Expression, purification, and characterization of the CuA–cytochrome c domain from subunit II of the Bacillus subtilis cytochrome \( \text{caa}_3 \) complex in Escherichia coli

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Abstract

Cytochrome \( \text{ca}_{\text{a}} \) from Bacillus subtilis is a member of the heme-copper oxidase family of integral membrane enzymes that includes mitochondrial cytochrome c oxidase. Subunit II of cytochrome \( \text{ca}_{\text{a}} \) has an extra 100 amino acids at its C-terminus, relative to its mitochondrial counterpart, and this extension encodes a heme C binding domain. Cytochrome \( \text{ca}_{\text{a}} \) has many of the properties of the complex formed between mitochondrial cytochrome c and mitochondrial cytochrome c oxidase. To examine more closely the interaction between cytochrome c and the oxidase we have cloned and expressed the Cu A-cytochrome c portion of subunit II from the cytochrome \( \text{ca}_{\text{a}} \) complex of B. subtilis. We are able to express about 2000 nmol, equivalent to 65 mg, of the Cu A-cytochrome c protein per litre of Escherichia coli culture. About 500 nmol is correctly targeted to the periplasmic space and we purify 50% of that by a combination of affinity chromatography and ammonium sulfate fractionation. The cytochrome c containing sub-domain is well-folded with a stable environment around the heme C center, as its mid-point potential and rates of reduction are indistinguishable from values for the cytochrome c domain of the holo-enzyme. However, the CuA site lacks copper leading to an inherent instability in this sub-domain. Expression of B. subtilis cytochrome c, as exemplified by the CuA-cytochrome c protein, can be achieved in E. coli, and we conclude that the cytochrome c and CuA sub-domains behave independently despite their close physical and functional association.

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Cytochrome c and cytochrome c oxidase combine during the process of respiration, in mitochondria and aerobic bacteria, to transfer electrons to molecular oxygen conserving some of the free energy of the redox process in the form of a proton electrochemical gradient [1].

Mitochondrial cytochrome c oxidase is an integral membrane protein complex composed of as many as 13 individual subunits. The three largest subunits (I, II, and III) are expressed by the mitochondrial genome and are conserved across members of the heme-copper oxidase family from humans to yeast, and bacterial species [2]. The other subunits of mitochondrial cytochrome c oxidase are encoded on the nuclear genome and their counterparts are not found in the much simpler bacterial homologs. The metal centers of cytochrome c oxidase that catalyse electron transfer from cytochrome c to oxygen are bound to subunits I and II. Subunit I contains both heme A centers, known as cytochrome a and cytochrome a\(_3\), and one of the two copper centers. Cu\(_B\). Cytochrome a\(_3\) and CuB are located in close proximity (Fe to Cu distance \( \sim 5.5 \) Å) and form a binuclear center that appears to cooperate in the activation and

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reduction of dioxygen [3]. Cytochrome a is located, at its closest heme edge, about 7 Å from the heme edge of cytochrome a$_2$. All three of these centers are within the membrane embedded portion of subunit I at a depth of 15–20 Å from the outer membrane surface. Subunit II is composed of two transmembrane helices at the N-terminal end followed by a soluble domain exposed on the outer membrane surface. The soluble domain of subunit II provides the inner sphere ligands for the dinuclear Cu$_A$ center. This domain is related in its protein fold to the simple blue copper proteins, but the copper site of Cu$_A$ is quite distinct [4]. There are two copper ions located within bonding distance of one another bridged on each side by sulfurs from two cysteine side chains [5]. Oxidized Cu$_A$ exists in a mixed valence state with one ion formally reduced (Cu$^{1+}$) and the other oxidized (Cu$^{2+}$). Reduction by one electron means both metal ions are Cu$^{1+}$ and overall the center is diamagnetic. In the functional cycle of Cu$_A$ one electron is received from ferrocytochrome c and is passed on to cytochrome a and then to the cytochrome a$_3$-Cu$_B$ binuclear site [6]. The binding site for cytochrome c is likely an acidic patch of residues on the surface of subunit II and is adjacent to the Cu$_A$ center [7]. However, it is as yet not possible to crystallize a complex of cytochrome c and cytochrome c oxidase and models of cytochrome c binding to the oxidase do not recognize an energetically unique site [8].

Cytochromes c are a diverse family of electron transfer proteins. Mitochondrial cytochrome c is a highly basic, soluble protein located in the intermembrane space that transfers electrons between integral membrane protein complexes, cytochrome bc$_1$ and cytochrome c oxidase. More recently, mitochondrial cytochrome c is found to play a central role in the process of programmed cell death [9]. In both respiratory and apoptotic pathways the interaction between cytochrome c and its protein partners, such as cytochrome c oxidase, are important in mediating cytochrome c’s activity. Mitochondrial cytochrome c forms an ionic-mediated complex with cytochrome c oxidase in which cytochrome c is proposed to dock with subunit II of cytochrome c oxidase such that it is in proximity to the Cu$_A$ center of the oxidase. The formation of this complex facilitates electron exchange between cytochrome c and Cu$_A$.

In Bacillus subtilis a version of cytochrome c oxidase is expressed in which a cytochrome c domain is covalently fused to subunit II of the oxidase to form a cytochrome caa$_3$ complex. The sequence of subunit II of this oxidase has an extension at the C-terminus of approximately 100 amino acids that specifies a cytochrome c-like protein including the canonical -CXXCH- heme C binding sequence [10]. In light of the covalent attachment of cytochrome c in cytochrome caa$_3$ it is somewhat surprising to find that the ionic character of the conventional subunit II, the Cu$_A$ domain and the cytochrome c domain, is retained (i.e., the cytochrome c domain has an alkaline pI) and the Cu$_A$ domain has an acidic pI). Single turnover experiments of reduced cytochrome caa$_3$ reacting with oxygen demonstrate that the reactivity of the covalent cytochrome c domain is identical to mitochondrial cytochrome c in its non-covalent complex with mitochondrial cytochrome c oxidase [11]. We anticipate that the B. subtilis cytochrome caa$_3$ will be a useful model of the transient complex formed during mitochondrial respiration.

Escherichia coli does not make a functional analog of mitochondrial cytochrome c and its aerobic respiratory chain does not have a cytochrome c oxidase. Wild-type E. coli only assembles cytochrome c proteins in the absence of oxygen as part of its anaerobic respiratory chain [12]. Cytochromes c from a variety of organisms have been efficiently over-expressed in E. coli that has been engineered to express the cytochrome c maturation proteins constitutively (e.g. [13]). However, there has been only limited success expressing B. subtilis cytochromes c in E. coli and it has been proposed that there is a significantly different mechanism for cytochrome c assembly in gram-positive organisms [14].

In this paper the Cu$_A$-cytochrome c domain of cytochrome caa$_3$ from B. subtilis has been cloned and conditions have been established for its expression in E. coli. The Cu$_A$-cytochrome c construct is targeted to the periplasmic space in E. coli and we find a substantial portion of the protein in this locale. The inclusion of a hexahistidine tag on the N-terminal portion of the molecule allows for isolation of the intact protein from the periplasmic fraction. A combination of metal-affinity chromatography and ammonium sulfate fractionation yields 5–7 mg of the Cu$_A$-cytochrome c domain per litre of E. coli culture. The protein has 1 Eq of cytochrome c, but is devoid of copper. The cytochrome c domain has native-like properties with a mid-point redox potential and rates of reactivity with the reductants ascorbate and TMPD$^2$ similar to those observed for the cytochrome c of the holo-enzyme. The cytochrome c and Cu$_A$ sub-domains behave independently, and our kinetic studies here support the view that the cytochrome c domain in the cytochrome caa$_3$ complex is the only avenue required for electron entry.

**Materials and methods**

**Cloning and expression of the Cu$_A$-cytochrome c domain**

The Cu$_A$-cytochrome c domain from subunit II was cloned using B. subtilis genomic DNA as a template and the following pair of primers (sequence of forward primer: GCGCGCATGGAGCTAGCGGACACAT CACC, reverse primer: TATCTCAGGGATCCAGC)

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2 Abbreviations used: BCA; bicinchoninic acid, Gdn·HCl; guanidinium hydrochloride, LB; Luria–Bertani, TMPD; N,N,N',N'-tetramethyl-p-phenylenediamine.
CGGAACCAGCTTGCTTCCCGTTTTAAGC, from Integrated DNA Technologies) and Taq polymerase (Novagen) to generate the Cu₅-cytochrome c protein with a hexahistidine tag at the C-terminus attached via a thrombin-cleavable linker. High level expression is achieved when this vector is used to transform E. coli BL21(DE3) harbouring pEC86 (kind gift from L. Thöny-Meyer), a plasmid expressing the cytochrome c maturation operon constitutively [15]. However, there is significant proteolysis of the recombinant protein at the earliest stages of the purification. This construct was modified to move the hexahistidine tag and thrombin-cleavable linker to the N-terminus between the pelB leader sequence and the structural gene for Cu₅-cytochrome c (Forward primer: CCATGGGCCACCATC ACCATCACCATCTGGTTCCGCGTGGATCCGAG, Reverse primer: CGCG CAAGCTTCTAACATCTCCCTTTCCGAG, Integrated DNA Technologies). Placement of the hexahistidine tag at the N-terminus did not alter the subsequent level of expression of Cu₅-cytochrome c relative to having the affinity tag at the C-terminus, but it did improve its stability and ultimate yield of purified protein. All expression constructs were verified by automated DNA sequencing (Robarts Sequencing Facility).

The E. coli strain transformed with pEC86 and the pET22b+ (Novagen) with the hexahis-Cu₅-cytochrome c insert was streaked from −80°C glycerol stocks and allowed to grow overnight at 37°C on Luria–Bertani plates supplemented with ampicillin (50 µg/mL) and chloramphenicol (10 µg/mL). Starter cultures (20 mL), in LB media supplemented with ampicillin and chloramphenicol, were inoculated from the fresh plate and allowed to grow overnight at 37 °C. The cultures were weighed and a portion (i.e., 50–100 mg) was resuspended by manual homogenization in 100mM Tris–HCl (pH 8.0) containing 20% sucrose in a volume of 80mL/litre culture. The suspension was incubated at 37°C with shaking for 10 min. Protease inhibitor cocktail (Roche) was added at a ratio of one tablet, dissolved in 2mL water, per 50mL suspension. Lysozyme (Sigma) was added to a final concentration of 0.1 mg/mL from a stock solution of 10mg/mL and the suspension was incubated for a further 10 min with shaking at 37°C. EDTA was added to a final concentration of 10mM and the suspension was shaken at 37°C for a further 10 min. The suspension was centrifuged for 10min at 15,000g and the supernatant was collected as the periplasmic fraction. The periplasmic fraction was applied to a Ni²⁺-equilibrated Fast Flow Sepharose column (40 mL, Amersham). Just prior to loading the sample on the Ni²⁺-column 20mM MgCl₂ was added to bind EDTA present in the solution. The Ni²⁺-column was previously equilibrated with 20mM sodium phosphate buffer with 0.2M NaCl (pH 7.6).

The column was eluted with a linear gradient of 0.2M imidazole in 20mM sodium phosphate (pH 7.6) with 0.2M NaCl. The coloured fractions eluting in the imidazole gradient were pooled, concentrated to about 25mL by ultrafiltration, and dialysed against 20mM sodium phosphate (pH 7.6) with 0.2M NaCl. This sample was then further purified using ammonium sulfate fractionation by taking the solution to 40, 60, and 80% with solid ammonium sulfate. At each stage the sample was centrifuged at 15,000g for 10 min. The vast majority of the Cu₅-cytochrome c protein precipitated at 80% ammonium sulfate and this pellet was resuspended in a minimal volume of (i.e., 4–5mL) of 20mM sodium phosphate, pH 7.6, with 0.2M NaCl. This was run over a second Ni²⁺-equilibrated Fast Flow Sepharose column (10mL). The column was eluted as above with a linear gradient up to 0.2M imidazole. The coloured fractions were pooled, dialysed to remove the imidazole, concentrated to 2–3mL, and stored frozen at 77 K.

Samples were taken from each stage of the purification for analysis by electrophoresis. The samples were denatured by incubation overnight at 25°C in Tris buffer (pH 6.8) with 1% SDS plus 2mM dithiothreitol. The samples were run on 15% polyacrylamide with 0.1% SDS and 2m Urea adjusted to pH 8.8. Molecular weight standards were the low molecular weight set from Bio-Rad.

**Spectroscopic assessment of Cu₅-cytochrome c**

Absorption spectra in the ultraviolet and visible regions were recorded using either a Shimadzu UV-160 or a Hewlett-Packard 8452A spectrometers. Fluorescence spectra were recorded on a Perkin–Elmer LS50
luminescence spectrometer. The samples were excited at 280 nm and emission collected from 300 to 450 nm with emission and excitation bandpasses set at 4 nm. The emission spectra were corrected for the inner filter effect [17] at each concentration of guanidinium hydrochloride. Circular dichroism spectra were recorded using a OLIS RSM-2000 spectrometer. CD spectra were recorded in the region of 180–260 nm with samples at 1 mg/mL concentration in 0.1 mm cells. Analysis of the secondary structure was done using the CDNN program [18] and the 23 protein set of basis spectra.

**Reactivity characteristics of Cu₄-cytochrome c**

The stability of the reduced protein was examined by first treating the oxidized protein with an excess of the reductant, sodium ascorbate. The excess reductant was removed by gel filtration on Sephadex G-25 superfine resin (Amersham) and absorption spectra were recorded as a function of time to assess the stability of reduced cytochrome c. The reactivity of the reduced protein with CO was determined by equilibrating a solution of the oxidized protein with argon in a sealed cuvette, reducing the protein with an excess of sodium dithionite and then replacing the argon atmosphere with CO. The spectrum of the reduced protein was recorded before and after the addition of CO and difference spectra constructed to look for evidence CO binding to reduced heme C.

Reduction kinetics of oxidized Cu₄-cytochrome c were measured by stopped-flow mixing using a Hi-tech stopped-flow cell coupled to a Cary 50 spectrophotometer. Rates were measured at 416 nm, characteristic for reduced cytochrome c, as a function of ascorbate concentration and with a fixed concentration of ascorbate and variable amounts of TMPD. The time courses of reduction were fit as single exponential processes to determine a pseudo-first order rate (i.e., k_{obs} s^{-1}). Non-linear least squares fitting was done using the Origin v6.0 software package.

**Redox potentials for cytochromes c**

Redox potentials were determined using a redox electrode (Corning) placed in a closed optical cuvette purged of O₂ by a continuous flow of argon. The optical cuvette was fitted into the cell holder of a Cary 50 spectrophotometer equipped with a constant temperature cell holder and magnetic stirrer. At the start of the experiment the cytochrome c solution was thoroughly equilibrated with argon prior to the addition of mediators. The following mediators were used: 4.0 μM phenazine methosulfate, 8.0 μM of 2,6 dichlorophenol indophenol, 4.0 μM potassium ferricyanide, 4.0 μM dimethyl ferrocene, 4.0 μM 2-hydroxy-1,4-naphthoquinone, and 6.0 μM dianisodurene. The electrode was calibrated with saturated quinhydrone at pH 7.0 before and after each use [19]. The solution potential was adjusted by titration with small amounts of sodium dithionite or potassium ferricyanide, the solution was kept at 25 °C and was stirred constantly [20].

**Results**

**Expression and yield of the Cu₄-cytochrome c protein**

Absorption spectra of whole cells reveal a high level of expression for the B. subtilis Cu₄-cytochrome c construct in E. coli (Table 1). Subsequent fractionation of E. coli cells shows substantial (~25% of total cytochrome c) levels in the periplasmic space, but also cytochrome c is observed in the cytosolic space, in inclusion bodies, as well as a fraction that is extractable from plasma membranes with detergent (data not shown). The membrane associated fraction remains soluble after removal of detergent reflecting the possibility that this fraction of the protein had been loosely associated with the assembly apparatus. The level of expression of cytochrome c corresponds to a protein expression of more than 60 mg/litre of liquid culture and about 12–14 mg of that is in the periplasmic space. Our purification procedure gives about a 50% yield from the periplasmic fraction (Table 1). Analysis of the preparation by electrophoresis shows a band above the 31 kDa marker that becomes more prominent as the purification proceeds (Fig. 1).

**UV-circular dichroism and secondary structural analysis**

Circular dichroism in the range from 180 to 260 nm provides an estimate of the extent of secondary struc-

<table>
<thead>
<tr>
<th>Step</th>
<th>nmol heme C</th>
<th>mg protein</th>
<th>nmol C/mg protein</th>
<th>% yield of C</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Whole cells</td>
<td>2500</td>
<td>1400</td>
<td>1.79</td>
<td>100</td>
</tr>
<tr>
<td>(2) Periplasm</td>
<td>550</td>
<td>100</td>
<td>5.5</td>
<td>22</td>
</tr>
<tr>
<td>(3) Ni-MAC</td>
<td>320</td>
<td>30</td>
<td>10.7</td>
<td>12.8 (58.2)</td>
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<tr>
<td>(4) AmSO₄</td>
<td>220</td>
<td>8.5</td>
<td>25.9</td>
<td>8.8 (68.8)</td>
</tr>
<tr>
<td>(5) Ni-MAC</td>
<td>200</td>
<td>7.0</td>
<td>28.6</td>
<td>8.0 (90.9)</td>
</tr>
</tbody>
</table>

Heme C concentrations were determined by reduced–oxidized difference spectra and protein was measured by BCA assay as described under Materials and methods. The values in parentheses are the yields from each individual step.
The balance between the content of α-helical and β-sheet elements is consistent with known structures of mitochondrial and bacterial cytochromes c [21], which have helical elements with little β-sheet structure, and CuA domains from cytochrome c oxidase complexes, which have large β-sheet content with little α-helical structure [22].

Spectral properties of Cu₄⁺-cytochrome c

UV–visible spectra of the protein show absorption from the aromatic amino acids with a peak at 280 nm and the visible spectrum of a typical low spin heme C with absorption maxima at 410 and 530 nm in the oxidized state and 416, 522, and 550 nm in the reduced state (Fig. 3). However, there is no evidence of the Cu–S charge transfer band that is expected from the Cu₄⁺ center [5]. In addition EPR spectra show features of the cytochrome c heme, but do not show signals characteristic of Cu₄⁺ (data not shown). It appears, therefore, that the recombinant protein has not incorporated copper into the Cu₄⁺ center. The copper content of the recombinant protein is not altered when the bacteria are cultured in media supplemented with copper, or when the purified protein is incubated with copper in native or denaturing conditions.
Heme C reactivity and protein stability

The lack of copper in the CuA center affords us the opportunity to investigate the reactivity of the cytochrome c sub-domain in isolation. The cytochrome c center is readily reduced by the addition of excess sodium ascorbate. Removal of excess reductant and subsequent monitoring of the redox state of the heme C center in aerobic buffer reveals an autooxidation rate of $4.1 \times 10^{-5}\text{s}^{-1}$. This is comparable with values reported for *B. subtilis* cytochrome $c_{550}$ ($8.25 \times 10^{-5}\text{s}^{-1}$) and horse heart mitochondrial cytochrome $c$ ($1.92 \times 10^{-6}\text{s}^{-1}$). In addition, incubation of the reduced protein with CO shows no further spectral change indicating that the cytochrome c heme is unreactive with CO. The stability of the reduced protein and its non-reactivity with CO indicate that the recombinant protein, despite the lack of copper ions in the CuA center, is folded around the heme C group to protect it from attack by $O_2$ and to generate a stable hexacoordinate ligand field.

The kinetics of reduction of the cytochrome c center by excess ascorbate occurs as a single exponential process, the rate of which is proportional to ascorbate concentration with a second order value of $14.6 \pm 1.71 \text{M}^{-1}\text{s}^{-1}$ (Fig. 4). When TMPD is added as a mediator the rate of reduction is enhanced, the process is also a single exponential and is now proportional to the TMPD concentration with a second order rate of $1.32 \pm 0.19 \times 10^3 \text{M}^{-1}\text{s}^{-1}$. The single exponential behaviour indicates that the cytochrome c site is kinetically homogeneous reflective of structural uniformity. The values for the rates are very close to those calculated for the cytochrome c domain in the holo-enzyme (Table 2), consistent with the proposition that the structure of the cytochrome c sub-domain is close to its native state despite the lack of a fully assembled CuA center.

Table 2

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k$ (M$^{-1}$s$^{-1}$)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate</td>
<td>Ascorbate + TMPD</td>
<td></td>
</tr>
<tr>
<td>CuA-cytochrome $c$</td>
<td>$14.6 \pm 1.71$</td>
<td>$1.32 \pm 0.19 \times 10^2$</td>
</tr>
<tr>
<td>$cau_3$-CN</td>
<td>20</td>
<td>$1.0 \times 10^5$</td>
</tr>
<tr>
<td>Cytochrome $c$</td>
<td>$25\text{–}725$</td>
<td>$3 \times 10^4$</td>
</tr>
</tbody>
</table>

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The equilibrium redox potential of the cytochrome c sub-domain was determined by measuring the redox state of the heme by UV–visible absorption spectroscopy as a function of solution potential (Fig. 5). The data shown are semi-log plots of the ratio of reduced to oxidized cytochrome c, derived from the absorption at 550–540 nm, versus potential. This has been determined for the cytochrome c center in three different proteins; CuA-cytochrome c, horse heart mitochondrial cytochrome c, and the cytochrome $cau_3$ complex. The redox transition for each center corresponds to a one-electron process with slopes between 52 and 60 mV, close to the expected value of 58 mV [20]. The mid-point potential for the cytochrome c sub-domain in either the CuA-cytochrome c protein or in the holo-enzyme is $\sim 192$ mV, which is significantly more reducing than mitochondrial
cytochrome \( c \) at 272 mV. The nearly equal values for the two forms of cytochrome \( c \) derived from cytochrome \( caa_3 \) means that the heme C environments in the recombinant CuA-cytochrome \( c \) protein and the native enzyme are similar.

**Denaturation and refolding of CuA-cytochrome \( c \)**

Denaturation of the CuA-cytochrome \( c \) domain was monitored by intrinsic fluorescence and visible absorption spectroscopies. Intrinsic fluorescence from CuA-cytochrome \( c \) is from five tryptophan and nine tyrosine residues. The three tyrosines and one tryptophan in the cytochrome \( c \) domain are probably heavily quenched via energy transfer to the heme [23]. Thus, the majority of the fluorescence under non-denaturing conditions probably originates from the four tryptophans of the CuA domain. The emission maximum from native CuA-cytochrome \( c \) is at 341 nm indicating a partially buried, hydrophobic environment for the tryptophan residues. At the highest Gdn·HCl concentration used here the emission maximum shifts to 350.5 nm as the tryptophans move to a more hydrophilic environment in the unfolded protein. The progress of the shift in the fluorescence emission maximum as a function of increasing concentrations of Gdn·HCl shows a sharp increase from 0 to 3 M Gdn·HCl, and a more gradual increase between 3 and 6 M Gdn·HCl (Fig. 6). Such behaviour is not consistent with two-state unfolding of a single domain protein. In contrast the absorption maximum of the Soret band, which is sensitive to the local environment of the heme C group, is observed to undergo a cooperative transition centered at a Gdn-HCl concentration of 3.5 M (Fig. 6). This indicates a classical two-state unfolding behaviour for the cytochrome \( c \) sub-domain similar to what has been reported for mitochondrial cytochrome \( c \) [24]. Thus the changes in fluorescence observed at low concentrations of Gdn·HCl could be due to unfolding of the CuA sub-domain.

**Discussion**

Earlier studies on the kinetics of reduction of cytochrome \( caa_3 \) proposed a model in which electrons enter the complex via the cytochrome \( c \) domain and are then passed to the CuA center [25]. This model allows for the calculation of rates of electron transfer to the cytochrome \( c \) domain from the artificial electron donor TMPD, but due to the rapid rate of intramolecular electron transfer this reaction cannot be measured directly. The purification of recombinant CuA-cytochrome \( c \) from subunit II of the heme-copper oxidase, cytochrome \( caa_3 \) from \( B. subtilis \), has allowed us to measure the reactivity of the cytochrome \( c \) domain in isolation from the rest of the oxidase complex. The second order rates for reaction of ascorbate and TMPD with the cytochrome \( c \) sub-domain are very close to those calculated from modelling the transient reduction kinetics of the holo-cytochrome \( caa_3 \) enzyme complex [17]. Such kinetic similarity implies an underlying structural similarity and suggests that the cytochrome \( c \) sub-domain of recombinant CuA-cytochrome \( c \) is in a native state.

Cytochrome \( caa_3 \) exhibits biphasic steady-state kinetics which are a hallmark feature of mitochondrial cytochrome \( c \) oxidase [26]. Explanations for the behaviour of the mitochondrial oxidase have suggested multiple binding sites for the reductive substrate cytochrome \( c \), or conformational changes in the oxidase that generate forms of the enzyme with distinct binding and turnover properties. We have argued that the observation of biphasic steady-state kinetics with the cytochrome \( caa_3 \) oxidase cannot be explained by multiple cytochrome \( c \) binding sites as there is by definition only one molecule of cytochrome \( c \) present and it is in a covalently fixed interaction. However, one simple explanation for biphasic kinetics with the cytochrome \( caa_3 \) complex is that there are two rates of reactivity of the electron donor TMPD with the cytochrome \( c \) domain, or another electron input site. The results reported here show that there is a single rate of reduction of the cytochrome \( c \) sub-domain in isolation, and it is indistinguishable from the rate calculated from studies of the holo-cytochrome \( caa_3 \) complex. This kinetic result strongly suggests that
there is a single rate for electron entry into cytochrome c

\( \text{ca}_{4} \) from TMPD, and therefore, the biphasic behaviour of the cytochrome c\( \text{ca}_{4} \) complex arises from an intrinsic step within the oxidase. Such kinetic behaviour could arise from a conformational change in the oxidase leading to a different intrinsic rate of electron transfer.

We have shown that the cytochrome c domain of the cytochrome c\( \text{ca}_{4} \) has kinetic properties that resemble the behaviour of mitochondrial cytochrome c when it is in complex with mitochondrial cytochrome c oxidase [11]. We have proposed that this functional similarity arises from a similar structural disposition of the cytochrome c domain in cytochrome c\( \text{ca}_{4} \) and mitochondrial cytochrome c in complex with its oxidase. In this work we have pursued the preparation of the soluble domain of subunit II from B. subtilis cytochrome c\( \text{ca}_{4} \) with the idea that it represents a good physical model of the electron transfer complex between mitochondrial cytochrome c and its oxidase. The recombinant protein as isolated here seems to have a functional cytochrome c domain, but is devoid of copper and preliminary efforts at reconstitution have not been successful. On the one hand, this is somewhat surprising as the CuA sub-domain alone has been produced before and it was possible to reconstitute the CuA site (e.g. [22]). In addition CuA proteins have been engineered from simple blue copper proteins (e.g. [27]). Reconstitution of the dinuclear CuA site in each of these proteins has required specific solution conditions with regard to ionic strength, pH, copper concentration, and redox potential. An alternative view is that the lack of assembly of CuA in E. coli in vivo is expected because E. coli lacks the specific proteins required for CuA assembly (e.g. [28]). A plentiful supply of recombinant CuA-cytochrome c will allow us to determine conditions necessary for reconstitution of the CuA center in this protein and this will open the way for comparative physical and structure studies.

The efficient expression of c-type cytochromes in E. coli requires the concerted action of a set of proteins expressed by the cytochrome c maturation, or Ccm, operon. In wild-type E. coli the genes of the Ccm operon are only expressed when the bacteria encounter anaerobic conditions. However, when the Ccm operon is placed under the control of a constitutive promoter the expression of a number of heterologous c-type cytochromes has been demonstrated [14]. However, it has been proposed that the expression of c-type cytochromes from gram-positive prokaryotes such as B. subtilis requires a different maturation process, and thus explains the lack of success in expressing these proteins in E. coli. We have shown here that high levels of expression of a c-type cytochrome originating from a gram-positive bacterium is possible in E. coli. The exclusive nature of the maturation process does not seem to be as absolute as originally proposed. However, the expression of the CuA-cytochrome c protein is somewhat unusual in that the heme containing protein is found in all fractions of the cell. An integral feature of the proposed cytochrome c maturation process in E. coli is proposed to involve the concomitant transport of the mature cytochrome out of the cell’s cytoplasm in a process coincident with heme attachment.

Irrespective of the mechanism for cytochrome c maturation, our work demonstrates that the E. coli system can be usefully employed to make large amounts of recombinant cytochrome c from a gram-positive species. We intend to further investigate the physical features of this protein and determine an efficient method for reconstituting the CuA domain so that this protein can be used to provide structural evidence as to the disposition of cytochrome c in the electron transfer complex with its oxidase. We conclude that the physical properties we have described for the cytochrome c sub-domain of the CuA-cytochrome c protein make this a good model of the interaction between mitochondrial cytochrome c and its oxidase.

Acknowledgments

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References


