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Expression and characterization of the diheme cytochrome *c* subunit of the cytochrome *bc* complex in *Heliobacterium modesticaldum*

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ABSTRACT

Heliobacterium modesticaldum is a Gram-positive, anaerobic, anoxygenic photoheterotrophic bacterium. Its cytochrome *bc* complex (Rieske/cyt *b* complex) has some similarities to cytochrome b_6f complexes from cyanobacteria and chloroplasts, and also shares some characteristics of typical bacterial cytochrome bc_1 complexes. One of the unique factors of the heliobacterial cytochrome *bc* complex is the presence of a diheme cytochrome *c* instead of the monoheme cytochrome *f* in the cytochrome b_6f complex or the monoheme cytochrome c_1 in the bc_1 complex. To understand the structure and function of this diheme cytochrome *c* protein, we expressed the N-terminal transmembrane-helix-truncated soluble *H. modesticaldum* diheme cytochrome *c* in *Escherichia coli*. This 25 kDa recombinant protein possesses two *c*-type hemes, confirmed by mass spectrometry and a variety of biochemical techniques. Sequence analysis of the *H. modesticaldum* diheme cytochrome *c* indicates that it may have originated from gene duplication and subsequent gene fusion, as in cytochrome c_4 proteins. The recombinant protein exhibits a single redox midpoint potential of +71 mV versus NHE, which indicates that the two hemes have very similar protein environments.

Introduction

Phototrophic heliobacteria are physiologically and metabolically unusual organisms [1–5]. They are the only phototrophs belong to the Gram-positive bacterial phylum *Firmicutes*. They are also the only phototrophs that produce the unique pigment bacteriochlorophyll *g*, which functions in electron transfer and as an antenna pigment in the reaction center (RC).[1] Heliobacteria contain a type I homodimeric RC, which is embedded in the cytoplasmic membrane [6]. They cannot grow photoautotrophically and grow best as photoheterotrophs. *Heliobacterium modesticaldum*, isolated from hot springs and volcanic soils in Iceland, is one of two known anoxygenic phototrophs that can fix nitrogen at temperatures higher than 50 °C. Based on the *H. modesticaldum* genome sequence, a putative photosystem electron transfer model has been proposed [2]. Fig. 1 shows the photosynthetic reaction chain core proteins, which have been the focus of many heliobacterial bioenergetic studies [1]. The RCs are the major proteins in membranes for cells grown photosynthetically [7]. The absence of separate antenna systems provides a certain advantage for spectroscopic investigations of the electron transfer chain, yet the organism's high sensitivity to oxygen complicates these studies [8]. Furthermore, there have also been reports that the rest of the electron transfer chain except the RCs can be only studied in whole cells, because any cell disturbance or membrane preparation would disrupt the function [9].

Cytochrome *bc* complex (Rieske/cytochrome *b* complex) in heliobacteria is a crucial component completing cyclic electron transfer and generating proton gradient. The genes proposed to code for *H. modesticaldum* cytochrome *bc* complex encode four protein subunits: Rieske iron sulfur protein (*petC*), cytochrome *b* (*petB*), subunit IV (*petD*) and diheme cytochrome *c* (*petA*, NC_010337, NCBI Genome database). Moreover, it has been shown that heliobacterial cytochrome *b* is a cytochrome b_6-like protein [10]. This suggests that *H. modesticaldum* cytochrome *bc* complex is structurally organized similarly to the cytochrome b_6f complex in cyanobacteria and chloroplasts with cytochrome b_6 and subunit IV [11]. However, instead of the cytochrome *f* found in the b_6f complex, heliobacterial *bc* complexes appear to use the diheme cytochrome *c* as a replacement, which is a unique feature also differentiating them from bacterial cytochrome bc_1 complexes possessing cytochrome c_1 [12]. Sequence alignment and structure modeling analysis [13] of the diheme cytochrome *c* show two important characters: (a) it is predicted to be α-

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[1] *Abbreviations used: H. modesticalsum, Heliobacterium modesticaldum*; RC, reaction center; Cyt, cytochrome; MQ, menaquinone; NADH, nicotinamide adenine dinucleotide; FNR, ferredoxin NAD^+ reductase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ESI, electrospray ionization; TEV, tobacco etch virus.

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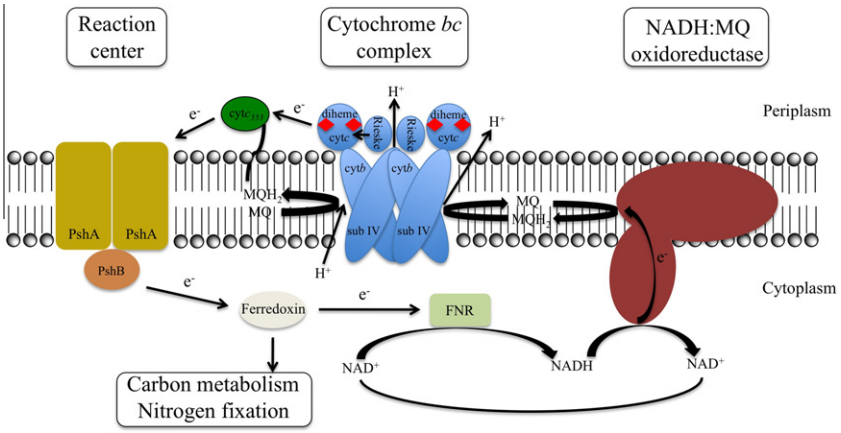


Fig. 1. Reaction center, cytochrome *bc* complex and NADH:MQ oxidoreductase (complex I) proteins in the membrane based on genetic components present in *Heliobacterium modesticaldum*. The cytochrome *bc* complex is here presented as a dimer based on its structural similarity to the cytochrome bc_1 and cytochrome b_6f complexes.

helix rich globular protein with an N-terminal transmembrane helix, which is similar to cytochrome c_1 rather than to the β-sheet rich cytochrome *f*; (b) the N- and C-terminal heme-binding domains share high sequence similarity, which makes it a cytochrome c_4-type protein. Cytochromes c_4 are diheme proteins believed to have originated from a gene duplication event, giving rise to two heme-binding domains with each domain resembling a typical type I monoheme cytochrome. Interestingly, cytochrome c_1 of proteobacteria and mitochondria also has been suggested to have evolved from cytochrome c_4 by the loss of one heme-binding domain followed by a collapse of part of the cytochrome c_4 structure [14]. This in turn suggests that the cytochrome *bc* complex of heliobacteria is potentially an evolutionary prototype for other similar complexes.

The possible shared origin of heliobacterial *bc* complex's diheme cytochrome *c* subunit and cytochrome c_1, and the general similarity of its rest subunits with those of the b_6f complex place it at a very interesting evolutionary position and make the structure, function and bioenergetics of diheme cytochrome *c* an important topic to explore. In this study, we report the successful expression of recombinant heliobacterial diheme cytochrome *c* in *Escherichia coli*. Its size and binding with *c*-type hemes were confirmed by gel electrophoresis, including SDS–PAGE, Western blot analysis and heme staining. Mass spectrometry and pyridine hemochrome analysis were used to investigate the details of the heme binding and extinction coefficient. The redox potential of the recombinant diheme cytochrome was determined by potentiometric titration.

Materials and methods

Growth of H. modesticaldum and cell lysis

H. modesticaldum was grown in PYE medium [1], which is composed of (per liter) K_2HPO_4 (1.0 g), $MgSO_4{\cdot}7H_2O$ (0.2 g), $CaCl_2{\cdot}2H_2O$ (20 mg), $Na_2S_2O_3{\cdot}5H_2O$ (0.2 g), sodium pyruvate (2.2 g), yeast extract (4.0 g), $(NH_4)_2SO_4$ (1.0 g), chelated iron solution [15] (2 mL), D-biotin (15 μg), vitamin B_{12} (20 μg) and trace element solution (1 mL) with the final pH adjusted to pH 6.9–7.0. The composition of the trace element solution was reported previously [3]. All cultures were grown anaerobically in a Coy anaerobic chamber at temperatures ranging from 46 to 50 °C in the light (10 ± 1 W/m^2). Five hundred microliters of overnight (18 h) culture was harvested by centrifugation at 13,000 rpm and re-suspended in 50 μL 20 mM Tris buffer (pH 8.0). The cell suspension was heated to 98 °C for 5 min and quickly chilled on ice to disrupt the cells. Another 5 min 13,000 rpm centrifugation was performed to remove unbroken cell debris. The cell lysate was frozen at −20 °C for further use.

E. coli cell strains and plasmids

E. coli stain DH5α from Invitrogen (Carlsbad, CA) was used for DNA ligation and sequencing steps. *E. coli* strain BL21 (DE3) Δ*ccm* (cytochrome *c* maturation genes knock-out), courtesy of Prof. Robert Kranz from Washington University in St. Louis, was used for the expression of the recombinant diheme cytochrome *c*. The plasmid pTEV [16], which bears T7 promoter, gentamicin resistance gene and the replication origin ColE1, was used to construct the expression vector. *H. modesticaldum* petA coding sequence was PCR amplified using TaKaRa Ex Taq (Takara Bio company) DNA polymerase. Genomic DNA in *H. modesticaldum* cell lysate acquired by the heat-shock method was used as PCR template. The recombinant diheme cytochrome *c* gene was constructed by replacing its original N-terminal transmembrane helix with the signal sequence of *Thiobacillus versutus* cytochrome c_{550} [17]. It was then digested and cloned into similarly digested pTEV to obtain the expression vector pTEV-petA, which has a sequence fused with the insertion allowing a C-terminal poly-histidine (His_6) tag to be co-expressed to facilitate purification. A plasmid containing *ccmABCDEFGH* (pSysI) genes was co-transformed with the expression vector to the host cells [17]. Routine techniques were performed to handle DNA. The final sequence was confirmed by determining the nucleotide sequences of both strands.

E. coli cell growth and harvest

E. coli cell strain BL21 (DE3) Δ*ccm*, carrying both pTEV-petA and pSysI plasmids, was grown aerobically in 1 L LB medium [18]. The culture was vigorously agitated at 16 °C until the optical density at 600 nm (OD_{600}) reached 0.7. The protein expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 300 μM. The cultivation was then continued for another 6 h. With this culture, cells were pelleted by centrifugation at 7100*g* for 20 min at 16 °C. The pellet was stored in −80 °C if not used immediately. Ampicillin, gentamicin and kanamycin were used to final concentrations of 100, 20 and 25 μg/mL, respectively, wherever was needed.

Purification of recombinant diheme cytochrome c

E. coli cells were re-suspended in buffer A (50 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, 1 mM phenylmethylsulfonyl

fluoride, pH 7.2) at 4 °C and disrupted by sonication. The cell extract was centrifuged at 7100*g* for 20 min to pellet the unbroken cell debris. The supernatant was ultracentrifuged for 2 h at 150,000*g* to pellet the membrane fraction from the supernatant of the previous step. The supernatant of the ultracentrifugation, containing the recombinant diheme cytochrome *c* and other *E. coli* soluble proteins, was filtered through a 0.45 μm filter and directly loaded onto a HisTrap HP (GE Healthcare) Co^{2+} chelated column. The column was washed with 10-column volumes of 20 mM imidazole in buffer A. The protein was then eluted with 50 mM imidazole in buffer A. The eluted protein was exchanged into buffer B (20 mM Tris–HCl, 800 mM ammonium sulfate, pH 8.0) and loaded onto a HiTrap phenyl HP column (GE Healthcare). A linear ammonium sulfate concentration gradient from 800 to 0 mM was performed and the pure recombinant diheme cytochrome *c* protein was obtained in the 450 to 350 mM ammonium sulfate fractions.

SDS–PAGE, Western blot and heme stain

SDS–PAGE analysis was performed to determine the purity of the samples, following the protocol described by Marshak et al. [19]. Samples dissolved in SDS–PAGE coomassie brilliant blue dye containing buffer were boiled for 5 min and used for heme stains and Western blots. Anti-His antibody was diluted 5000 times (Sigma–Aldrich, USA). Chemiluminescence was used for both heme stain and anti-His Western blot using the SuperSignal Femto kit (Pierce) and detected in an LAS-1000plus detection system (Fujifilm).

Protein concentration assay and heme analysis

The concentration of recombinant protein was determined by the bicinchoninic acid protein assay (BCA assay kit, Pierce). The pyridine hemochrome analysis, as previously by Berry and Trumpower [20] was used in this study.

In-gel protein digestion

After SDS–PAGE and gel staining, the bands were carefully excised and cut into small pieces with a diameter roughly 1 mm. The samples were then washed with 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate (NH_4HCO_3) aqueous solution. Proteins were reduced with 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 for 30 min and then the cysteines in the proteins were alkylated by 55 mM iodoacetamide in 100 mM NH_4HCO_3 for another 30 min. Trypsin (20 μg/mL, Sigma–Aldrich) in 9% ACN 40 mM NH_4HCO_3 aqueous solution was added to the excised gel mixture and the digestion reaction was conducted at 37 °C for 12 h. The peptides were then extracted by 0.5% trifluoroacetic acid (TFA) 60% ACN buffer at 37 °C for 1 h. The solution containing the extracted peptides was dried in a bench-top speed-vacuum and stored at −20 °C for further analysis.

Mass spectrometry

In-gel digested protein samples were used for MALDI-TOF mass spectroscopic analysis. The protocol was reported previously and followed in this work [21]. For ESI mass spectrometry, the protein sample was desalted by a home-packed C18 tip. The eluate with 75% acetonitrile, 25% water, 0.1% formic acid was collected. The protein solution was delivered by syringe pump at a flow rate of 400 nL/min. Mass spectra were acquired with a Waters SYNAPT G2 Q-TOF (Waters Corporation, Milford, MA) equipped with a nanoESI source. The protein spectrum was acquired at sensitive mode ("V" mode) with the capillary voltage 1.8 kV, cone voltage 30 V and source temperature 100 °C.

Room temperature and low temperature UV–vis spectroscopy

The UV–vis spectra of oxidized and reduced forms of recombinant diheme cytochrome *c* at room temperature and 78 K (liquid nitrogen cooling) were taken with a Perkin-Elmer Lambda 950 UV–vis spectrophotometer and analyzed by Perkin Lambda UV WinLab Explorer software.

Potentiometric titration

Potentiometric titrations were performed to determine the midpoint potential (E_m) of the recombinant diheme cytochrome *c*. The general protocol was described previously [22] and was followed in this study with some modifications. The redox potential of the sample was controlled by a CH 620C potentiostat (CH instruments), and the spectral changes of diheme cytochrome *c* upon oxidation and reduction were monitored at 550 nm in a Perkin-Elmer Lambda 950 UV–vis spectrophotometer. The electrodes used for this analysis were platinum gauze working electrode, Ag/AgCl reference electrode and a platinum wire auxiliary electrode. The titration was performed at room temperature with the following redox mediators: anthraquinone-2-sulfonic acid (E_m = −255 mV), anthraquinone-2,6-disulfonic acid disodium salt (E_m = −184 mV), 2-hydroxy-1,4-naphthoquinone (E_m = −145 mV), 2,5-dihydroxy-*p*-benzoquinone (E_m = −60 mV), phenazine methosulfate (E_m = 8 mV), phenazine ethosulfate (E_m = 60 mV), Fe (III) EDTA (E_m = 117 mV), 1,2-naphthoquinone-4-sulfonic acid (E_m = 215 mV), 2,3,5,6-tetramethyl-*p*-phenylenediamine (E_m = 260 mV), and *N*,*N*-dimethyl-1,4-phenylenediamine dihydrochloride (E_m = 371 mV). Recombinant diheme cytochrome *c* was dissolved in 50 mM sodium phosphate pH 7.2 buffer at a final concentration of 2 mg/mL. Then a final concentration of 150 mM NaCl and 40 μM each of the mediators were added to the working solution. The calculation and equations used for fitting the data were described in Bell et al. [22].

Results

Recombinant diheme cytochrome c expression, purification and identification

The coding sequence of the diheme cytochrome *c* of *H. modesticaldum*'s cytochrome *bc* complex was directly obtained from the NCBI genome database (NC_010337). Based on secondary structure prediction (SOSUI engine version 1.11, Nagoya University), starting from the N-terminus, the first 22 amino acids form a single transmembrane helix, and the rest of the protein is hydrophilic (Fig. 2a), which is consistent with the fact that this cytochrome *c* is part of the cytochrome *bc* complex and does not function independently as some other soluble diheme cytochrome *c* proteins do, which therefore do not require such a structural feature [23–25]. This N-terminal transmembrane helix probably functions only as an anchor to bind the diheme cytochrome *c* with the cytochrome *bc* complex, but its presence will likely complicate both protein expression and purification. We therefore replaced the codons for

(a) **MFGSTGLLLAGILLAPTWQAQG**------ADGDKKQ

(b) **MKISIYATLAALSLALPAGA**----------ADGDKKQ***ENLYFQGHHHHHH***

Fig. 2. Partial amino acid sequence from N- to C-terminus of (a) original *H. modesticaldum* diheme cytochrome *c* of the cytochrome *bc* complex and (b) recombinant diheme cytochrome *c*. The amino acids forming the transmembrane helix ((a) in bold characters) are replaced by *T. thermophilus* cytochrome c_{552} signal peptide ((b) in bold characters). The TEV cutting site and the poly-histidine tag (His_6) are shown in bold italic and italic forms, respectively.

the first 22 amino acids with the ones that will be translated as the signal peptide from *T. versutus* cytochrome c_{552} (Fig. 2b). Two previous reported successful cases regarding the expression of *c*-type cytochrome proteins in *E. coli* using the *T. versutus* signal peptide made it a favored choice [17,26]. To facilitate detection and purification of the recombinant protein, a poly-histidine tag (His_6) was added to the C-terminus of the protein linked with a TEV-protease cutting site (Fig. 2b).

A Co^{2+} chelated affinity column was used in the first purification step. Compared to Ni^{2+} columns, Co^{2+} columns showed better resolution and less protein impurities in our studies (data not shown). We also compared the affinity column purification result at 4 °C with ones at room temperature (25 °C) and found no difference, which is an indication of the stability of the recombinant diheme cytochrome *c* protein. After affinity chromatography, anti-His Western blot and heme staining were used to confirm the expression of a *c*-type heme-binding protein with poly-histidine tag (Fig. 3a and b). The samples for both tests were under denatured conditions. Therefore, the existence of *c*-type hemes in His-tagged proteins was detected. The molecular mass of the protein showed on both anti-His Western blot and heme staining gels are 25 kDa (under gel electrophoresis resolution), which is consistent with the protein peptide mass without the exogenous *T. versutus* signal peptide plus two heme molecules [16,25,26]. After the Co^{2+} affinity chromatography, phenyl sepharose (hydrophobic) column was used to further purify the cytochrome *c* enriched samples and pure recombinant protein was obtained. The coomassie blue stained SDS–PAGE gel is shown in Fig. 3c. In the gel, a single 25 kDa band confirmed the high purity of the final product.

UV–vis spectral analysis

UV–vis absorption spectra of both reduced and oxidized recombinant diheme cytochrome *c* at room temperature are shown in Fig. 4. Upon reduction by adding dithionite to 0.5 mM, the reduced protein exhibits a characteristic *c* type cytochrome γ (Soret) band at 418 nm, β band at 521 nm and α band at 550 nm. In the oxidized protein spectra, the Soret band becomes broader and is blue shifted to 411 nm, and the structured α and β bands are lost. To minimize the influence of the protein backbone vibration on the heme molecules and further investigate the diheme binding pockets, 78 K

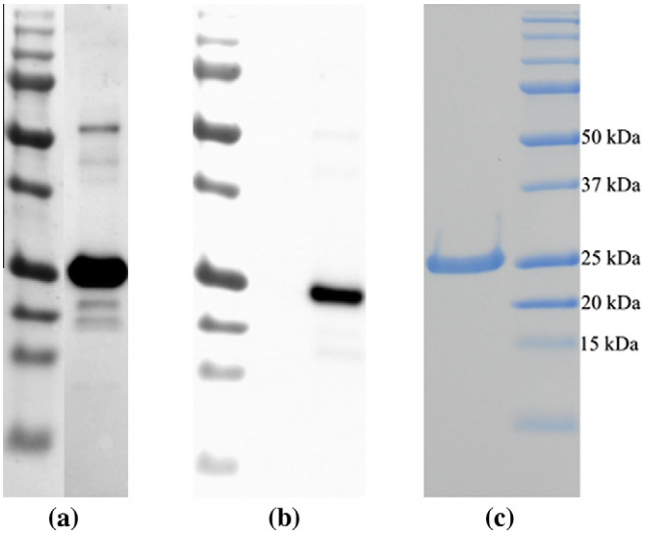


Fig. 3. Affinity column enriched recombinant diheme cytochrome *c* sample was subjected to SDS–PAGE, then (a) anti-His Western blot and (b) heme staining were performed. The strongest bands on the gels correspond to the recombinant proteins. (c) Coomassie blue stained SDS–PAGE gel of the purified protein.

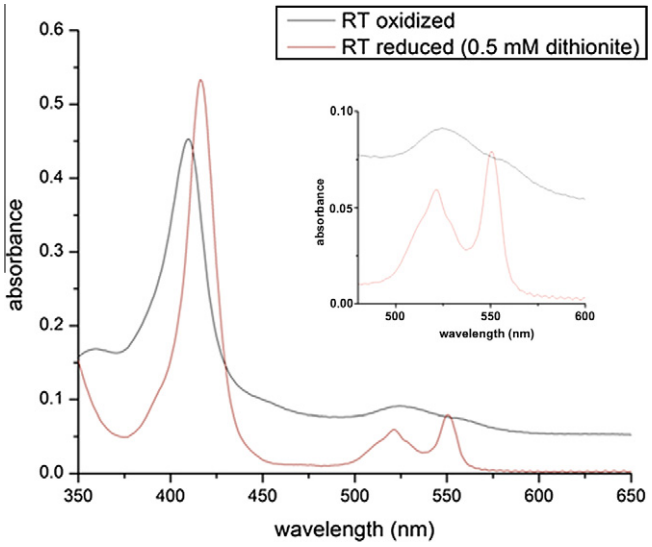


Fig. 4. UV–vis absorption spectra of both reduced and oxidized recombinant diheme cytochrome *c* at room temperature (RT).

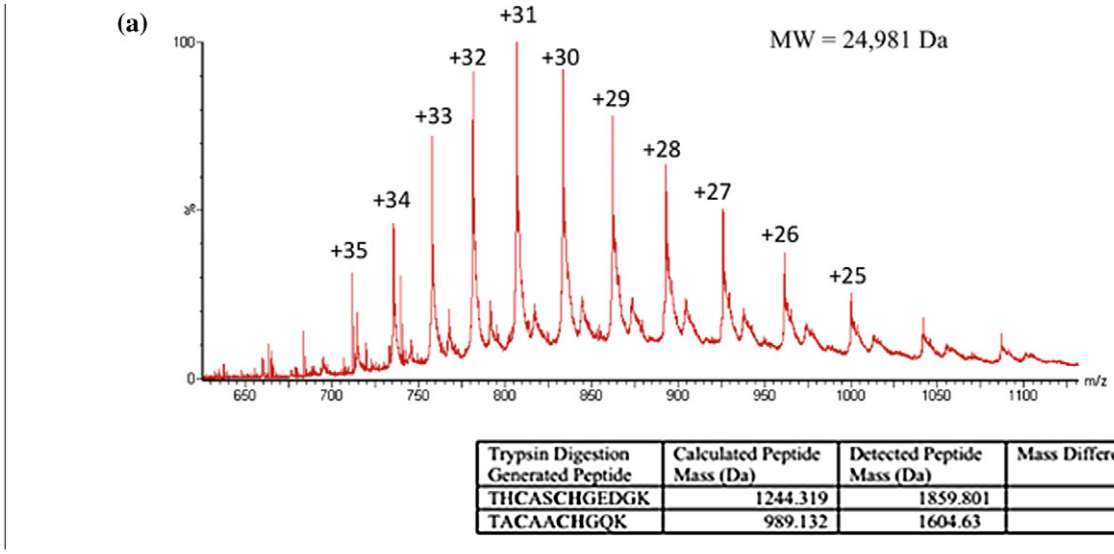
spectra were also recorded. Compared to its room temperature counterpart, the spectrum of reduced cytochrome *c* at 78 K has an overall blue shift. Although it exhibits more detailed β and α band fine structures (Supplementary Fig. 1), no obvious splitting was observed, which suggests that the two heme binding pockets are very similar.

Determination of c-type heme stoichiometry by pyridine hemochrome analysis

To determine weather the recombinant cytochrome *c* has two covalently bound hemes, pyridine hemochrome analysis was performed as described in Materials and methods. The concentration of hemes was quantified by using an extinction coefficient of 23.97 mM^{-1} cm^{-1} for *c*-type heme. The protein concentration was determined by BCA assay. By comparing the concentration of hemes and protein, two hemes (rounded from 2.1 ± 0.1) are present in each protein molecule. Moreover, by comparing the reduced minus oxidized absorption spectrum of the diheme cytochrome *c* with that of the pyridine hemochrome, we determined that the reduced minus oxidized extinction coefficient is 37.4 mM^{-1} cm^{-1} for the wavelength pairs 551–536 nm. And the extinction coefficient for the reduced form of diheme cytochrome *c* is 46.9 mM^{-1} cm^{-1} for the wavelength pairs 551–580 nm. This value reflects both heme groups.

Mass spectrometry

An ESI mass spectrum of the recombinant diheme cytochrome *c* is shown in Fig. 5a. The mass of the protein is 24,981 Da. The calculated mass of the apoprotein (without signal peptide and hemes) is 23,751 Da. The mass difference is 1230 Da, which equals to two hemes molecules (615 Da per heme). To further investigate whether the hemes are correctly linked to the protein backbone, trypsin digested protein was subjected to MALDI-TOF mass spectroscopic analysis (Fig. 5b). In the resulting spectrum, only the two peptides, each bearing a *c*-type heme-binding motif and actually linked with heme, were detected (summarized in the table in Fig. 5b). The mass of just the two peptides without hemes, on the other hand, did not appear in the spectrum. Taken together, this evidence is a strong indication of the presence of correctly expressed and assembled diheme cytochrome *c*.



Trypsin Digestion Generated Peptide	Calculated Peptide Mass (Da)	Detected Peptide Mass (Da)	Mass Difference (Da)
THCASCHGEDGK	1244.319	1859.801	615.482
TACAACHGQK	989.132	1604.63	615.498

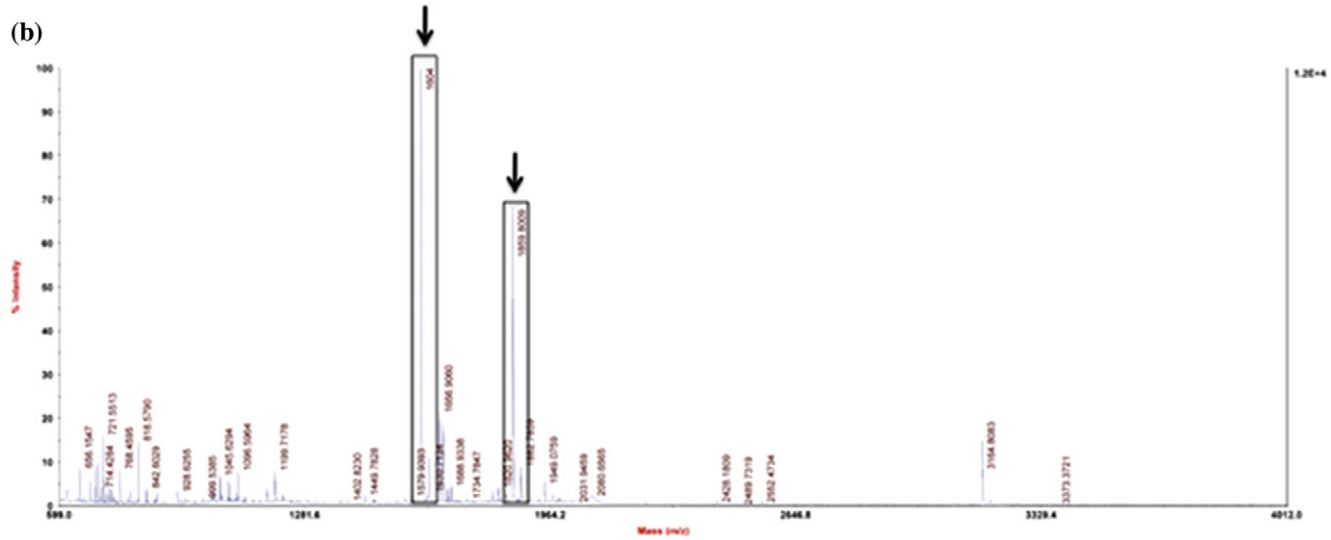


Fig. 5. (a) ESI mass spectrum of diheme cytochrome *c*. The spectrum range is from 650 to 1125 *m*/*z* and multiple charged protein peaks were observed and labeled in this spectrum. The molecular mass of the protein is 24,981 Da. (b) MALDI-TOF mass spectrum of the trypsin-digested protein. Two heme-binding peptide mass peaks are circled. The table summarizes the calculated and observed results.

Redox potentiometry

The midpoint redox potential of recombinant diheme cytochrome *c* was determined by using an optically transparent thin film electrode with a potentiostat as described in Materials and methods. The data were analyzed by the method reported by Bell et al. [22]. With this method, *c*-type hemes were assayed spectrally as a function of the potential. The titration plot (Fig. 6) shows a single E_m for cytochrome *c*, which is +71 mV versus NHE. The standard error in the midpoint potential is ±2 mV obtained from the ORIGIN fitting analysis. However, the overall error including systematic errors for all sources is estimated to be ±10 mV. The plot shows a reversible Nernstian behavior corresponding to a one-electron reduction/oxidation reaction.

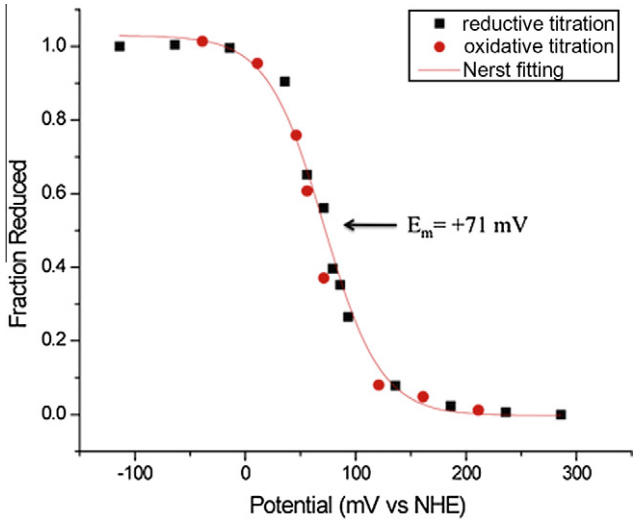


Fig. 6. Potentiometric titration of recombinant diheme cytochrome *c* purified from *E. coli* with squares representing the reductive titration, circles representing oxidative titration and the solid line representing the Nernst fitting. Standard error for E_m is 1.93 mV, adjusted R^2 value is 0.99 and the reduced chi-squared number is 0.0012.

Discussion

Similar to that found in the cytochrome c_1 in bc_1 complex and cytochrome *f* in b_6f complex, the N-terminal helix of the heliobacterial diheme cytochrome *c* functions as an anchor to fix the protein in the membrane and binds it with the other parts of the complex [12,28–30]. Moreover, this helix is spatially removed from

```
N-: VRGHELYKTHCASCHGEDGKGVQGVKAATLNNEGFLKVASDDYLLKSIRLGRPSLATNAM
    V+G   YKT CA+CHG+ G+G  G+ AA L + G+L  ASD+++L+SI +GRP   TN
C-: VKGEAFYKTACAACHGQKGEG--GIGAALL-DPGYLNSASDEFILQSIIMGRP--GTNMP

N-: PHFDTQKLPDEKVNLIIKNMRSWHPEISAPVETGEK
    P+ D+   PD      I+N+ ++      P +  EK
C-: PYPDS---PD------IRNVVAFLRSKQVPYDPAEK
```

Fig. 7. The alignment of the N- and C-terminal domains of the heliobacterial diheme cytochrome *c*. The two heme-binding motifs are highlighted in bold characters.

the protein core and so probably does not have a significant effect on the optical and redox properties of the hemes. Therefore, during the construction of the expression vector, we replaced it with the signal peptide from *T. versutus* cytochrome c_{552}, which can be removed by leader peptidase *in vivo* in *E. coli* [26,27]. One interesting feature is that a small amount of diheme cytochrome *c* was found in the membrane, indicating that the signal peptides of some proteins were not cut off and therefore the protein stayed with the membrane. One explanation is that during the over-expression, the leader peptidases were overwhelmed by the amount of precursor diheme cytochrome *c*. Based on the same logic, the co-transformation of pSysI (*ccmABCDEFGH*) and subsequent *ccm* machinery over-expression are necessary to make the productivity level of the recombinant proteins suitable for biochemical and biophysical studies and lower their misfolding rate [17]. Because of the recombinant cytochrome *c*'s location in the periplasm and electron transfer function, its toxicity (final OD_{600} 1.1–1.2) to host cells is significant during the over-expression. To counteract this negative effect, a low induction temperature, 16 °C, and low IPTG concentration, 300 μM were used. The purified protein yield was 0.2 mg per gram of *E. coli* cell.

In Heliobacteria, menaquinones and membrane-attached cytochrome c_{553} are the electron transfer partners of the cytochrome *bc* complex. The redox potential of menaquinone is −60 mV [31] and cytochrome c_{553} is +180 mV in *Heliobacerium mobilis* [32]. *H. modesticaldum* diheme cytochrome *c*, as final step of the inner electron transfer chain of the *bc* complex, passes electrons from the Rieske protein to cytochrome c_{553}. Therefore, a reasonable speculation is that the redox potentials of these three partners would form a gradient from a high value (cytochrome c_{553}) to a low value (Rieske protein) to allow efficient electron transfer. Based on our optical redox titration result, a single E_m of +71 mV was detected for the diheme cytochrome *c*, which implies that the Rieske protein in *H. modesticaldum* cytochrome *bc* complex should have a lower midpoint potential, assuming that the recombinant cytochrome *c* has the same or similar potential as its native form. A previous whole cell redox titration study of flash-induced heme *c* oxidation in *H. mobilis* revealed that three *c*-type hemes with midpoint potentials of +190, +170 and +90 mV became oxidized by the flash-oxidized primary donor [33]. According to our result, the +90 mV value is probably the one midpoint potential reflecting oxidation of the diheme cytochrome *c* of the *H. mobilis* cytochrome *bc* complex. The +190 and +170 mV hemes might both be due to cytochrome c_{553}, possibly in slightly different environments.

Heliobacterial diheme cytochrome *c* is a cytochrome c_4 type protein, which is found in many different species [25]. It is believed that cytochromes c_4 originated from gene duplication of a monoheme type I cytochrome *c* followed by fusion of the two genes [13]. The same feature can be seen from the sequence similarity of the N- and C-terminal domains of the heliobacterial diheme cytochrome *c* (Fig. 7). Therefore, the structure of its globular heme-binding domains has been modeled based on the crystal structure of cytochrome c_4 from *Pseudomonas stutzerii* [13]. Unlike cytochrome *f* and cytochrome c_1, whose hemes have distinctive double edges allowing them to accept electrons from one side and pass them to the partners on the other side, heliobacterial diheme cytochrome *c*, as in cytochrome c_4 in general, are predicted to have only one edge of each heme that is accessible toward different sides of the protein, respectively. This implies that there must be an intramolecular inter-heme electron transfer in the diheme cytochrome *c*. Some kinetic studies showed that this inter-heme electron transfer is a much faster process compared to the *ms* time range for the electron transfer between diheme cytochrome *c* protein and its partners [23,34]. In *P. stutzerii* cytochrome c_4, this fast inter-heme electron transfer was attributed to the strong heme–heme hydrogen bond via their propionates, and the same reason was also believed to be responsible for the two different redox midpoint potentials of the two hemes [34]. Whereas, in our study, the heliobacterial diheme cytochrome *c* has only a single observable midpoint potential, indicating two identical or at least spectrally and thermodynamically very similar heme binding pockets. This interesting difference may originate from their different relative geometry and thus different interactions between the two hemes among a variety of diheme cytochromes *c*. The structure of the heliobacterial diheme cytochrome *c* will be an important piece of information to resolve this question.

In conclusion, as part of the effort to understand the properties of the heliobacterial cytochrome *bc* complex, its unique subunit, the diheme cytochrome *c*, was expressed in *E. coli* and purified. Western blot, heme staining and mass spectrometry results confirmed that two heme-binding motifs covalently linked two *c*-type hemes. The single redox midpoint potential of the recombinant diheme cytochrome *c* strongly suggests that the two hemes are in extremely similar binding environments. This result is quite different from the two distinct midpoint potentials of many cytochromes c_4, which may share the same origin with heliobacterial diheme cytochrome *c*. This is a hint that in heliobacterial diheme cytochrome *c*, the two hemes interact with each in a different way compared to some other cytochromes c_4. To answer this question and a more intriguing one: what is the relationship between the diheme cytochrome *c* and the rest of the whole cytochrome *bc* complex, crystallographic analysis and more effort trying to purify the intact complex in its native form from heliobacteria are underway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2011.11.012.

References

[1] K.H. Tang, H. Yue, R.E. Blankenship, BMC Microbiol. 10 (2010) 150.
[2] W.M. Sattley, M.T. Madigan, W.D. Swingley, P.C. Cheung, K.M. Clocksin, A.L. Conrad, L.C. Dejesa, B.M. Honchak, D.O. Jung, L.E. Karbach, A. Kurdoglu, S. Lahiri, S.D. Mastrian, L.E. Page, H.L. Taylor, Z.T. Wang, J. Raymond, M. Chen, R.E. Blankenship, J.W. Touchman, J. Bacteriol. 190 (2008) 4687–4696.
[3] M.T. Madigan, Prokaryotes 4 (2006) 951–964.
[4] M.T. Madigan, Bergey's Manual of Systematic Bacteriology vol. 1, second ed., Springer-Verlag, New York, 2001. pp. 625–630.
[5] M. Heinnickel, J.H. Golbeck, Photosynth. Res. 92 (2007) 35–53.
[6] H. Oh-oka, Photochem. Photobiol. 83 (2007) 177–186.
[7] J.T. Trost, R.E. Blankenship, Biochemistry 28 (1989) 9898–9904.
[8] T.J. Michalsky, J.E. Hunt, M.K. Bowman, U. Smith, K. Bardeen, H. Gest, J.R. Norris, J.J. Katz, Proc. Natl. Acad. Sci. USA 84 (1987) 2570–2574.
[9] M.H. Vos, H.E. Klaasen, H.J. Gorkom, Biochim. Biophys. Acta 973 (1989) 163–169.
[10] A.L. Ducluzeau, E. Chenu, L. Capowiez, F. Baymann, Biochim. Biophys. Acta 1777 (2008) 1140–1146.
[11] D. Baniulis, H. Zhang, T. Zakharova, S.S. Hasan, W.A. Cramer, Methods Mol. Biol. 684 (2011) 65–77.
[12] D. Xia, C.A. Yu, H. Kim, J.Z. Xia, A.M. Kachurin, L. Zhang, L. Yu, J. Deisenhofer, Science 277 (1997) 60–66.
[13] F. Baymann, W. Nitschke, Photosynth. Res. 104 (2010) 177–187.
[14] F. Baymann, E. Lebrun, W. Nitschke, Proc. Natl. Acad. Sci. USA 101 (2004) 17737–17740.
[15] L.K. Kimble, A.K. Stevenson, M.T. Madigan, FEMS Microbiol. Lett. 115 (1994) 51–55.
[16] C.J. Rocco, K.L. Dennison, V.A. Klenchin, I. Rayment, J.C. Escalante-Semerena, Plasmid 59 (2008) 231–237.
[17] J.A. Fee, Y. Chen, et al., Protein Sci. 9 (2000) 2074–2084.
[18] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[19] D.R. Marshak, J.T. Kadonaga, et al., Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, 1996.
[20] E.A. Berry, B.L. Trumpower, Anal. Biochem. 161 (1987) 1–15.
[21] X. Gao, Y. Xin, P.D. Bell, J. Wen, R.E. Blankenship, Biochemistry 49 (2010) 6670–6679.
[22] P.D. Bell, Y. Xin, R.E. Blankenship, Photosynth. Res. 102 (2009) 43–51.
[23] H.R. Gibson, C.G. Mowat, et al., Biochemistry 45 (2006) 6363–6371.
[24] G.D. Rocco, G. Battistuzzi, et al., J. Biol. Inorg. Chem. 16 (2011) 461–471.
[25] J.V. Beeumen, Biochim. Biophys. Acta 1058 (1991) 56–60.
[26] M. Ubbink, J.V. Beeumen, G.W. Canters, J. Bacteriol. 174 (1992) 3707–3714.
[27] R.G. Kranz, C.R. Fogal, J.S. Taylor, E.R. Frawley, MMBR 73 (2009) 510–528.
[28] C. Lange, C. Hunte, Proc. Natl. Acad. Sci. USA 99 (2002) 2800–2805.
[29] D. Stroebel, Y. Choquet, J.L. Popot, D. Picot, Nature 426 (2003) 413–418.
[30] G. Kurisu, H. Zhang, J. Smith, W.A. Cramer, Science 302 (2003) 1009–1014.
[31] D.M. Kramer, B. Schoepp, U. Liebl, W. Nitschke, Biochemistry 36 (1997) 4203–4211.
[32] W. Nitschke, B. Schoepp, B. Floss, A. Schricker, A.W. Rutherford, U. Liebl, Eur. J. Biochem. 242 (1996) 695–702.
[33] W. Nitschke, U. Liebl, K. Matsuura, D.M. Kramer, Biochemistry 37 (1995) 11831–11839.
[34] A.C. Raffalt, L. Schmidt, et al., J. Inorg. Biochem. 103 (2009) 717–722.