Published in final edited form as:

Biochemistry. 2010 August 10; 49(31): 6670–6679. doi:10.1021/bi100858k.

STRUCTURAL ANALYSIS OF ALTERNATIVE COMPLEX III IN THE PHOTOSYNTHETIC ELECTRON TRANSFER CHAIN OF *CHLOROFLEXUS AURANTIACUS*

Xinliu Gao¹, Yueyong Xin^{1,2}, Patrick D. Bell¹, Jianzhong Wen¹, and Robert E. Blankenship^{1,*}

¹Departments of Biology and Chemistry, Washington University in St. Louis, St. Louis, MO, 63130 USA

²College of Life and Environmental Sciences, HangZhou Normal University, HangZhou, 310036 P.R. China

Abstract

The green photosynthetic bacterium *Chloroflexus aurantiacus*, which belongs to the phylum of filamentous anoxygenic phototrophs, does not contain a cytochrome *bc* or *bf* type complex as is found in all other known groups of phototrophs. This suggests that a functional replacement exists to link the reaction center photochemistry to cyclic electron transfer as well as respiration. Earlier work identified a potential substitute of the cytochrome *bc* complex, now named alternative complex III (ACIII), which has been purified, identified and characterized from *C. aurantiacus*. ACIII functions as a menaquinol:auracyanin oxidoreductase in the photosynthetic electron transfer chain, and a related but distinct complex functions in respiratory electron flow to a terminal oxidase. In this work, we focus on elucidating the structure of the photosynthetic ACIII. We found that AC III is an integral-membrane protein complex of around 300 kDa that consists of 8 subunits of 7 different types. Among them, there are 4 metalloprotein subunits, including a 113 kDa iron-sulfur cluster-containing polypeptide, a 25 kDa penta-heme *c*-containing subunit and two 20 kDa mono-heme *c*-containing subunits in the form of a homodimer. A variety of analytical techniques were employed in determining the ACIII substructure, including HPLC combined with ESI-MS, metal analysis, potentiometric titration and intensity analysis of heme-staining SDS-PAGE. A preliminary structural model of the ACIII complex is proposed based on the analytical data and chemical cross-linking in tandem with mass analysis using MALDI-TOF, as well as transmembrane and transit peptide analysis.

Bacterial electron transport pathways largely fall into two major categories: the light-driven photosynthetic electron transfer chain and the aerobic or anaerobic respiratory electron transfer chain. Despite the vast differences between photo- and oxidative phosphorylations, they both couple the chemical reactions between electron donors and electron acceptors to the translocation of protons across the membrane, which then drives ATP formation and other energy-dependent processes (1). As a result, the common feature of all electron transport chains is the presence of a proton pump to create the transmembrane proton gradient. In respiratory electron transfer pathways, there may be as many as three types of proton pumping protein complexes reminiscent of mitochondria, depending on

*Address correspondence to: Robert E. Blankenship, Washington University in St. Louis, One Brookings Dr., CB 1137, St. Louis, MO 63130 USA. Tel: 314-935-7971. Fax: 314-935-4432. blankenship@wustl.edu.

Supporting information available

The identification of the cross-linked products. This supplementary material is available free of charge via the internet at <http://pubs.acs.org>.

environmental factors (2). In contrast, the proton pump in all the photosynthetic electron transfer chains was until recently believed to involve a cytochrome *bc*₁ or *b₆f* complex, which resemble mitochondrial complex III in terms of overall structure and mechanism (3).

In the species tree of bacteria based on 16S rRNA analysis (4), the phylum of filamentous anoxygenic phototrophs (FAPs) is not closely related to the other phyla that contain organisms that carry out chlorophyll-based photosynthesis; purple bacteria, cyanobacteria, heliobacteria, green sulfur bacteria and chloroacidobacteria. Instead, it exhibits a much deeper branching position to the other five bacterial phyla that contain phototrophic representatives (5,6). Because of this distinctive feature, the study of FAPs may shed an interesting light on the evolutionary development of photosynthesis. The FAPs are a very diverse and unique phylum of bacteria including several genera: *Chloroflexus* (7), *Oscillochloris* (8), *Chloronema* (9), *Heliothrix* (10) and several *Chloroflexus*-like bacteria found in marine environment (11). Among them, *Chloroflexus aurantiacus*, a prominent microorganism of hot spring microbial mat communities, was the first described and is the most extensively studied representative of FAPs in terms of its photosynthetic and other metabolic pathways. The photosynthetic apparatus of *Chloroflexus aurantiacus* exhibits an interesting combination of characteristics found in very different and diverse groups of phototrophic prokaryotes. They have a type II photoreaction center and integral membrane antenna complex reminiscent of purple bacteria (12,13). In addition, they have a peripheral chlorosome antenna complex (14) and a chlorophyll biosynthesis pathway that are both similar to those found in green sulfur bacteria (15,16). *Chloroflexus aurantiacus* also contains a unique autotrophic carbon fixation pathway different from that found in any other phototrophs, the 3-hydroxypropionate cycle (17,18). Therefore, the phylogenetic characterization and the versatile photosynthetic apparatus of *Chloroflexus aurantiacus* suggest that it occupies an important place in the origin and evolution of photosynthesis (19).

An intriguing characteristic of *Chloroflexus aurantiacus* is its extraordinary electron transfer pathway. For all types of photosynthetic organisms, following the initial process where the light energy transforms into chemical energy, the electrons pass through a series of electron carriers and ultimately either return to the electron donor side of the photosystem *via* a cyclic electron transfer pathway, or reduce a terminal electron acceptor in a non-cyclic electron transfer process (1). The overall pattern of electron transfer depends critically on the type of the organism, the environment it occupies, whether aerobic or anaerobic metabolism takes place and what type of terminal oxidants and reductants are present. While the electron transfer pathways appear to be quite different in various groups of phototrophs, one component was until recently believed to be a constant constituent in all photosynthetic system: the cytochrome *bc*₁ or *b₆f* complex, which transfers electrons from quinol to soluble cytochrome *c* or plastocyanin and at the same time translocates protons across the membrane, creating a transmembrane proton motive force (20,21). However, *Chloroflexus aurantiacus*, like other members of the FAP phylum, does not exhibit either biochemical or genomic evidence for the existence of a related cytochrome *bc*₁ or *b₆f* complex. The lack of a cytochrome *bc*₁ or *b₆f* complex suggests that this group of organisms contains an unusual photosynthetic electron transfer pathway.

A multi-subunit protein complex containing *c*-type cytochromes but no characteristic features of a cytochrome *bc*₁ complex was isolated from *C. aurantiacus* by Yanyushin (22). A similar complex from *Rhodothermus marinus* is now named alternative complex III (ACIII) (23,24). These two complexes have been proposed to be the functional substitute of the cytochrome *bc*₁ complex based on gene analysis of sequenced genomes of various species (25) and an enzymatic study of ACIII from *R. marinus* (23). Recent enzyme kinetic analysis showing that ACIII performs the function of a quinol:auracyanin oxidoreductase

strongly supports the hypothesis that ACIII fulfills the functional role of cytochrome *bc*₁ complex in the photosynthetic electron transfer chain in *Chloroflexus aurantiacus* (26). Figure 1 shows the proposed cyclic electron transfer pathway in *Chloroflexus aurantiacus*.

Based on the genome arrangement of ACIII genes and early fundamental structural studies, the organization of ACIII was revealed to be entirely different from that of cytochrome *bc*₁ or *b₆f* complexes. However, a challenging question emerges - how does ACIII, a complex with structure vastly different from the cytochrome *bc* complex, carry out the same function in the electron transfer pathway in photosynthesis? A complete picture of the structure and role of ACIII complex is still missing. The key to elucidating this system is therefore believed to reside in understanding the ACIII complex in terms of its substructure and how this relates to its function in photosynthesis and respiration. In this work, a schematic structural model of the photosynthetic ACIII complex is proposed based on chemical cross-linking of subunits in tandem with MALDI-TOF mass spectrometry. The size and type of each subunit was determined by gel electrophoresis including one and two dimensional SDS-PAGE and native PAGE. The type and number of cofactors existing in the ACIII complex was investigated using metal analysis, HPLC combined with ESI-MS and potentiometric titrations.

Materials and methods

Bacterial Strains and Growth Conditions

Chloroflexus aurantiacus strain J-10-fl was grown in batch culture anaerobically and photosynthetically in modified medium D (13) at 55 °C for ~72 hours. Cells were harvested by centrifugation at 12,000×g and the pellet was washed by 20mM Tris-HCl buffer, pH 8.0 (buffer A) and then stored at -20 °C. A large-scale growth in a 16-liter fermentor typically yields approximately 80 g wet-packed cells.

Whole membrane isolation

C. aurantiacus cells were thawed and suspended in buffer A to a concentration of 1 g of wet cells per 4 ml buffer. The cells were broken by sonication for 5 min for three times in a pre-cooled sonicator (Branson Sonifier 450) in the presence of DNase and MgCl₂ at 4 °C. Unbroken cells and large debris were precipitated by centrifugation at 12,000×g for 15 min. The supernatant was then ultra-centrifuged at 200,000×g for 2 h. The resulting pellet, referring to as whole membranes, was re-suspended in buffer A to a concentration of OD = 10 at 866 nm and stored at -20 °C until further use.

Protein Purification

The whole membrane sample was thawed to room temperature and treated with reduced Triton X-100 by drop-wise addition to a final concentration of 4% (w/v). Solid NaCl was added to a concentration of 0.1 M. After a 1-hour incubation at RT with stirring, a subsequent ultracentrifugation at 200,000×g for 2 h was performed. The collected supernatant was filtered through a 0.2 µm filter and loaded on to a Q Sepharose Fast Flow 50/10 (mm/cm) column pre-equilibrated with buffer A containing 0.25% reduced Triton X-100 and 0.1 M NaCl (buffer B). The column was washed extensively with buffer B until the green color due to solubilized free BChl *c* pigment was washed out. Afterwards, the column was washed with buffer A containing a higher NaCl concentration (0.4 M) and 0.25% reduced Triton X-100. Proteins including ACIII, ATP synthase and menaquinone fumarate reductase (mQFR) were eluted (27). The eluant was diluted with buffer A to 3x of its volume and loaded onto a 75-ml Q Sepharose High Performance (QSHP) (GE Healthcare) column. The crude ACIII complex and other proteins, e.g., mQFR complex and ATP synthase complex, were separated with a linear gradient of NaCl from 0.1 M to 0.5 M

in 20 column volumes. The fractions containing crude ACIII complex were combined, diluted with buffer A and loaded onto a third ion exchange column, 5-ml QSHP. Afterwards, further purification was performed on an S-300 (26 × 70 cm) gel filtration column in buffer B using the concentrated crude ACIII complex fractions. A final purification step using a Mono Q1 column with a Bio-Rad Duo-Flow chromatography system was carried out to obtain the purified ACIII complex for use in subsequent experiments. Detergent exchange was required for the further purification. The detergent exchange process was similar to the gel filtration column purification step described above except for the change of the detergent to 0.1% dodecyl maltoside (DDM).

Electrophoresis

Tricine SDS-PAGE was performed on the purified ACIII complex as described previously (28) with 12.5% T/3% C. Urea (8 M) was added to both the separating layer of the gel and the loading buffer to obtain sharp bands. Blue-Native gel electrophoresis and a following second dimensional tricine SDS-PAGE were carried out using the method of Schagger and von Jagow (29). The subunit bands were tested for *c*-type heme on the SDS-PAGE gel by a colorimetric heme-staining assay (30). A series of horse heart cytochrome *c* samples of different concentrations and a purified ACIII sample were subjected to SDS-PAGE and subsequent heme-staining, followed by intensity analysis using an image processing program, ImageJ (developed by NIH, <http://rsbweb.nih.gov/ij>).

In-Gel Protein Digestion

Stained bands of the subunits were excised, in-gel digested with trypsin, and extracted from the gel as described previously (31) with modifications. The excised protein bands were de-stained and washed with 50% acetonitrile in 50 mM aqueous NH_4HCO_3 . Proteins were then reduced by 10 mM dithiothreitol in 100 mM NH_4HCO_3 for 30 min. Cysteines in the protein peptides were further alkylated by 55 mM iodoacetamide in 100 mM NH_4HCO_3 for another 30 min. Trypsin (Promega Trypsin Gold, TPCK treated) in 40 mM NH_4HCO_3 was added to the gel pieces and the enzymatic reaction was carried out overnight at 37 °C. Afterwards, peptides were extracted twice by 1% trifluoroacetic acid in 60% acetonitrile for 30 min. Extracted solutions were collected, dried completely in a speed-vac and then re-dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid for analysis.

MALDI-TOF Analysis and Database Search

MALDI-TOF analyses were used to determine protein identities by peptide mass fingerprinting. Analysis was performed using an ABI 4700 MALDI-TOF mass spectrometer (Applied Biosystems, USA). Approximately a 0.5 ml mixture of the peptide sample and freshly prepared matrix solution (10 mg/ml of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% TFA aqueous solution) was spotted on a stainless steel sample plate. Each mass spectrum was the average of at least 100 laser shots and was calibrated by Data Explorer software (Applied Biosystems, USA). Peptide mass value searches were performed against the National Center for Biotechnology Information (NCBI) sequence (NCBI nr) database using MASCOT Peptide Mass Fingerprint database search software (www.matrixscience.com). The alkylation of cysteine was included as a possible modification. One missed tryptic cleavage was considered, and the mass tolerance for the monoisotopic peptide masses was set to ± 0.6 Da.

Metal and cofactor analysis

The concentration of ACIII complex was determined by the bicinchoninic acid protein assay (BCA assay) (Pierce). The number and type of metal atoms were determined using an AA600 atomic absorption spectrophotometer (PerkinElmer Life Sciences, Wellesley, MA).

The amount of heme was quantified by pyridine hemochrome analysis using the extinction coefficients of $23.97 \text{ mM}^{-1}\text{cm}^{-1}$ for *c*-type heme (32). The amount of acid labile sulfur was measured by the modified methylene blue method (33).

Heme determination by HPLC and Electrospray Ionization-Mass Spectrometry (ESI-MS)

The separation of heme-containing subunits in ACIII was performed on an Agilent 1100 Series HPLC. Isolated ACIII complex (10 mg/ml, 10 μ l) was applied at 1.0 ml/min to a C4 reverse-phase HPLC column. The mobile phase consisted of water with 0.1% TFA (solvent A) and 80% acetonitrile/20% water containing 0.09% TFA (solvent B). The following gradients were applied at a flow rate of 1.0 ml/min: linear gradient from 20% B to 60% B in 30 min, linear gradient from 60% B to 100% B in 50 min, 100% B for 10 min, linear gradient from 100% B to 20% B in 5 min, 95 min in total, and then an isocratic elution with 20% B for at least 30 min to re-equilibrate the column for the next injection. Elution of the protein subunits was monitored at 214 nm, 280 nm, 415 nm and 525 nm. The eluant fractions were collected based on the peak slope and threshold absorbance at 3-min intervals. The fractions containing targeted subunits were then concentrated to about 20 μ l by vacuum centrifugation at room temperature and injected on to a C18 guard column (1 mm $\times$ 15 mm, Optimize Technologies, Oregon City, OR). Proteins were then eluted by gradient and analyzed using a Thermo LTQ-FT mass spectrometer (Thermo Fisher, San Jose, CA).

Potentiometric titration

Anaerobic potentiometric titrations were performed to determine the midpoint potentials (E_m) of redox-active cytochromes in ACIII. The potential was controlled using a CH 620C potentiostat (CH Instruments) and spectral changes of ACIII upon reduction and oxidation were monitored at 555 nm by a Perkin Elmer Lambda 950 UV-Vis spectrophotometer. The titration was carried out at room temperature in the presence of a mixture of the following electron mediators: methyl viologen (E_m , γ = -475 mV), benzyl viologen (E_m , γ = -357 mV), anthraquinone-2-sulfonic acid (AQS) (E_m , γ = -255 mV), anthraquinone 2, 6 disulfonic acid disodium salt (AQDS) (E_m , γ = -184 mV), 2-hydroxy-1,4-naphthoquinone (E_m , γ = -145 mV), 2,5-dihydroxy-p-benzoquinone (E_m , γ = -60 mV), phenazine methosulfate (E_m , γ = +8 mV), phenazine ethosulfate (E_m , γ = +60 mV), Fe(III) EDTA (E_m , γ = +117 mV), 1,2-naphthoquinone-4-sulfonic acid (E_m , γ = +215 mV), 2,3,5,6-tetramethyl-p-phenylenediamine (DAD) (E_m , γ = +260 mV), N, N-dimethyl-1,4-phenylene diamine dihydrochloride (DMPD) (E_m , γ = +371 mV), potassium ferricyanide (E_m , γ = +435 mV). Very concentrated ACIII was diluted into 20 mM Tris buffer (pH 7.0) to a final concentration around 5 mg/ml, and each mediator was added to a final concentration of 50 μ M.

Low temperature and room temperature UV-Vis spectroscopy

UV-Vis spectra of reduced and oxidized ACIII were recorded, at both room temperature and liquid nitrogen temperature, using a Perkin Elmer Lambda 950 UV-Vis spectrophotometer and a cryostat (Optistat DN, Oxford Instruments).

Transmembrane topology analysis and transit peptide analysis

The subunits containing transmembrane helices and the transmembrane topology were predicted by hydropathy plots accessed at <http://www.expasy.ch/cgi-bin/protscale.pl>. The transit peptide analysis was conducted by CBS SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>).

Chemical cross-linking

Chemical cross-linking was utilized to probe the subunit spatial arrangement of the ACIII complex. The purified complex was cross-linked with EGS (ethylene glycol bis[succinimidylsuccinate]) (16.1 Å), DSP (dithiobis (succinimidylpropionate)) (12 Å), DSG (disuccinimidyl glutarate) (7.7 Å), NHS-ASA (*N*-hydroxysuccinimidyl-4-azidosalicylic acid) (5.7 Å), DFDNB (1,5-difluoro-2,4-dinitrobenzene) (3.0 Å) and sulfo-NHS (*N*-hydroxysulfosuccinimide) assisted EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) (0 Å) (all cross-linkers were obtained from Pierce, Rockford, IL) according to the instructions of the supplier with modifications of the reaction time or the concentration ratio between protein and cross-linker. Before performing cross-linking, the Tris-HCl buffer (pH 8.0) used for dissolving and stabilizing ACIII was changed into 20 mM HEPES buffer (pH 7.5) using a centrifugal filter unit (Millipore). Following incubation of the protein with various cross-linkers, the reaction was quenched for 20 minutes with 1M Tris-HCl pH 7.5 to give a final concentration of 100 mM. Subsequently, SDS-PAGE was applied to the cross-linked protein (non-reducing conditions for DSP cross-linked products) and a control ACIII sample without cross-linking. The bands of potentially cross-linked subunits were then cut out of the gel, in-gel digested with trypsin as described above and the resulting peptides were analyzed by using an ABI 4700 MALDI-TOF mass spectrometer (Applied Biosystems, USA) and a Thermo LTQOrbitrap (MS/MS) mass spectrometer (Thermo Fisher, San Jose, CA).

Results

Purification and protein chemistry of ACIII

The isolation and purification of *C. aurantiacus* ACIII was achieved by several steps of column chromatography as described in the Materials and Methods section. The procedure is based on a modification of the method described by Yanyushin *et al.* (22,25). The purified ACIII was subjected to urea SDS-PAGE, which confirmed that the protein is comprised of 7 different subunits, as shown in Figure 2A. The molecular masses of the subunits range from approximately 10 kDa to 110 kDa, consistent with the size as expected from the corresponding gene sequences of the completed *C. aurantiacus* genome. Blue native gel electrophoresis gives a single band (Figure 2B), which corresponds to a molecular mass of *ca.* 300 kDa by plotting the distance of migration versus log *M_r* of marker proteins. This value roughly coincides with the sum of the molecular weight of each subunit (*ca.* 294 kDa), suggesting that the ACIII complex is a monomer and contains at least one copy of each subunit, but it doesn't eliminate the possibility that the ACIII complex may possess more than one copy of certain smaller subunits. The second dimensional tricine SDS-PAGE of the excised lane from the blue native gel (Figure 2C) also confirmed that the ACIII complex was comprised of 7 different subunits. By heme-staining the SDS-PAGE, it was shown that two of the subunits contained *c*-type heme, having molecular masses of around 25 kDa and 23 kDa, respectively (Figure 2D).

Each subunit of the ACIII complex observed in SDS-PAGE was excised, digested with trypsin, and analyzed by peptide fingerprinting MALDI-TOF mass-spectrometry, as described in previous studies (25). The peptide fragment mass spectra were used for a search by the MASCOT (Matrix Science) software for protein identification, against the *C. aurantiacus* genome and the NCBI protein database. All the seven subunits were conclusively identified and the search results are summarized in Table 1. Both the MASCOT score and the percent sequence coverage indicate reliable correspondence with respect to each subunit sequence of ACIII.

UV-Vis spectral analysis

UV-Vis absorption spectroscopy at room temperature for both reduced and oxidized ACIII complex were carried out, and the results are shown in Figure 3A, 3B. The oxidized complex showed amino acid absorption at 280 nm and a *c*-type cytochrome γ (Soret) band at 411 nm. Addition of dithionite resulted in reduction of the heme, and gave rise to two absorption maxima at 525 nm (β band) and 555 nm (α band) and a red shift of the γ band to 418 nm. The low temperature absorption spectra revealed more details regarding individual hemes and their different redox potentials (Figure 3D-F). When the hemes of ACIII were in their oxidized state, their UV absorbances at both room temperature and reduced temperature showed no discernible difference in the heme wavelengths (418, 525 and 555 nm). However, in their reduced state, the environmentally-different hemes could be partially resolved at low temperatures, indicating that ACIII is a multi-heme-containing protein complex. Moreover, ascorbic acid ($E^0=58$ mV) was unable to fully reduce the ACIII hemes, suggesting that some of the hemes in ACIII have mid-point potentials lower than that of ascorbic acid.

Metal and cofactor analysis

Determining the numbers and types of metal centers and cofactors is an essential aspect of the characterization of ACIII. Graphite furnace atomic absorption spectroscopy (AA) revealed *ca.* 17 (rounded from 16.9 ± 0.3) iron in one single complex and undetectable amounts of Mo and Mn. AA spectroscopy, however, does not distinguish iron in various forms, such as heme and non-heme iron. To identify the types of iron, pyridine hemochrome difference spectra (Figure 3C) were used to calculate the relative stoichiometry for *c*-type hemes, the only type of heme in ACIII (22), which was 7 (rounded from 6.8 ± 0.1) in each complex. The remaining 10 out of 17 irons were thought to be in the form of iron-sulfur clusters. To confirm this assignment, the amount of acid-labile sulfur was measured using a modified methylene blue method. It was found that there were *ca.* 10 (rounded from 10.3 ± 0.1) acid-labile sulfur atoms per ACIII complex, which gave an iron to sulfur ratio of 1:1. This value is consistent with analysis of the gene sequences of the seven ACIII subunits. By comparing the arrangement of cysteines in all ACIII subunits with a number of known [2Fe2S] and [4Fe4S] cluster-binding motifs (34), it was found that the largest subunit (s1) contained 3 apparent iron-sulfur binding motifs, while all other subunits did not contain such binding sites. Therefore, it is estimated that in the ACIII complex, only one subunit contains iron-sulfur clusters, which are suggested to be in the form of one [2Fe2S] cluster and two [4Fe4S] clusters. Nevertheless, the presence of [3Fe4S] clusters, as observed for the ACIII complex from *R. marinus* (23) cannot be excluded. Future work will characterize the FeS clusters in subunit s1 using EPR spectroscopy.

Redox potentiometry

The midpoint redox potentials of the heme cofactors of ACIII were determined using an optically transparent thin film electrode along with a potentiostat and the data were analyzed by a method previously described (35). Although ACIII has more than one type of redox unit (e.g., heme and iron-sulfur cluster) functioning as electron transfer centers, in this method the *c*-type hemes were selectively assayed spectrally as a function of potential. From the titration plot (Figure 4), four distinct E_m values were obtained, which were -228 mV, -110 mV, +94 mV and +391 mV vs. NHE, respectively, with intensity ratio 3:1:1:2. Assuming that the extinction coefficients are similar for all the hemes, this indicates that about 7 hemes are present in the ACIII complex, and that three hemes are of the lowest redox potential, two hemes are of the highest redox potential, while the other two have distinct and intermediate redox potentials. This result is consistent with the finding of seven *c*-type hemes per ACIII from the pyridine hemochrome analysis. The redox titration plot

also showed a reversible Nernstian behavior corresponding to one-electron oxidation/reduction reactions.

Heme quantification by HPLC and ESI-MS

A novel approach was introduced to quantify heme in ACIII. By monitoring UV-Vis absorption at 415 nm, which is characteristic of heme-containing species, two fractions were collected at 34.3 min and 35.3 min from the HPLC (Figure 5A). SDS-PAGE with silver staining indicated that the two fractions corresponded with the two heme-containing subunits (s4, s5) of ACIII (Figure 5B). Peak integration ratio of the two subunits measured at 415 nm was 2:5, giving a total of 7 hemes, again consistent with the number of hemes determined by pyridine hemochrome analysis and potentiometric titration. The accurate molecular masses of both of the subunits containing covalently-bound heme were determined by ESI mass spectrometry to be 20,168.0 (s5) and 28,224.0 (s4), respectively (Figure 5C, 5D). By comparing the measured molecular mass against the calculated molecular mass of the peptide based on the gene sequence (s5: 19,496.7; s4: 25,222.0), which does not include the molecular weight of any cofactors, the number of hemes for each subunit was calculated by Equation 1.

$$\text{Number of hemes} = (\text{MW}_{\text{MS}} - \text{MW}_{\text{calc.}}) / \text{MW}_{\text{heme-c}} \quad \text{Eq. 1}$$

From the calculation, the first fraction (34.3 min), which corresponds to the smaller heme-containing subunit (s5), contained one heme (calc. 1.09, considering the subtraction of the predicted signal peptide). The second fraction (35.3 min) possessed five hemes (calc. 4.86), corresponding to the larger multi-heme-containing subunit (s4) (Figure 5A).

This result is consistent with the genome analysis, which showed only one *c*-type heme binding motif (CXXCH) in s5 and 5 *c*-type heme binding sites in s4. Because the s5 subunit contains only 1 heme, the 2:5 ratio of the hemes of the two subunits as determined by HPLC strongly suggests that there are two copies of s5 existing in ACIII complex to match the stoichiometry of the hemes as determined by metal analysis, potentiometric titration and pyridine hemochrome analysis.

Heme quantification by heme staining and intensity analysis

The intensities of the stained bands from the two heme-containing subunits (s4 and s5, Figure 6A) of the ACIII complex were quantified using ImageJ, which were then compared against a serially-diluted horse heart cytochrome *c* parallel control. The intensity of the two bands from the ACIII complex gave close to a 2:5 ratio (2.00:4.92), indicating that the heme number in the two types of subunits are 2 and 5 respectively, assuming that the three proteins stain with equal efficiency. From Figure 6B, a calibration curve of intensity against concentration was created from the stained bands of horse heart cytochrome *c*, to examine the concentrations of the two heme-containing subunits of ACIII. The concentration of s4 determined by the calibration curve ($29.23/5 = 5.85 \mu\text{M}$) roughly matches the concentration of ACIII complex determined by the BCA assay ($6.12 \mu\text{M}$), while the concentration of s5 from the calibration curve ($11.88 \mu\text{M}$) is nearly twice of the concentration of ACIII complex ($6.12 \mu\text{M}$). These results again point toward the conclusion that ACIII contains two copies of s5 and one copy of s4.

Chemical cross-linking analysis

Intra-complex cross-linking of the purified ACIII complex was conducted with a series of bi-functional cross-linkers of various lengths (from 0 Å to 16 Å), including EDC/sulfo-NHS, DFDNB, NHS-ASA, DSG, DSP and EGS. These cross-linkers are amine-reactive or can

form amine-reactive intermediates, and are able to react with the ϵ -amino group of lysine or the protein N-terminus. With the exception of EDC/NHS and NHS-ASA, all other cross-linkers are homo-bifunctional, effecting amine group couplings. EDC/NHS is a hetero-bifunctional, zero-length crosslinker, linking carboxylic acids and amines. NSHASA contains two functional ends, an amine-reactive NHS ester and a photoreactive azidosalicylic acid for which the binding site is nonspecific. Intermolecular reactions were not considered as competing reactions due to the relatively low concentration of protein sample. Figure 7 displays the cross-linked products detected by SDS-PAGE with coomassie blue-staining using different cross-linkers. The cross-linking treatment gave rise to several new bands compared with the protein control. These new bands were then identified by MALDI-TOF and LC-MS/MS following trypsin digestion. The results are listed in Table 2. Cross-linking with DSP and EDC both produced the same pattern of 120 kDa products, which were then identified as cross-linked s1 and s7 (Figure 7A, 7B). EDC cross-linking also gave rise to two low-molecular weight products of *ca.* 65 kDa and 45 kDa, and one high-molecular weight product of around 150 kDa (Figure 7B). The two low molecular weight products were determined to be the complex of s5 and s3 and the dimer of two s5's. The high molecular weight species was the trimer of s1, s7 and s4. The finding of the homo-dimer of s5 is a convincing support that there are 2 copies of the mono-heme subunit existing in ACIII. Incubation of ACIII with EGS, the longest cross-linker, produced the most complex cross-linked product, a tetramer of around 160 kDa formed by s1, s7, s4 and s5 (Figure 7C). DFDNB cross-linking gave another high-molecular weight pattern of approximately 130 kDa by linking with s1 and s6 (Figure 7D). A low-molecular weight product of around 30 kDa was observed when DSG was used, which was identified to be the dimer of s7 and s4 (Figure 7E). Cross-linking with the hetero-bifunctional cross-linker NHS-ASA gave two low-molecular weight bands, at *ca.* 70 kDa and 50 kDa, recognized as the dimer of s2 and s4 and the dimer of s3 and s6, respectively (Figure 7F). Figure 7G shows the identification using mass spectrometry of the DSP cross-linked product as a typical example. All other spectra and identification results are included in the supplementary section.

Discussion

Instead of a cytochrome *bc*₁ or cytochrome *b₆f* complex, the phototrophic bacterium *C. aurantiacus* contains a newly-discovered multisubunit integral membrane protein complex called alternative complex III. ACIII is thought to be the menaquinone: auracyanin oxidoreductase, playing a critical role in the electron transfer chain (25,26). This entirely new bioenergetic system not only provides us with an opportunity to understand *C. aurantiacus*, the earliest branching group of bacteria that have the ability to perform photosynthesis (36), but also gives us a tool to investigate different mechanisms and evolutionary pathways involved in the electron transfer chain in various species. To better understand the intriguing role of ACIII in *C. aurantiacus*, two main problems need to be solved: the structure of the complex and the mechanism of its function in photosynthesis and respiration. We have previously investigated the function of ACIII by studying its enzymatic activities as the menaquinol:auracyanin oxidoreductase (26). Herein, we provide the detailed subunit and cofactor structure of the alternative complex III, which was revealed by use of various biochemical methods.

The number and size of the subunits in ACIII were determined utilizing urea SDS-PAGE, blue native gel electrophoresis in combination with 2nd dimensional SDS-PAGE. These experiments consistently showed 7 different subunits of a wide range of molecular weights, from *ca.* 10 kDa to 100 kDa, suggesting a much more complicated structure of ACIII than the cytochrome *bc*₁ or *b₆f* complex in other bacterial species, which usually contain 3-4 subunits (20,37).

Identifying the cofactors of an unknown protein is an essential aspect in protein structural and functional studies. It therefore has been one of our primary focuses in the investigation of the ACIII complex. Among the 7 subunits, s1 is the only subunit that possesses specific binding motifs for iron-sulfur clusters, while s4 and s5 contain *c*-type heme-binding motifs (CXXCH), possessing five and one heme-binding sites, respectively. As such, the cofactors existing in the ACIII complex should comprise both heme groups and Fe-S clusters. This hypothesis was unequivocally confirmed by atomic absorption spectroscopy, pyridine hemochrome analysis and an acid labile sulfur assay. In addition, from low temperature UV-Vis absorption measurement, it was found that the ACIII complex contained more than one copy of *c*-type hemes. During the investigation of the number of *c*-type heme in the ACIII complex, an apparent conflict emerged between the number of hemes determined by potentiometric titration experiments and by binding-motif searching in the ACIII gene. One explanation is that there are 2 copies of s5 in the ACIII complex to account for the extra heme found by potentiometric titration. This hypothesis was tested by two different experiments, one being a novel analytical method developed in our lab (HPLC in tandem with ESI MS), and the other being the intensity analysis of heme-staining SDS-PAGE. The results of these two studies agreed very well, independently showing that there are two copies of s5 in the ACIII complex.

The ACIII complex in *C. aurantiacus* has many structural analogs in non-photosynthetic species regarding subunit and cofactor composition at both the biochemical and genomic levels. For example, in *Rhodothermus marinus*, an aerobic non-phototrophic Gram-negative bacterium, an atypical complex III, which is believed to be the functional equivalent of quinol: HiPIP/cytochrome *c* oxidoreductase, has been isolated and characterized (23,24,38,39). However, none of these analogs was found to possess more than one copy of their respective mono-heme containing subunit. This unique feature of ACIII in *C. aurantiacus* may suggest a specific functional use of this extra heme-containing subunit for electron transfer in the photosynthetic electron transfer chain compared with oxidative electron transfer pathways.

In addition to identifying the cofactors, we were also interested in understanding the spatial arrangements of the various subunits of ACIII, which may provide useful information on interpreting and understanding the connection between structure and function. To investigate the subunit topology and establish the subunit architecture of ACIII, a chemical cross-linking approach was employed. Using a series of homo- and hetero-bifunctional chemical cross-linkers of various spacer arm length, we obtained insights into the subunit arrangement of the ACIII complex. The largest subunit (s1) was found to be in close proximity of s7, s6 and s4. Moreover, it showed no transmembrane helices (Table 3) in transmembrane topology analysis. Also lacking any transit peptide sequences, this subunit is most likely located on the cytoplasmic side of the membrane, attached to other transmembrane subunits. S7 is the only other cytoplasmic-side membrane-attached subunit of ACIII, lacking transmembrane helices and signal peptide sequences (Table 3). As a result, the cross-linking yield between s1 and s7 is significant as the specific cross-linked band (*ca.* 120 kDa) can be observed by using several different cross-linkers. Besides being adjacent to s1, s7 was also found to be close to s4, as evidenced by cross-linking with DSG. The second and third largest subunits (s2, s3), which contain 10 and 9 transmembrane helices, respectively (Table 3), are the two most hydrophobic subunits, as determined from gene sequence analysis. In addition, among the seven subunits, s2 is the only subunit that was found to possess a possible quinone-binding motif, which was previously summarized by Fisher *et al* (40). Therefore, it is hypothesized that s2 is a transmembrane subunit and contains the quinone binding site. Using the chemical cross-linking approach, s2 was found to be in direct contact with s4, the multi-heme containing subunit, indicating that the electrons of quinol may be delivered to the heme cofactors of s4 from the quinone-binding subunit, s2. The other highly

hydrophobic transmembrane subunit, s3, is adjacent to s5 and s6, as detected by cross-linking with EDC and NHS-ASA, respectively. The fourth and fifth largest subunits (s4, s5), which are found to be heme-containing subunits, are shown by genomic analysis to contain one transmembrane helix each, suggesting that they could be partially transmembrane. However, for s5, it was found that the first 20 to 30 amino acids starting from the N-terminus serves both as transmembrane helix and transit peptide sequence, the latter of which is usually cleaved from the protein after the protein is transported to the membrane. This hypothesis was confirmed by the ESI-MS of s5 separated from the intact ACIII by HPLC, which shows that the mass of the s5 including one heme is 20,168.0 Da, 3,444 Da less than the value predicted from gene sequencing (23612.0 Da). This difference in mass matches that of a transit peptide sequence. These results suggest that the two copies of s5 are transported to the outside of the membrane and attached to the periplasmic side of the membrane. This scenario was supported by the chemical cross-linking with EDC and examination with MALDI-TOF, showing a cross-linked product around twice of the molecular weight of s5 (dimeric s5). In addition, no chemical cross-linking of s5 with cytoplasm-attached subunits (s1, s7) was found by any cross-linker used, suggesting s5 is well separated from s1 and s7, agreeing with the proposed model that they are positioned on opposite sides of the membrane. Based on all the above information, a preliminary structural model of ACIII is proposed, shown in Figure 8.

In conclusion, in this work, as part of the effort to explore the fundamental structural characteristics of the ACIII complex in *C. aurantiacus*, the number of hemes compared to the number of heme-binding motifs, was confirmed by various biochemical methods. A structural model of ACIII complex interpreting the subunit-subunit interactions was proposed by utilizing chemical cross-linking and mass spectrometry. The similarity between the two ACIII complexes from *C. aurantiacus* and *R. marinus* in regard to structure and function strongly suggests that the ACIII from *C. aurantiacus* is another member of the alternative complex III family performing the same function as the *bc₁* complex but not belonging to its family (38).

Many intriguing questions remain to be answered to complete the entire picture of the ACIII complex in the electron transfer pathway. Ongoing work includes establishing the manner in which the cofactors of the ACIII complex are tuned to transfer electrons and determining if the ACIII complex pumps protons through the cytoplasmic membrane as is done by the cytochrome *bc₁* complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Grant #MCB-0646621 to R.E.B. from the Molecular Biochemistry Program of NSF. The mass spectrometry research was supported by the National Center for Research Resources (NCRR) of the National Institutes of Health (Grant #2P41RR00954).

Abbreviations

ACIII	alternative complex III
<i>C. aurantiacus</i>	<i>Cloroflexus aurantiacus</i>
<i>R. marinu</i>	<i>Rhodothermus marinu</i>
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

MALDI-TOF MS	matrix-assisted laser desorption/ionization-time of flight mass spectrometry
HPLC	high performance liquid chromatography
ESI-MS	electrospray ionization mass spectrometry
DDM	dodecyl maltoside
EGS	ethylene glycol bis[succinimidylsuccinate]
DSP	dithiobis (succinimidylpropionate)
DSG	disuccinimidyl glutarate
NHS-ASA	<i>N</i> -hydroxysuccinimidyl-4-azidosalicylic acid
DFDNB	1,5-difluoro-2,4-dinitrobenzene
EDC	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

References

- Blankenship, RE. Molecular Mechanisms of Photosynthesis. Blackwell; Oxford: 2002.
- Anraku Y. Bacterial electron transport chains Ann. Rev. of Biochem. 1988; 57:101–132.
- Trumpower BL. Cytochrome *bc*₁ complexes of microorganisms. Microbiol. Rev. 1990; 54:101–129. [PubMed: 2163487]
- Woese CR, Kandler O, Wheelis ML. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. USA. 1990; 87:4576–4579. [PubMed: 2112744]
- Blankenship, RE. Molecular Mechanisms of Photosynthesis. Blackwell Science; Oxford UK: 2002.
- Pierson, BK.; Castenholz, RW. Taxonomy and physiology of filamentous anoxygenic phototrophs. In: Blankenship, RE.; Madigan, MT.; Bauer, CE., editors. Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers; New York, Boston, Dordrecht, London, Moscow: 1995. p. 31-47.
- Pierson BK, Castenholz RW. A phototrophic gliding filamentous bacterium of hot springs, *Chloroflexus aurantiacus*, gen. and sp. nov. Arch. Microbiol. 1974; 100:5–24. [PubMed: 4374148]
- Keppen OI, Baulina OI, Lysenko AM, Kondratieva EN. New green bacterium belonging to family Chloroflexaceae. Mikrobiologiya (Eng transl). 1993; 62:179–185.
- Gorlenko, VM. Ecological niches of green sulfur and gliding bacteria. In: Olson, JM.; Ormerod, JG.; Ames, J.; Stackebrandt, E.; Trüper, HG., editors. Green Photosynthetic Bacteria. Plenum Press; New York: 1988. p. 257-267.
- Pierson BK, Giovannoni SJ, Stahl DA, Castenholz RW. *Heliothrix oregonensis*, gen. nov., sp. nov., a phototrophic filamentous gliding bacterium containing bacteriochlorophyll *a*. Arch Microbiol. 1985; 142:164–167. [PubMed: 2412519]
- Pierson BK, Valdez D, Larsen M, Morgan E, Mack EE. *Chloroflexus*-like organisms from marine and hypersaline environments: distribution and diversity. Photosynth. Res. 1994; 41:35–52.
- Bruce BD, Fuller RC, Blankenship RE. Primary photochemistry in the facultatively aerobic green photosynthetic bacterium *Chloroflexus aurantiacus*. Proc. Natl. Acad. Sci. USA. 1982; 79:6532–6536. [PubMed: 16593246]
- Pierson BK, Thornber JP. Isolation and spectral characterization of photochemical reaction centers from the thermophilic green bacterium *Chloroflexus aurantiacus* strain J-10-fi. Proc. Natl. Acad. Sci. U.S.A. 1983; 80:80–84. [PubMed: 16593269]
- Blankenship, RE.; Matsuura, K. Antenna complexes from green photosynthetic bacteria. In: Green, BR.; Parson, WW., editors. Light-Harvesting Antenna. Kluwer; Dordrecht: 2003. p. 195-217.
- Xiong J, Fischer WM, Inoue K, Nakahara M, Bauer CE. Molecular evidence for the early evolution of photosynthesis. Science. 2000; 289:1724–1730. [PubMed: 10976061]

16. Raymond J, Zhaxybayeva O, Gogarten JP, Gerdes SY, Blankenship RE. Whole-genome analysis of photosynthetic prokaryotes. *Science*. 2002; 298:1616–1620. [PubMed: 12446909]
17. Holo H, Sirevåg H. Autotrophic growth and CO₂ fixation of *Chloroflexus aurantiacus*. *Arch Microbiol*. 1986; 148:292–297.
18. Herter S, Fuchs G, Bacher A, Eisenreich W. A bicyclic autotrophic CO₂ fixation pathway in *Chloroflexus aurantiacus*. *J. Biol. Chem*. 2002; 277:20227–20283.
19. Blankenship RE. Origin and early evolution of photosynthesis. *Photosynth. Res*. 1992; 33:91–111. [PubMed: 11538390]
20. Berry EA, Guergova-Kuras M, Huang L-S, Crofts AR. Structure and function of cytochrome *bc* complexes. *Annu. Rev. of Biochem*. 2000; 69:1005–1075. [PubMed: 10966481]
21. Smith JL, Zhang H, Yan J, Kurisu G, Cramer WA. Cytochrome *bc* complexes: A common core of structure and function surrounded by diversity in the outlying provinces. *Curr. Opin. Struc. Biol*. 2004; 14:432–439.
22. Yanyushin MF. Fractionation of cytochromes of phototrophically grown *Chloroflexus aurantiacus*. Is there a cytochrome *bc* complex among them? *FEBS Lett*. 2002; 512:125–128. [PubMed: 11852065]
23. Pereira MM, Carita JN, Teixeira M. Membrane-bound electron transfer chain of the thermohalophilic bacterium *Rhodothermus marinus*: a novel multihemic cytochrome *bc*, a new complex III. *Biochemistry*. 1999; 38:1268–1275. [PubMed: 9930987]
24. Pereira MM, Refojo PN, Hreggvidsson GO, Hjorleifsdottir S, Teixeira M. The alternative complex III from *Rhodothermus marinus* – A prototype of a new family of quinol:electron acceptor oxidoreductases. *FEBS Lett*. 2007; 581:4831–4835. [PubMed: 17888426]
25. Yanyushin MF, del Rosario MC, Brune DC, Blankenship RE. New class of bacterial membrane oxidoreductases. *Biochemistry*. 2005; 44:10037–10045. [PubMed: 16042380]
26. Gao X, Xin Y, Blankenship RE. Enzymatic activity of the alternative complex III as a menaquinol:auracyanin oxidoreductase in the electron transfer chain of *Chloroflexus aurantiacus*. *FEBS Lett*. 2009; 583:3275–3279. [PubMed: 19755122]
27. Xin Y, Lu Y-K, Fromme P, Blankenship RE. Purification, characterization and crystallization of menaquinol:fumarate oxidoreductase from the green filamentous photosynthetic bacterium *Chloroflexus aurantiacus*. *Biochim. Biophys. Acta*. 2009; 1787:86–96. [PubMed: 19103151]
28. Schägger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. *Anal. Biochem*. 1987; 166:368–379. [PubMed: 2449095]
29. Schägger H, von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem*. 1991; 199:223–231. [PubMed: 1812789]
30. Goodhew CF, Brown KR, Pettigrew GW. Haem staining in gels, a useful tool in the study of bacterial c-type cytochromes. *Biochim. Biophys. Acta*. 1984; 852:288–294.
31. Henzel WJ, Billeci TM, Stults JT, Wong SC, Grimley C, Watanabe C. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. *Proc. Natl. Acad. Sci. U.S.A.* 1993; 90:5011–5015. [PubMed: 8506346]
32. Berry EA, Trumpower BL. Simultaneous determination of hemes *a*, *b*, and *c* from pyridine hemochrome spectra. *Anal. Biochem*. 1987; 161:1–15. [PubMed: 3578775]
33. Brumby PE, Miller RW, Massey V. The content and possible catalytic significance of labile sulfide in some of metalloflavoproteins. *J. Biol. Chem*. 1965; 240:2222–2228. [PubMed: 14299651]
34. Johnson, MK. *Encyclopedia of Inorganic Chemistry*. Wiley; New York: 1994.
35. Bell PD, Xin Y, Blankenship RE. Purification and characterization of cytochrome *c₆* from *Acaryochloris marina*. *Photosynth. Res*. 2009; 98:131–140.
36. Woese CR. Bacterial evolution. *Microbiol. Rev*. 1987; 51:221–271. [PubMed: 2439888]
37. Baniulis D, Yamashita E, Zhang H, Hasan SS, Cramer WA. Structure-function of the cytochrome *b₆f* complex. *Photochem. Photobiol*. 2008; 84:1349–1358. [PubMed: 19067956]
38. Refojo PN, Sousa FL, Teixeira M, Pereira MM. The alternative complex III: A different architecture using known building modules. *Biochim. Biophys. Acta*. 2010 (In press) DOI: 10.1016/j.bbabi.2010.04.012.

39. Refojo PN, Teixeira M, Pereira MM. The alternative complex III of *Rhodothermus marinus* and its structural and functional association with *caa3* oxygen reductase Biochim. Biophys. Acta. 2010; 1797:1477–1482.
40. Fisher N, Rich PR. A motif for quinone binding sites in respiratory and photosynthetic systems. J. Mol. Biol. 2000; 294:1153–1162. [PubMed: 10686111]

Figure 1.
The proposed photosynthetic cyclic electron transfer pathway in *Chloroflexus aurantiacus*.

Figure 2.

Electrophoresis analysis of isolated ACIII complex from *C. aurantiacus*. The graphic shown at the bottom represents the gene encoding for ACIII (DOE Joint Genome Institute) database. (A) Urea SDS-PAGE of purified ACIII complex using a 12.5%T, 3%C and 8M urea gel system. Right lane is molecular weight standards (Bio-Rad), and the molecular masses of the standard proteins from the bottom to the top are 10 kDa, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 200 kDa. (B) Blue native PAGE of ACIII complex. Molecular mass standards on the right lane include BSA (66 kDa for a monomer and 132 kDa for a dimer) and ferritin (440 kDa for a monomer and 880 kDa for a dimer). (C) The second dimensional tricine SDS-PAGE of the excised lane from the blue native gel. (D) Heme-stained tricine SDS-PAGE of the purified ACIII complex. Right lane is molecular weight standards (Bio-Rad), and the molecular masses of the standard proteins from the bottom to the top are 10 kDa, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa and 75 kDa.

Figure 3.

(A) UV-vis absorption spectrum of the ACIII complex purified from *C. aurantiacus* at room temperature. (B) Spectra of the air-oxidized (dashed lines) and dithionite-reduced forms (solid lines) from 350 nm to 650 nm. The insert shows the reduced-minus-oxidized difference spectrum. (C) Spectra of pyridine hemichromes *c* (dashed lines) and pyridine hemochrome *c* (solid lines) at room temperature. (D) Spectra of air-oxidized ACIII complex at room temperature (dashed lines) and 77 K (solid lines). (E) Spectra of dithionite-reduced ACIII complex at room temperature (dashed lines) and 77 K (solid lines). (F) Spectra of ascorbic acid-reduced ACIII complex at room temperature (dashed lines) and 77 K (solid lines).

Figure 4. Potentiometric titration of ACIII complex purified from *C. aurantiacus* with squares representing the oxidative titration, black circles representing the reductive titration and solid line representing the Nernst fitting.

Figure 5.

(A) Separation of the two heme-containing subunits, s4 (blue) and s5 (red), by reverse phase HPLC on a Vydac C4 column. The UV-Vis absorptions of s4 and s5 are shown in the inset. (B) Analysis of the two HPLC elution peaks containing s4 (left lane) and s5 (middle lane) by silver stained SDS-PAGE. (C) ESI mass spectrum of the ACIII s5 subunit isolated by RP-HPLC. (D) ESI mass spectrum of the ACIII s4 subunit isolated by RP-HPLC.

Figure 6.

(A) Heme-stained SDS-PAGE of a series of horse heart cytochrome *c* of gradually increasing concentrations and purified ACIII complex (right lane). Left lane is molecular weight standards (Bio-Rad), and the molecular masses of the standard proteins from the bottom to the top are 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 200 kDa. (B) The calibration curve of gel band intensity against concentration of cytochrome *c* (black squares) generated from (A). The blue circle represents the intensity of s4 and the red triangle represents the intensity of s5.

Figure 7.

Chemical cross-linking of ACIII subunits and identification of subunits interaction. (A) Cross-linking of s1 and s7 (120 kDa) with DSP. Left lane is cross-linked ACIII, middle lane is ACIII control without cross-linker and right lane is protein standard marker (from the bottom to the top, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 200 kDa). (B) Cross-linking of s1, s4 and s7 (150 kDa), s1 and s7 (120 kDa), s3 and s5 (65 kDa), two s5 (40 kDa) with sulfo-NHS assisted EDC. Right lane is cross-linked ACIII, middle lane is ACIII control without cross-linker and left lane is protein standard marker (from the bottom to the top, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 200 kDa). (C) Cross-linking of s1, s4, s5 and s7 (160 kDa) with EGS. Middle lane is cross-linked ACIII, left lane is ACIII control without cross-linker and right lane is protein standard marker (from the bottom to the top, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 200 kDa). (D) Cross-linking of s1 and s6 (125 kDa) with DFDNB. Middle lane is cross-linked ACIII, right lane is ACIII control without cross-linker and left lane is protein standard marker (from the bottom to the top, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 200 kDa). (E) Cross-linking of s4 and s7 (30 kDa) with DSG. Middle lane is cross-linked ACIII, left lane is ACIII control without cross-linker and right lane is protein standard marker (from the bottom to the top, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 200 kDa). (F) Cross-linking of s2 and s4 (70 kDa) and s3 and s6 (50 kDa) with NHS-ASA. Middle lane is cross-linked ACIII, right lane is ACIII control without cross-linker and left lane is protein standard marker (from the bottom to the top, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 200 kDa). (G) MALDI-TOF mass spectrum of cross-linked s1 and s7 using DSP, the peaks with red arrows are the characteristic peptides of s1 after trypsin digestion and the peaks with blue arrows are the characteristic peptides of s7 after trypsin digestion.

Figure 8. Proposed structural model of the ACIII complex subunit architecture from *C. aurantiacus* as revealed by structural and functional studies and gene sequence analysis.

Table 1

The result of peptide mass fingerprinting for the identification of the ACIII complex in *C. aurantiacus* genome.

ACIII Subunit	M _r ^a (kDa)	Fragments identified ^b	MASCOT score ^b	Percent sequence coverage ^b
s1	113.0	49	124	53
s2	55.2	10	76	20
s3	45.7	7	79	25
s4	25.2	8	52	61
s5	23.0	8	61	49
s6	19.6	8	32	48
s7	12.5	9	52	58

^a Provided by National Center for Biotechnology Information (NCBI) database

^b Provided by MASCOT Peptide Mass Fingerprint database search software (www.matrixscience.com)

Table 2

Identified cross-linked products and their constituent polypeptides. The method is described under the Materials and Methods section.

Cross-linker	Apparent M_r of cross-linking (kDa)	Subunits identified
DSP	120	s1+s7
EDC	150	s1+s4+s7
	120	s1+s7
	65	s3+s5
	45	s5+s5
	130	s1+s6
DFDNB	130	s1+s6
DSG	30	s4+s7
EGS	160	s1+s4+s5+s7
NHS-ASA	70	s2+s4
	50	s3+s6

Table 3

Transmembrane helix topology analysis and transit peptides analysis of the subunits of the ACIII complex from *C. aurantiacus*. The method is described under the Materials and Methods section.

	s1	s2	s3	s4	s5	s6	s7
TM ^a	0	10	9	1	1	2	0
TP ^b	N	N	N	N	Y	N	N

^aTM: transmembrane helix

^bTP: transit peptide