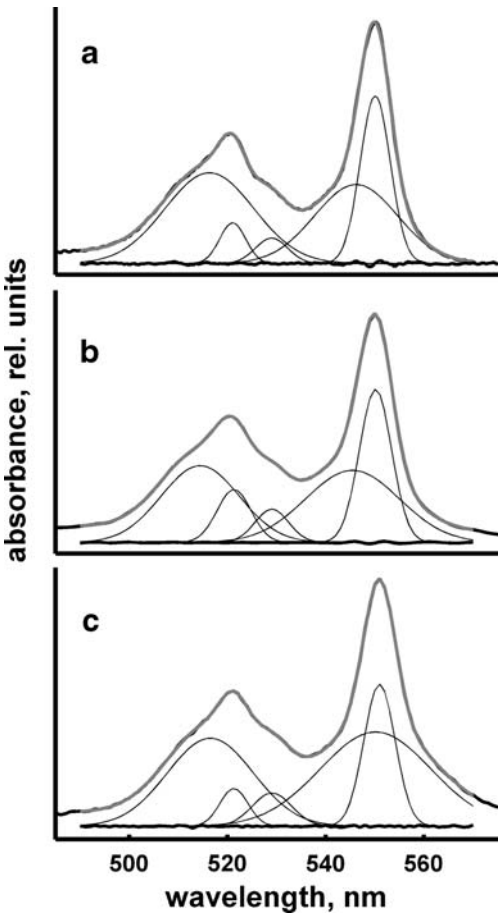

Fig. 5 Resolution of the Q band of reduced cytochrome *c* into Gaussians. Authentic cytochrome *c* **a**, purified from BL21-AI *E. coli* strain when co-expressed with yeast CCHL **b**, and non-enzymatically matured in BL21-AI *E. coli* strain **c**. Measured spectra (*black*), mostly masked by the fit (*gray*), residuals of the fit (*thick horizontal black*) and the Gaussian components (*thin black*) are plotted. A linear baseline of small amplitude is not shown

Table 1 Results of the Gaussian resolution of the Q band measured on various reduced holocytochrome species

Sample		Authentic	CCHL co-expressed	Non-enzymatically matured
Component 1	Position, nm	516.3 (0.59)	514.4 (0.47)	516.5 (0.61)
	Height, %	22.5 (0.63)	19.7 (0.64)	22.2 (0.78)
	Width, nm	21.2 (0.9)	18.6 (0.6)	20.5 (0.8)
Component 2	Position, nm	521.0 (0.13)	521.3 (0.19)	521.3 (0.19)
	Height, %	10.1 (1.05)	13.7 (1.50)	9.6 (1.03)
	Width, nm	6.1 (0.4)	8.0 (0.5)	6.6 (0.5)
Component 3	Position, nm	529.0 (0.24)	529.0 (0.28)	529.0 (0.31)
	Height, %	6.3 (1.26)	8.6 (0.64)	8.5 (1.55)
	Width, nm	7.8 (0.8)	8.0 (0.6)	9.4 (0.9)
Component 4	Position, nm	546.2 (0.30)	545.5 (0.24)	550.1 (0.12)
	Height, %	19.5 (0.63)	18.6 (0.43)	23.8 (0.52)
	Width, nm	19.8 (0.6)	22.4 (0.6)	26.6 (1.5)
Component 5	Position, nm	550.1 (0.02)	550.1 (0.02)	551.0 (0.02)
	Height, %	41.6 (0.63)	39.4 (0.43)	35.9 (0.78)
	Width, nm	7.30 (0.07)	7.77 (0.07)	7.54 (0.09)

Errors of the values are in parentheses. The width is expressed as full width at half maximum

maturation and, therefore, the degradation of the apoprotein appears to be kinetically competitive. An approximate comparison can be made between the holocytochrome yields with and without CCHL co-expression. The yield of non-enzymatically matured cytochrome *c*, as determined from the absorption spectrum after protein purification, was 14±7 μg protein / g wet cell mass. This is approximately 2% of the yield of the enzymatically matured cytochrome *c* obtained in a similar way (i.e. after protein purification).

The non-enzymatically matured cytochrome *c* is chemically identical to the native holocytochrome *c* with appropriate covalent heme binding to both cysteines of the binding motif, as demonstrated by its pyridine hemochrome spectrum. Incorrect orientation of the heme relative to its rotation around the α, γ meso-axis can be ruled out since it would result in a single thioether bond to the protein for reasons of stereo-specificity (Bowman and Bren 2008), and this would show up as a ~3 nm red shifted contribution in the pyridine hemochrome spectrum (Tomlinson and Ferguson 2000), which was not observed.

The Q band in the visible region of reduced cytochrome absorption spectra is characteristic of the electronic and vibrational energies of the heme, the central Fe ion, and the axial ligands, and it is also affected by the environment provided by the heme pocket. The spectrum of the non-enzymatically matured cytochrome *c* is typical of a 6-coordinated, strong axial ligand—low spin c type cytochrome, but exhibits a 1 nm red-shift relative to the authentic cytochrome *c*. Red-shifts of similar magnitude have been reported and theoretically explained in certain site directed mutants of yeast iso-1 cytochrome *c* where the mutations close to the heme affected mainly the polarity of the heme environment and, as a result, the electronic perturbation of the heme macrocycle (Schweitzer-Stenner et al. 2006). It has been shown in cryogenic temperature absorption experiments and by vibrational spectroscopy (Levantino et al. 2005), that the lowest energy transition (at 551 nm in our non-enzymatically matured cytochrome *c*) corresponds to the 0-0 vibronic transition to the first excited state, and the rest of the Q band reflects the higher vibronic sub-bands of this electronic excitation. At room temperature homogeneous and inhomogeneous broadening of the low temperature Voigtian bands result in broader Gaussian bands, whose assignment is difficult. Nevertheless, a five-Gaussian decomposition of the Q bands of the reduced authentic, CCHL-matured, and non-enzymatically assembled cytochromes yielded consistent results, with similar sub-bands in all three cases. The decomposition of the Q band therefore supports the conclusion about the homogeneity of the non-enzymatically matured cytochrome *c*, and its overall structural similarity to the authentic protein. The 1 nm red-shift of the lowest energy transition reflects a small decrease in the energy gap between the ground and the excited states in the non-enzymatically matured cytochrome. The broadening of the next Gaussian component, which is observable as a "tail" of the 551 nm peak, may be the sign of a conformational heterogeneity not present (or present to a lesser extent) in the two enzymatically matured cytochrome *c* species. In addition, it may also indicate a different population of vibrational levels in the ground state of the chromophore, again reflecting a slightly different heme environment and/or geometry in the non-enzymatically matured protein.

The 695 nm charge transfer band in the spectra of oxidized cytochromes *c* is characteristic of the Met80-Fe axial ligand bond. Its absence has been explained by the disruption of this bond during the alkaline transition or the unfolding of cytochrome *c* (Greenwood and Wilson 1971). However, the lack of the 695 nm band does not necessarily

reflect the absence of methionine ligation, but rather a change in the position of Met80 relative to the heme plane (Angstrom et al. 1982). Very recently it has been shown that this band is also sensitive to the taxonomic conformational substates of the protein, i.e. to small conformational variations in the heme pocket (Spilotros et al. 2010).

A substantial increase of the autoxidation rate would be expected if major structural changes took place in the heme pocket. This is the case when cytochrome *c* is perturbed by extreme acidic or alkaline pH, by the interaction with (poly) anions or as a result of polymerization (Harrington and Carrier 1985; Margoliash and Lustgarten 1962; Margoliash and Schejter 1966). We have instead observed a slight deceleration of the autoxidation of the non-enzymatically matured cytochrome *c*. This indicates again that only minor structural differences may remain in the heme environment during lyase-free maturation as compared to the authentic cytochrome *c*. Dimerization or polymerization of the cytochrome would also result in a substantially lower activity in the COX system (Margoliash and Lustgarten 1962), but this was not observed.

The midpoint reduction potential of the non-enzymatically matured cytochrome *c* is slightly lower than that of the authentic or the recombinant, CCHL matured proteins. This relative stabilization of the oxidized (Fe^{III}) form may be the result of a small change in the interaction of the iron with the axial ligands. A slightly altered position of Met80 was already substantiated by the oxidized absorption spectrum, whereas a complete disruption of this bond would result in a much larger negative shift of the midpoint reduction potential. It has also been reported that the proximal histidine-Fe interaction is especially sensitive to the geometry of the heme, which is usually substantially distorted in c-type cytochromes. Small changes in this distorted geometry are expected to result in spectral changes as well as reduction potential changes of the heme as a result of altered Fe-His interactions (Bowman and Bren 2008). Alterations of the polarity of the heme pocket and the electric perturbation of the heme may also be the reasons behind the shift of the midpoint reduction potential. The lower reduction potential also means that the driving force for electron transfer between cytochrome *c* and the first redox center, Cu_A of COX, increases. Since this electron transfer is in the normal region of the non-adiabatic Marcusian process, an increased driving force should result in faster electron transfer—if other parameters are unchanged. The activity of COX with the various cytochromes was measured by following the consumption of oxygen, and no direct connection is expected between this and the rate of the first electron transfer step. Nevertheless, the calculated maximal rate of turnover is higher, although not significantly, with the non-enzymatically matured cytochrome. A more sensible difference was observed in K_m, indicating that the cytochrome-COX interaction is also affected by certain structural differences between the enzymatically and non-enzymatically matured cytochromes.

The covalent heme binding reaction, normally catalyzed by CCHL, is hereby demonstrated to also take place with low efficiency in horse cytochrome *c* in the cytoplasm of *E. coli* without the assistance by known maturation factor(s). The spectral and physicochemical parameters of the non-enzymatically matured cytochrome *c* are only slightly, but significantly different from those of the authentic holocytochrome. Structural differences in the heme pocket seem to affect in particular the Met80-Fe axial ligand bond. Our results confirm, therefore, that i) a lyase-free assembly of functional holocytochrome *c* occurs—although with low efficiency; ii) CCHL is indispensable in the folding of horse holocytochrome *c* to its native conformation during the process of maturation, while the heme is covalently attached to the apoprotein.

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