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Characterization of a blue-copper protein, auracyanin, of the filamentous anoxygenic phototrophic bacterium *Roseiflexus castenholzii*

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ABSTRACT

A blue-copper protein auracyanin of the filamentous anoxygenic phototroph *Roseiflexus castenholzii* was purified and characterized. Genomic sequence analysis showed that *R. castenholzii* has only one auracyanin, whereas *Chloroflexus aurantiacus* is known to have two auracyanins, A and B. Absorption spectrum of the *Roseiflexus* auracyanin was similar to that of auracyanin B of *C. aurantiacus*. On the other hand, ESR spectrum of the *Roseiflexus* auracyanin resembles that of auracyanin A of *C. aurantiacus*. These results suggest that the blue-copper protein auracyanin from *R. castenholzii* shares features with each of auracyanin A and B. Amino acid sequence alignment of auracyanins from filamentous anoxygenic phototrophs also demonstrated the chimeral feature of the primary structure of the *Roseiflexus* auracyanin, i.e., auracyanin A-like amino-terminal characteristics and auracyanin B-like one-residue spacing at the Cu-binding loop in the carboxyl-terminus.

Introduction

Filamentous anoxygenic phototrophs (FAPs: formerly called green non-sulfur bacteria or green filamentous bacteria) thrive usually in thermal microbial mats in hot springs. They belong to the phylum *Chloroflexi* [1], which is positioned at the most deeply branching lineage in the 16S rRNA phylogenetic tree amongst the six phyla containing phototrophic prokaryotes. Exploring the mechanisms of photosynthesis in FAPs is important in order to trace the early evolution of photosynthesis. However, little had been known about photosynthetic apparatuses and electron transfers of FAPs, probably because of their resistance to genetic manipulation, lack of genomic information, and the limited number of species. There had been only one isolated thermophile in FAP, *Chloroflexus aurantiacus* [2], for more than 20 years until Hanada and coworkers discovered two new FAP species, *Chloroflexus aggregans* [3] and *Roseiflexus castenholzii* [4] from Japanese hot springs.

FAP have a type-2 reaction center (RC)[1] complex which is similar to that of purple photosynthetic bacteria and photosystem II in plants and cyanobacteria [5,6]. Photosynthetic energy conversion in purple bacteria is based on the cyclic electron transfer, in which membranous quinones, cytochrome *bc* complexes, and soluble electron carriers (e.g., cytochrome c_2) cooperate together with the RC complex. Photosynthetic cyclic electron transfer leads to the formation of a proton-motive force across the membrane which is used for ATP synthesis. However, in FAPs, components other than the RC complex that function in photosynthetic electron transfer have not been fully characterized. In particular, cytochrome *bc* complex and soluble cytochrome *c* have neither been found in biochemical nor molecular biological studies.

Instead of soluble cytochromes, two types of a blue copper-containing protein have been discovered in *C. aurantiacus*, namely auracyanin A and auracyanin B [7,8]. Auracyanin A is comprised of an N-terminal signal peptide region, a glycine-rich linker region and a soluble Cu-containing domain. The soluble domain is anchored to the cytoplasmic membrane via glycerol fatty-acid esters which are attached to the first-residue cysteine of the linker region [9]. In contrast, auracyanin B has a transmembrane helix in its N-terminus to anchor the C-terminal soluble copper-containing domain to the membrane [8,9]. Crystal structures of soluble domains of auracyanins A and B from *C. aurantiacus* have revealed that the auracyanin molecules have close structural homologies with bacterial blue-copper protein, azurin [10,11]. The auracyanin proteins have been candidates for electron donors to the RC complex of *C. aurantiacus*, although there has been no direct evidence for this speculation.

Since *C. aurantiacus* has anomalously large light-harvesting antennae called chlorosomes, large absorbance and fluorescence emission from chlorosomes would interfere spectroscopic studies

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[1] *Abbreviations used:* FAPs, filamentous anoxygenic phototrophs; RC, reaction center; PCR, polymerase chain reaction; IPTG, isopropyl β-D-1-thiogalactopyranoside.

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of flash-induced absorption changes of electron-transfer components. The newly isolated FAP species *R. castenholzii* naturally lacks chlorosome [4], and may therefore serve as a model organism to study the photosynthetic electron-transfer reaction in FAP. Although *R. castenholzii* contains a type-2 reaction center complex comprised of L, M, and cytochrome subunits [6,12], it has not yet been determined if *R. castenholzii* has an auracyanin protein as *C. aurantiacus* does.

The genome sequencing project revealed that an open reading frame encoding a putative copper-containing protein occurs in the genome of *R. castenholzii*. In this study, we cloned the gene, and overexpressed it in *Escherichia coli*. The resulting protein was then purified and compared with auracyanins A and B of *C. aurantiacus*. The blue-copper auracyanin protein of *R. castenholzii* was shown to share characteristics with each of auracyanin A and B from *C. aurantiacus*.

Materials and methods

Cloning of the gene encoding the blue-copper protein

Roseiflexus castenholzii strain HLO8 (DSM 13941) was photosynthetically grown in PE medium [3] at 50 °C. Genomic DNA was extracted from *R. castenholzii* basically according to the method supplied by JGI, Joint Genome Institute. Genome sequence information of *R. castenholzii* was obtained by the genome sequencing project in collaboration with Drs. Bryant (The Pennsylvania State University) and Raymond (JGI) although the genomic information of this organism is now publicly available at the JGI DNA database [13]. A DNA fragment encoding the soluble domain of the putative auracyanin of *R. castenholzii* was amplified by polymerase chain reaction (PCR) using the isolated genomic DNA and primers RAu-F5 (5′-GGGCATATGACAACGATCGAGATCGCG; NdeI site underlined) and RAu-R1 (5′-TTTTGGATCCTCACGGCGCGACCGTCAT; BamHI site underlined). The amplified fragment was digested with NdeI and BamHI and cloned into the same sites of pET15b. The resulting plasmid, pET15-RAcore, was then transformed into *E. coli* strain BL21(DE3).

Purification of heterologously expressed auracyanin

The recombinant amino-terminal histidine-tagged auracyanin was overexpressed in *E. coli* by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25 °C for 15 h. Before harvesting cells, the grown culture of *E. coli* was incubated with 0.25 mM copper sulfate for 30 min. Harvested cells were suspended in an incubation buffer (20 mM Tris–HCl, pH 8.0, 250 mM NaCl, 5 mM Imidazole), disrupted by sonication, and centrifuged at 10,000*g* for 15 min. The supernatant was mixed with His-select resin (Sigma–Aldrich, USA) and the expressed protein was isolated according to instructions supplied by the manufacturer. The fraction enriched with histidine-tagged auracyanin after affinity resin purification was dialyzed against 20 mM Tris–HCl buffer, pH 8.0, containing 0.25 mM copper sulfate. The blue-colored copper protein fraction was concentrated by ultrafiltration (Viva-spin 5000 MW cut-off, Sartorius, Germany), treated with thrombin protease (Novagen, USA) to remove the histidine-tag, and then applied to an anion-exchange column (DEAE-Toyopearl 650 M) equilibrated with 20 mM Tris–HCl buffer, pH 8.0. The blue-colored auracyanin was eluted by a gradient of 50–100 mM NaCl. After the histidine-tag was removed, four residues (GSHM) derived from the pET15b sequence still remain at the N-terminus of the recombinant auracyanin. Measurements of absorption spectrum, redox titration, and flash photolysis were done with tag-removed auracyanin, whereas ESR spectrum were measured with histidine-tagged auracyanin.

SDS–PAGE analysis and protein assay

SDS–PAGE was performed by the method of Laemmli [14]. After electrophoresis, protein bands were stained with Coomassie brilliant blue.

Spectroscopic analyses

Absorption spectrum was measured with a U-1900 spectrophotometer (Hitachi, Japan). The sample was suspended in 10 mM MOPS–NaOH, pH 7.0.

Redox titration was performed using the U-1900 spectrophotometer as described previously [15]. Ten micromolar of auracyanin was suspended in 10 mM MOPS–NaOH, pH 7.0. The redox mediators employed in the measurements were 50 μM Fe–EDTA, 20 μM 2,3,5,6-tetramethyl-*p*-phenylene-diamine (DAD). The suspension was continuously purged by nitrogen gas. Potassium ferricyanide and sodium ascorbate were used as oxidative and reductive titrants, respectively.

ESR spectrum was recorded using a Bruker ESP-300 EPR spectrometer equipped with a standard resonator (TE102), and a gas flow temperature control system (CF935; Oxford Instruments) was used.

Results

The gene encoding a blue-copper protein in R. castenholzii

A simple tBLASTn analysis demonstrated that *R. castenholzii* has a gene showing significantly high sequence similarities to those for auracyanins A and B of *C. aurantiacus* (Fig. 1B). The amino acid sequence deduced from this gene (*Rcas3112*, locus tag of the gene numbered by JGI) shares 51% identity and 65% similarity with auracyanin A, and 41% identity and 60% similarity with auracyanin B of *C. aurantiacus*. The BLAST search using auracyanin A or B as a query showed that *R. castenholzii* contain a single gene for an auracyanin protein, even when the *E*-value threshold is set at $<10^{-2}$. The same type of analysis demonstrated that *Chloroflexus* species (*C. aurantiacus* and *C. aggregans*) contain two auracyanins, A and B.

Expression and purification of the putative auracyanin of R. castenholzii

Auracyanin of *R. castenholzii* has an N-terminal peptide sequence characteristic for signal sequences of bacterial lipoprotein [16], similar to that of auracyanin A. The following "linker" region is extremely rich in glycine (Fig. 1A and B). The first residue of the linker region is a cysteine residue which must be modified as acetyl-*N*-cysteine-*S*-glycerol possibly working as an anchor to the membrane. Residues of the glycine-rich linker region were disordered in the crystal structure of auracyanin A [11]. The linker region might act as a tether that links the soluble domain to the membrane and swings the soluble domain in the periplasmic space. In this study, we cloned the DNA fragment encoding the soluble domain (122 of 159 total amino acid residues). In the preliminary experiment, the *E. coli* strain BL21(DE3) carrying pET15-RAcore produced a large amount of apoprotein and smaller amount of holoprotein of the copper protein, as judged from the combination of SDS–PAGE analysis and absorption spectra (data not shown). Then we improved the procedure, in which apoprotein of the potential auracyanin was dialyzed against the buffer containing 0.25 mM copper sulfate, and retrieved a large amount of the bright-blue-colored holoprotein showing the large absorption band at around 600 nm typical for a blue-copper protein. The blue-colored holoprotein solution was further purified by the DEAE

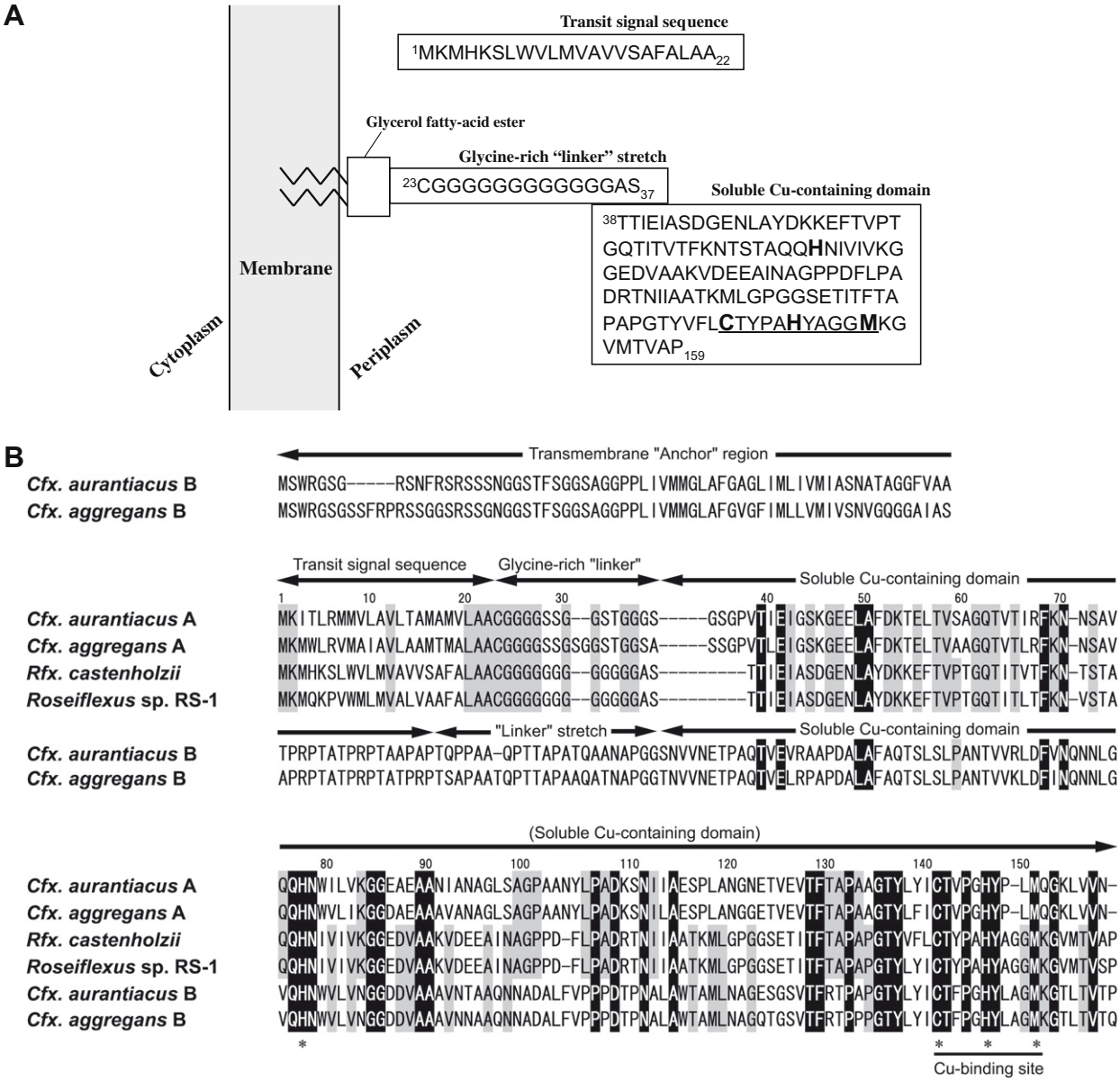

Fig. 1. (A) Structure model of the *R. castenholzii* auracyanin based on the deduced amino acid sequence. Auracyanin of *R. castenholzii* has a transit signal sequence in its N-terminal region followed by a glycine-rich linker region, and a soluble Cu-containing domain. The first residue of the linker region is a cysteine residue which is thought to be modified as acetyl-*N*-cysteine-*S*-glycerol. Using glycerol fatty-acid esters, the modified cysteine probably anchors the soluble domain to the membrane. Cu-binding motif is underlined, and four residues responsible for binding copper are in bold. The oligonucleotide primers used for the cloning were designed to amplify Cu-containing-domain region which corresponds residues from 38th to 159th. (B) Alignment of the amino acid sequences of auracyanins of *Roseiflexus* and *Chloroflexus* species. Conserved residues among all auracyanins are shown by reverse contrast. Conserved residues between auracyanins A and the *Roseiflexus* auracyanins or between auracyanins B and the *Roseiflexus* auracyanins are highlighted by gray. Numbering of residues follows that of the *R. castenholzii* auracyanin. Four residues responsible for binding copper are marked by asterisks. Concerning *Chloroflexus* species, A and B represent auracyanins A and B, respectively.

ion-exchange column and eluted by 50–100 mM NaCl (for details, see "Materials and methods").

As shown in Fig. 2, the purified auracyanin protein appeared as a single band on a Coomassie blue-stained SDS–PAGE gel. The molecular mass predicted from the SDS–PAGE analysis was about 13 kDa, which corresponds to the mass predicted by the amino acid sequence of soluble domain of auracyanin (12.7 kDa).

Spectroscopic properties and redox potential of auracyanin

Auracyanin of *R. castenholzii* showed an absorption spectrum typical of type-1 blue-copper proteins with a broad primary peak at 603 nm and a second peak at around 465 nm (Fig. 3). Auracyanins A and B from *C. aurantiacus* have their primary absorption peaks at 596 nm and 600 nm, respectively [7,8]. The second peak of auracyanin A at 450 nm is higher than that of auracyanin B at 450 nm. The ratios of $\varepsilon_{450}/\varepsilon_{600}$ of auracyanins A and B are 0.31 and 0.12, respectively (summarized in Ref. [17] and also in Table 1). As for the *R. castenholzii* auracyanin, the ratio of absorbance at 465 nm to that at 603 nm was calculated as 0.16, which is close to the auracyanin-B value.

Fig. 4 shows the ESR spectrum of the purified auracyanin protein of *R. castenholzii*. Contrary to the case of the absorption spectrum, the shape of the obtained ESR spectrum was basically identical to that reported for auracyanin A of *C. aurantiacus*. The spectrum is asymmetric at around $g = 2.06$ with a shoulder at

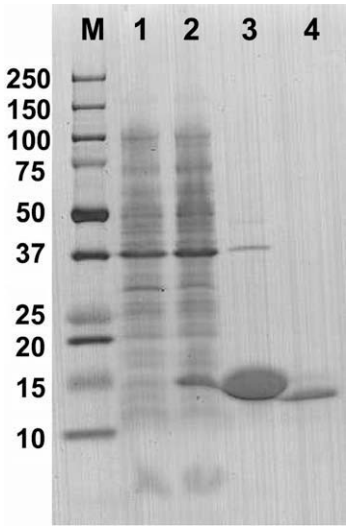

Fig. 2. SDS–PAGE analysis of the purified auracyanin of *R. castenholzii*. Proteins were stained with Coomassie brilliant blue. Lane 1, cell extracts from BL21(DE3) strain; lane 2, cell extracts from BL21(DE3) carrying pET15-RAcore induced with 1 mM IPTG; lane 3, histidine-tagged auracyanin purified from affinity chromatography; lane 4, purified histidine-tag-cleaved auracyanin after DEAE column chromatography. The numbers indicate the molecular weights of proteins in kilodalton.

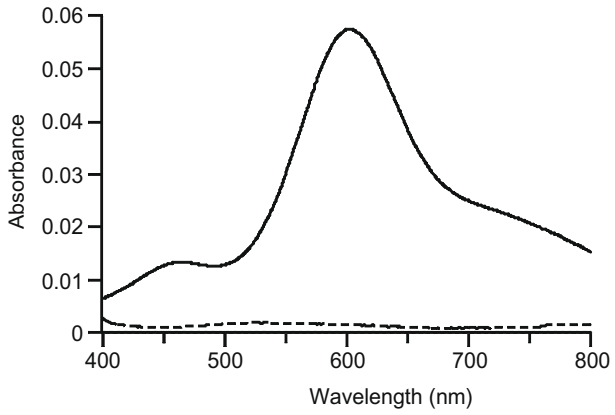

Fig. 3. Absorption spectra of the *R. castenholzii* auracyanin in 10 mM MOPS–NaOH, pH 7.0. The auracyanin was oxidized by air (solid line). The reduced auracyanin by ascorbate showed no characteristic absorption band (dashed line).

around $g = 2.21$. As shown in the ESR spectrum of auracyanin A in [7,8], the ESR spectrum of the *R. castenholzii* auracyanin indicated that the geometry about the copper center has rhombic rather than axial distortion. On the other hand, auracyanin B of *C. aurantiacus*

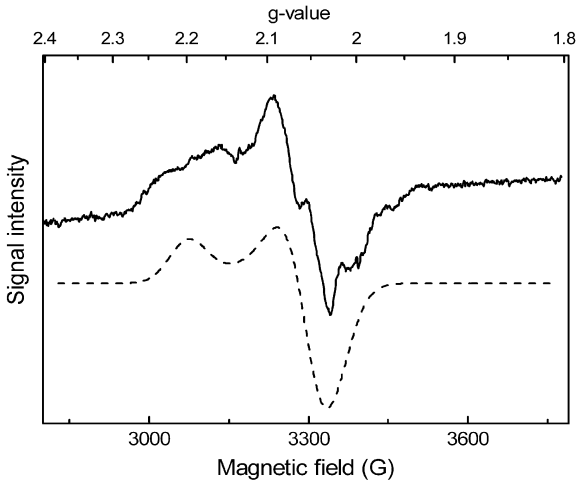

Fig. 4. ESR spectrum of auracyanin of *R. castenholzii*. Experimental conditions for the measurement are as follows: temperature, 8 K; microwave power, 10 mW; microwave frequency, 9.526 GHz; modulation amplitude, 5 G at 100 kHz; time constant, 20 ms. The experimental spectrum is the solid line, and the simulated spectrum is the dashed line. The parameters $g// = 2.06$ and $g\perp = 2.21$ are used for simulation. The well-resolved hyperfine splitting of Cu^{2+} nucleus is not observed. Therefore, the hyperfine interaction of Cu^{2+} is replaced by the Gaussian line shape width of 40 G.

has been known to show an axial ESR spectrum [8]. It is noteworthy that type-1 copper proteins with a rhombic EPR spectrum tend to have a high $\varepsilon_{450}/\varepsilon_{600}$ ratio and a tetragonal Cu-site geometry [18]. Apart from this tendency, auracyanin of *R. castenholzii* showed the rhombic ESR spectrum but the low $\varepsilon_{465}/\varepsilon_{603}$ ratio. Spectroscopic properties of these three auracyanins are summarized in Table 1.

We measured the redox midpoint potential of auracyanin purified in this study (Fig. 5). When the peak height at 603 nm was plotted against E_h, the data obtained was best fit to a one-electron Nernst curve with E_m of +246 mV (pH 7). This value is close to the midpoint potentials of auracyanins A and B from *C. aurantiacus* (+205 and +215 mV at pH 7, respectively) [17].

Comparison of the primary structure of auracyanins

An alignment of amino acid sequences of auracyanins from the two *Roseiflexus* species and the two *Chloroflexus* species was shown in Fig. 1B. Genome sequence of *C. aggregans* also demonstrated that this organism has two homologous genes encoding auracyanins A and B as *C. aurantiacus* does. The primary structure of auracyanin of *R. castenholzii* is similar to that of auracyanin A in the N-terminal signal sequence region, but is similar to that of auracyanin B in terms of one-residue spacing in Cu-binding site.

Table 1
Comparisons between properties of the *R. castenholzii* auracyanin and auracyanins A and B.

	Auracyanin of *R. castenholzii*	Auracyanin A	Auracyanin B
No. of amino acid residues	159	162	235
N-terminal tail	22	22	95
Putative membrane anchor	Fatty-acid esters	Fatty-acid esters	Transmembrane helix
Cu-binding loop	Cys-x-x-x-x-His-x-x-x-x-Met	Cys-x-x-x-x-His-x-x-x-Met	Cys-x-x-x-x-His-x-x-x-x-Met
UV–vis spectra			
Primary peak (nm)	603	596	600
Secondary peak (nm)	465	450	450
$\varepsilon_{450}/\varepsilon_{600}$	0.16	0.31	0.12
ESR spectra	Rhombic	Rhombic	Axial
Redox potential $E_{m,7}$ (mV)	246	205	215

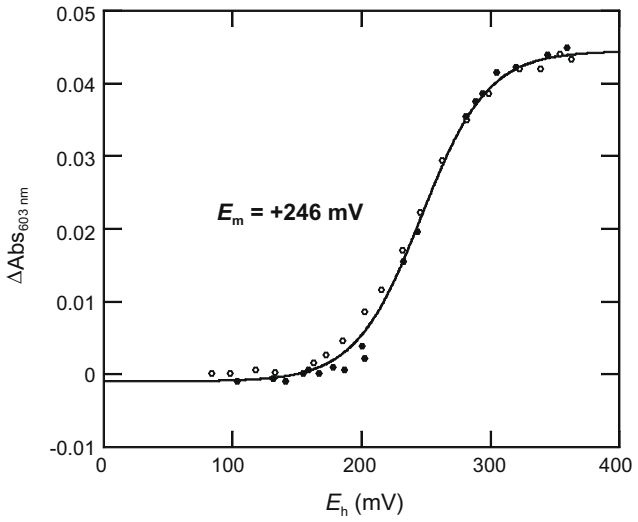

Fig. 5. Redox titration of auracyanin of *R. castenholzii*. The absorption peak heights at 603 nm were plotted against E_h. Redox midpoint potential was obtained by fitting to a Nernst curve with $n = 1$. The redox mediators employed in the titration were described in Materials and methods. The titration was performed in both the oxidizing (open symbols) and reducing (closed symbols) direction. The standard deviation for the E_m value obtained was 5.5.

The phylogenetic tree based on the amino acid sequences of auracyanins shows that the auracyanin proteins of *R. castenholzii* and *Roseiflexus* sp. RS-1 cluster with auracyanin A (Fig. 6). Auracyanin of the *Roseiflexus* species branched off from the auracyanin-A lineage after the divergence of auracyanins A and B occurred in the course of evolution. Phylogenetic analysis using amino acid sequences of auracyanins and other blue-copper proteins, i.e., azurin, pseudoazurin, plastocyanin, and rusticyanin demonstrated that auracyanin is most closely related to azurin (data not shown). Molecular superposition analysis using the known auracyanin and azurin structures has also demonstrated that these proteins are structurally homologous [10,11].

Discussion

The "chimeric" auracyanin protein of R. castenholzii

Auracyanins A and B of the *Chloroflexus* species have different methods of anchoring the soluble Cu-containing domain to the membrane. Whereas auracyanin B has a transmembrane α-helix in its N-terminus, auracyanin A uses glycerol fatty-acid esters which are thought to be attached to the first cysteine residue of mature forms of the protein [9]. In addition, auracyanins A and B have different size Cu-binding polypeptide loops. The Cu-binding motif in the C-terminus contains three residues (Cys, His and Met) out of four residues responsible for binding the copper cofactor. Auracyanin A has a one-residue gap in the Cu-binding motif in the alignment (i.e., Cys-x-x-x-x-His-x-x-x-Met in auracyanin A, Cys-x-x-x-x-His-x-x-x-x-Met in auracyanin B). Most bacterial azurins have auracyanin-A type Cu-binding motifs [19]. Based on the alignment, the *R. castenholzii* auracyanin is likely to employ an auracyanin-A type membrane anchoring system, but have an auracyanin-B type Cu-binding polypeptide loop.

The auracyanin protein from *R. castenholzii* shares features with each of auracyanin A and B from *C. aurantiacus*, not only in terms of primary structures but also spectroscopic properties. The *Roseiflexus* auracyanin has the auracyanin-B type absorption spectrum (Fig. 3). On the other hand, the shape of the ESR spectrum of the *R. castenholzii* auracyanin resembles that of auracyanin A (Fig. 4). These results, combined with the alignment data, suggest that similarities/differences between ESR spectra of auracyanins may result from the overall structural properties, not just from the structural properties around Cu-binding site. In fact, the recent study comparing crystal structures of auracyanins A and B demonstrated the close similarity between the Cu-binding geometries of both auracyanins in spite of the one-residue spacing in the Cu-site. [11]. The Cu-binding geometry of the *Roseiflexus* auracyanin is also likely to be similar to those of auracyanins A and B.

Electron transfer to and from auracyanin

In *C. aurantiacus*, tetraheme cytochrome c_{554} bound to the RC complex has been thought of as a direct electron acceptor from auracyanin. This cytochrome subunit was found to have the redox midpoint potential of around +260 mV as a total of contributions from four hemes [20]. The E_m values of auracyanins A and B (205 ± 7 and 215 ± 7 mV at pH 7, respectively [17]) are low enough to re-reduce the photo-oxidized RC-bound cytochrome. However, direct evidence from reconstitution experiments using purified auracyanins and membrane preparations of *C. aurantiacus* has never been reported, possibly due to the spectroscopic overlapping from the chlorosome antenna.

Flash-induced absorption changes were measured using reconstitution of auracyanin and membrane preparations of *R. castenholzii* in order to investigate whether the blue-copper protein works as an electron donor to the RC complex. However, reduction of the photo-bleached cytochrome subunit bound to the RC complex was not observed after a flash excitation (data not shown). We employed typical experimental conditions for the reconstitution that allow us to monitor the reduction of

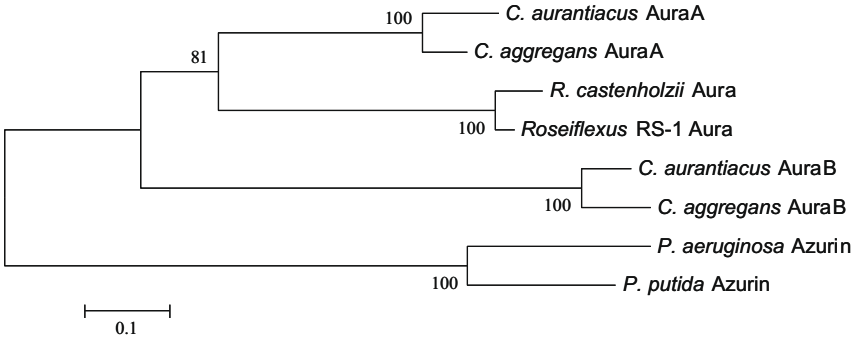

Fig. 6. Phylogenetic tree based on the amino acid sequences of auracyanins. Bootstrap values from 1500 replicates are indicated beside nodes. Phylogenetic analysis was carried out by using the ClustalX [23] and MEGA4 [24] programs. Construction of the tree was performed by the neighbor-joining method [25] applying Poisson Correction distance parameter. Azurin of *Pseudonomas* species was used as an outgroup.

the photo-oxidized RC-bound cytochrome in purple photosynthetic bacteria. Although we also tested the isolated RC complex of *R. castenholzii* for the reconstitution experiment, we could not get the evidence so far to support the hypothesis that auracyanin is an electron donor to the RC. Possible reasons why we were unable to do this are as follows: the electron transfer to FAP RCs requires certain reaction conditions different from those for purple bacterial RCs, the auracyanin protein used in this study was slightly different from that in nature, or auracyanin might not be an electron donor to the RC.

C. aurantiacus is known to lack a typical cytochrome *bc* complex. Yanyushin et al. showed that *C. aurantiacus*, instead, expresses two other types of membranous multi-subunit complexes, called cytochrome C_p and C_r complexes [21,22]. The cytochrome C_p complex is expressed under photosynthetic conditions, while the cytochrome C_r complex is expressed mainly under respiratory conditions. Similarly, Lee and coworkers [11] recently revealed that auracyanin A is expressed only when *C. aurantiacus* is grown under photosynthetic conditions but auracyanin B is expressed constitutively under both photosynthetic and respiratory conditions. Thus, they claimed that auracyanin A is an electron acceptor from the C_p complex in photosynthesis while auracyanin B works as an electron acceptor from the C_r complex in respiration [11]. *R. castenholzii* expresses the RC complex under both anaerobic photosynthetic and aerobic respiratory conditions [4]. Thus, *R. castenholzii* does not seem to definitely regulate expression patterns of electron-transfer proteins between photosynthetic and respiratory growth conditions. In fact, genomic analysis revealed that the two *Roseiflexus* species possess only cytochrome C_p complex (not C_r complex) and only single auracyanin. This also supports the conclusion that the cytochrome C_p complex works together with auracyanin.

The shared spectroscopic properties of the *R. castenholzii* auracyanin and the fact that *C. aurantiacus* has two auracyanins suggest that *Roseiflexus* auracyanin might be bifunctional and work in multiple metabolic pathways, e.g., respiration and photosynthesis. Comprehensive analyses such as transcriptome study on FAP species and meta-transcriptome study on samples from microbial mats in hot springs would be useful to address this hypothesis. Moreover, mutational analysis would also be useful to clarify the hypothesis that auracyanin is the sole electron donor to the RC of FAPs, although it has not yet been determined if *R. castenholzii* or *C. aurantiacus* is transformable.

Acknowledgments

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