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Soluble cytochrome *c*-554, CycA, is not essential for photosynthetic electron transfer in *Chlorobium tepidum*

Yusuke Tsukatani[a,1], Ryo Miyamoto[b], Shigeru Itoh[b], Hirozo Oh-oka[a,*]

[a] *Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan*
[b] *Division of Material Science (Physics), Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan*

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Abstract **We constructed a mutant lacking soluble cytochrome *c*-554 (CycA) by disruption of the *cycA* gene in the green sulfur bacterium *Chlorobium tepidum*. The mutant grew phototrophically with a growth rate slower than that of the wild type, suggesting that CycA is not essential for photosynthetic electron transfer even though CycA is known to work as an electron donor to the reaction center. The re-reduction of photo-oxidized cytochrome c_z by quinol oxidoreductase was inhibited almost completely by the addition of stigmatellin in the mutant cells. This result indicates that, in the mutant cells, the linear electron transfer can occur from the quinol oxidoreductase to cytochrome c_z, and to reaction center P840 with no participation of CycA.**

Keywords: Cytochrome *c*-554 (CycA); Cytochrome c_z; Green sulfur bacteria; Quinol oxidoreductase (cytochrome *bc* complex); Reaction center

1. Introduction

In the photosynthesis of plants and bacteria, light energy is converted into electrochemical energy in the light-induced electron transfer driven by the reaction center (RC) complex. In most purple photosynthetic bacteria, soluble cytochrome c_2 mediates electrons from quinol oxidoreductase (cytochrome bc_1 complex) to type-II RC. In some species, such as *Rhodobacter capsulatus*, membrane-anchored cytochrome c_y is also known to function in parallel with cytochrome c_2 [1,2]. In green sulfur photosynthetic bacteria, which have type-I RC, water-soluble cytochrome *c*-554 (denoted CycA in this study) donates electrons to the RC complex [3,4]. We conducted studies to clarify the role of CycA by the gene manipulation of *Chlorobium tepidum*, which is a thermophilic transformable green sulfur bacterium.

The type-1 RC complex of green sulfur bacteria is comprised of only four subunits, PscA–PscD, and seems to have a much simpler architecture than that of the photosystem I RC of plants and cyanobacteria [5]. The PscC subunit (denoted as cytochrome c_z in this study) is a membrane-anchored monoheme *c*-type cytochrome and functions as an electron donor to P840, the primary electron donor in the RC complex [6,7]. The C-terminal heme-containing moiety of cytochrome c_z is exposed to the periplasmic space and seems to mediate the electron transfer reaction from quinol oxidoreductase to the P840 [8,9]. CycA with an apparent molecular mass of 10 kDa has been shown to reduce cytochrome c_z in an in vitro system reconstituted with the isolated RC complex [4].

Green sulfur bacteria use reduced sulfur compounds (sulfide, thiosulfate, and elemental sulfur) as the electron donors for photosynthetic carbon dioxide fixation. In in vitro biochemical experiments using *Chl. limicola* f. *thiosulfatophilum*, membrane-bound sulfide-quinone reductase (SQR) and flavocytochrome *c*-sulfide dehydrogenase (SoxEF) were determined to participate in sulfide oxidation [10–12], and thiosulfate-cytochrome *c* reductase in thiosulfate oxidation [3,12]. CycA is likely to act as an electron carrier that is involved in electron transfer reaction from SoxEF and/or thiosulfate-cytochrome *c* reductase to the RC [3, 11–13; for reviews, 14, 15].

In this study, we constructed a deletion mutant of CycA in *Chl. tepidum* and detected tight coupling between quinol oxidoreductase and the RC complex in the mutant. The photosynthetic growth capability of the mutant implied that electrons from reduced sulfur compounds could be supplied to the P840 RC even without involvement of CycA.

2. Materials and methods

2.1. Plasmid construction for natural transformation

The strain WT2321 [16] of *Chl. tepidum* was used as the wild type and host for transformation. A CL medium and a CP plate [17] were prepared for the liquid cultivation and plating incubation, respectively. A *cycA* gene coding for cytochrome *c*-554 (CycA) was amplified by polymerase chain reaction (PCR) using primers C554F (5′-GAATTCGAAAAGGCCGATCAGCCCAAG) and C554R (5′-AAGCTTCGGATCATCTGGATCGCCTGG) and cloned into a plasmid, pCR2.1-TOPO (a TA cloning kit by Invitrogen), producing pRCT75. An *Eco*RI/*Hin*dIII-digested fragment from pRCT75, harboring the cloned *cycA* region, was again cloned into the same site of pUC18, producing pCT75. Plasmid pCT75 was cut at the PstI site, which is located at the center of the CycA gene, blunt-ended, and then ligated with the streptomycin/spectinomycin resistance gene [18] (Fig. 1A). The resultant plasmid, pCT75A, was purified in a large amount by using a MIDI-prep kit (Invitrogen). About 1 μg of pCT75A was digested at

*Corresponding author. Fax: +81 6 6850 5425.
E-mail address: ohoka@bio.sci.osaka-u.ac.jp (H. Oh-oka).

[1] Present address: Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan.

Abbreviations: Chl., *Chlorobium*; P840, a primary electron donor in green sulfur bacteria; PCR, polymerase chain reaction; *Rba.*, *Rhodobacter*; RC, reaction center; SQR, sulfide-quinone reductase

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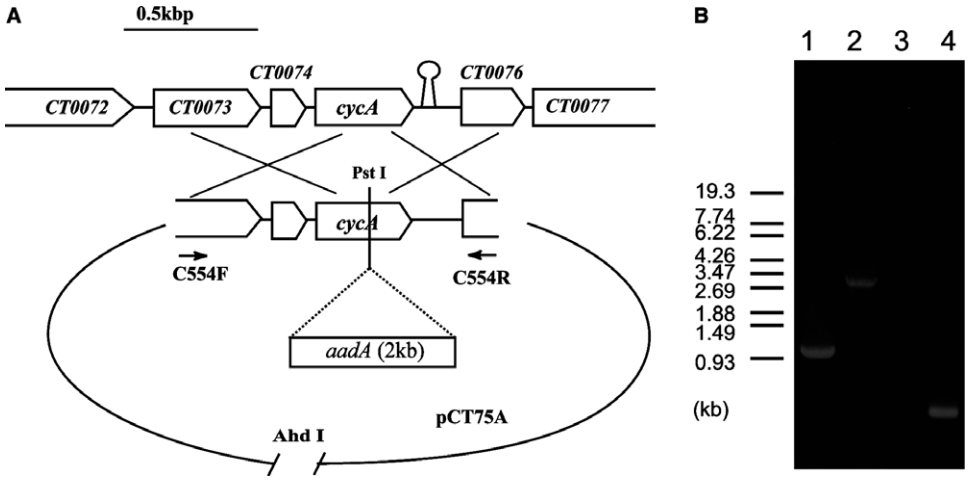

Fig. 1. (A) Schematic map of the construction of the mutant. Genes are indicated by rectangles. Arrows represent the oligonucleotide primers used for the cloning of the *cycA* gene. Plasmid pCT75A for natural transformation was digested at the AhdI site and then introduced into *Chl. tepidum*. The loop structure designed downstream of the *cycA* gene indicates the inverted repeat sequence (*AAAACGGGAG*CCAGC*CTCCCGTTTT*: the complementary sequences are italicized). CT0073 appears to be a membrane-bound *c*-type cytochrome. The homologues of *CT0073* are found in other green sulfur bacteria, but are located immediately upstream of the homologues of *cycA*. (B) PCR analysis of the genomes from wild-type *Chl. tepidum* (lanes 1 and 3) and the mutant (lanes 2 and 4). A primer set of C554F and C554R to amplify the *cycA* locus was used in lanes 1 and 2, and another primer set of aadA946 and aadA947 to amplify the *aadA* (Sm^r/Sp^r) gene was used in lanes 3 and 4. The numbers indicate the lengths of the DNA fragments in kilobase(s).

the AhdI site and applied to natural transformation in *Chl. tepidum* as described previously [17]. Transformants grown on selective (Sm^r/Sp^r) CP plates were re-streaked three times onto Sm^r/Sp^r CP plates. A single colony on the third CP plate was inoculated into the liquid CL medium, and the growth culture was then used for further studies. The genomic DNAs of the Sm^r/Sp^r transformants were purified and used for genetic confirmation by Southern hybridization and PCR analyses, as described previously [19]. Using the same primers as those used for cloning, DNA fragments containing the *cycA* gene were amplified. To amplify the *aadA* marker, the primers aadA946 and aadA947 [20] were used.

2.2. Preparation of chlorosome-depleted crude cell extract

Ten liters of wild-type and mutant cells of *Chl. tepidum* were grown photosynthetically in a CL medium for 2–3 days and harvested by centrifugation. The harvested cells were suspended in a 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 2 mM DTT, homogenized, and then disrupted by passing three times through a French pressure cell at 20 000 psi. The unbroken cells were removed by centrifugation at $23\,500 \times g$ for 20 min. After the supernatant was again centrifuged at $40\,000 \times g$ for 60 min, large amounts of chlorosomes that had detached from the cytoplasmic membrane were precipitated. The resultant supernatant contained fragmented chlorosome-free membranes and water-soluble proteins. This crude cell extract was transferred into a vial, degassed and flushed with a N_2 gas three times, and left in an anaerobic chamber (Coy Laboratory Products) overnight before use for a subsequent spectral analysis, which is described below.

2.3. Spectral analysis

Kinetics measurements of the flash-induced absorption changes of cytochrome c_z were carried out using a single-beam spectrophotometer under anaerobic conditions as described previously [21]. A crude cell fraction was diluted with a 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 15 mM sodium ascorbate to give an absorbance of 1.0 at 810 nm. Samples were placed in air-tight anaerobic cuvettes to avoid oxidative degradation. Stigmatellin was purchased from Fluka. All of the buffers used in the present study were prepared and stored under anaerobic conditions. Almost all redox cofactors with midpoint potentials above approx. 100 mV, including CycA, cytochrome c_z (PscC), P840 and/or other electron carries probably present in the crude fraction [4,5], were kept in the reduced states because of the presence of 15 mM sodium ascorbate in the reaction mixtures under anaerobiosis.

3. Results and discussion

3.1. Cloning the gene encoding CycA and construction of the mutant

Low-molecular-weight water-soluble *c*-type cytochrome, namely, cytochrome *c*-554 of *Chl. tepidum* or cytochrome *c*-555 of *Chl. limicola*, works as the electron donor to the RC in green sulfur bacteria and is denoted as CycA in this study. We cloned the *cycA* gene, *CT0075* (the number designated by TIGR (The Institute for Genomic Research, USA)), from the *Chl. tepidum* genome and performed the mutational inactivation of the gene by inserting the streptomycin/spectinomycin resistance gene (see Fig. 1A).

The insertion of the Sm^r/Sp^r resistance gene (*aadA* gene) into the genomic DNA of the mutant was verified by PCR analyses. Fig. 1B shows the result of PCR using primer sets, C554F-R and aadA946-947, to amplify the *cycA* locus and the internal region of the *aadA* gene, respectively. Using the primers C554F and C554R, the size of the PCR product for the wild-type *Chl. tepidum* was expected to be 1108 bp (see lane 1, Fig. 1B). The same primer set amplified an about 3.1-kbp fragment from the mutant strain. A primer set specific for the *aadA* gene (aadA946 and aadA947) did not produce any fragment from the wild type but amplified a 0.4-kbp fragment from the mutant. These results indicated that the *aadA* gene was correctly introduced into the targeted gene, *cycA*, in the mutant strain.

3.2. Growth capability of the Δcyt c_{554} mutant

The growth profiles of the wild type and the mutant lacking CycA were measured (see Fig. 2). The mutant grew phototrophically in the CL medium [17] at a rate about two times slower than that of the wild type. The result indicated that CycA is not indispensable for photosynthetic growth.

CycA is considered to accept electrons from sulfur-oxidation pathways operated by SoxEF and/or thiosulfate-cytochrome *c*