Expression and One-Step Purification of a Fully Active Poly-Histidine Tagged Cytochrome $b_{c_1}$-Complex from *Rhodobacter sphaeroides*.


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Running Head: Purification of Histidine Tagged $b_{c_1}$-Complex

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**ABSTRACT.** The $fbcB$ and $fbcC$ genes encoding cytochromes $b$ and $c_1$ of the $bc_1$-complex, were extended with a segment to encode a poly-histidine tag linked to their C-terminal sequence allowing a one-step affinity purification of the complex. Constructions were made *in vitro* in a pUC-derived background using PCR amplification. The modified $fbc$ operons were transferred to a pRK-derivative plasmid, and this was used to transform the $fbc^-$ strain of *Rhodobacter sphaeroides*, BC17. The transformants showed normal rates of growth. Chromatophores prepared from these cells showed kinetics of turnover of the $bc_1$-complex on flash activation which were essentially the same as those from wild-type strains, and analysis of the cytochrome complement, spectral and thermodynamic properties by redox potentiometry showed no marked difference from the wild-type. Chromatophores were solubilized and mixed with Ni-NTA Sepharose resin. A modification of the standard elution protocol in which histidine replaced imidazole, increased the activity 20-fold. Imidazole modified the redox properties of heme $c_1$, suggesting ligand displacement and inactivation when this reagent is used at high concentration. The purified enzyme contained all four subunits in an active dimeric complex. This construction provides a facile method for preparation of wild type or mutant $bc_1$-complex, for spectroscopy and structural studies.
The ubiquinol:cytochrome $c_2$ oxidoreductase ($bc_1$-complex) of *Rb. sphaeroides* is the central enzyme of the photosynthetic and respiratory electron transfer chains of this bacterium, and performs the same catalytic function as the equivalent enzyme in the mitochondrial respiratory chain. Kinetic studies of the function are more tractable in the photosynthetic bacteria because of the ease with which the reactions can be initiated by flash illumination. A considerable body of work on characterization of modified function in mutant strains and interpretation in terms of mechanism and structure has been reported (see Brasseur et al. (1), for a recent summary). With the recently reported progress in structural characterization of the $bc_1$-complex from beef and chicken heart mitochondria (2-4), the field is poised for dramatic advances in our understanding of the interface between structure and function.

Protocols for isolation and purification of an active complex have been established, but these have been relatively time consuming, and this has discouraged extensive work on crystallization, detailed spectroscopy and characterization of mutant strains, where these have required substantial concentrations of the complex. In the past few years, simple and rapid protocols using an affinity tag, usually a six histidine repeat on one subunit, have been reported as a means to isolate membrane protein complexes. Several respiratory or photosynthetic enzymes such as cytochrome oxidases (5-7), ATP-synthases (8,9), and photosynthetic reaction centers (10,11) have been successfully isolated from a broad range of organisms using this technique. In this paper we report the construction and expression of two versions of the *Rb. sphaeroides* $bc_1$-complex in which histidine extensions (his-tag) have been added to the C-terminal ends of cyt $b$ and cyt $c_1$ subunits, respectively. This has allowed the development of protocols for one-step purification of the complex in active form. The presence of the his-tag appears to have no
significant effect on the kinetics of turnover of the complex in the photosynthetic chain, nor on the thermodynamic and spectrophotometric characteristics of the complex in situ or after isolation. However, isolation of an active complex required modification of the protocol for elution of the his-tagged complex, in which histidine was used instead of imidazole.

MATERIALS AND METHODS

Bacteria and Plasmids. The Escherichia coli strains were grown at 37°C in LB broth. Plasmids pUC9 (GenBank accession number L09128), pT7T319U (GenBank accession number U13870) and their derivatives were maintained in the presence of 100 µg/mL ampicillin, pRK415 (12) and its derivatives with 10 µg/mL tetracycline. Rb. sphaeroides strains were grown in Sistrom medium. This medium was supplied with 10 µg/mL kanamycin when growing the recipient strain BC17 (13). For all of the derivatives, 2 µg/mL tetracycline was also added in order to maintain the mutant plasmids. Cells were grown either under anaerobic photosynthetic conditions or aerobically. When large volumes of cell culture were needed, Rb. sphaeroides cells were grown in a 20 liter fermentation tank equipped with lights. The temperature was kept at 29°C and nitrogen gas was used to maintain anaerobiosis in the fermentor.

Strategy for the insertion of histidine tag. Prior to the introduction of the his-tag at the C-terminus of either cyt b or cyt c1, the 1.9 kb Xcm I- EcoR I DNA fragment of plasmid pBC9 (13) located after the putative transcription termination site of the fbc operon was deleted and replaced by the ECO linker (see Table 1). The resulting plasmid, which contains the whole fbc operon without ancillary sequences, was called pGB1 (Fig. 1). Subsequently, the 2.4 kb Hind III-Bgl II fragment of pGB1 was removed and replaced by the HIND linker (Table 1). The Hind III-EcoR I
fragment was then subcloned into the pT7T319U cloning vector yielding the pGB2T plasmid. This plasmid contains the end of the \textit{fbcB} coding frame and the complete sequence of \textit{fbcC} gene.

In order to introduce the histidine tag sequence at the C-terminus of cyt \textit{b}, we first introduced an unique Sal I restriction site in place of the \textit{fbcB} stop codon. To this end, we amplified a 241 bp fragment using the primers BSAL and BRPDOWN, with pGB2T as template. This PCR product was used as a primer in combination with BGFB CPRUP primer to amplify a 475 bp fragment. This fragment was digested by Bgl II and Pst I and used to replace the original Bgl II-Pst I fragment of pGB2T leading to plasmid pGB3BSAL. The his-tag sequence was introduced by PCR amplification between the primers BGFBLPRUP and BGBPRH6, using pGB3BSAL as a template. The resulting product was digested by Bgl II and Sal I and used to replace the Bgl II-Sal I fragment of pGB3BSAL leading to plasmid pGB10BH6. Primer BGBPRH6 allowed the insertion of both the factor Xa proteolytic site and the hexahistidine motif (see Table 1). The factor Xa site provided a way to remove \textit{in vitro} the histidine tail from the recombinant protein. This feature was not used in our study. Finally the Bgl II-EcoR I fragment of pGB10BH6 was cloned back into pGB1 to yield the complete \textit{fbc} operon carrying a his-tag sequence fused to the end of the \textit{fbcB} coding frame. The construction of the \textit{fbcC} gene carrying the sequence encoding a his-tag was performed using a similar approach. A unique Sal I restriction site was introduced in place of the \textit{fbcC} stop codon. To this end, we amplified a 216 bp fragment using the primers C1PRUP and C1SALDOWN, with pGB2T as template. This fragment was digested by Aat II and Xma I and used to replace the original Aat II-Xma I fragment of pGB2T leading to plasmid pGB3CSAL. The his-tag sequence was introduced by PCR amplification between the primers C1PRUP and BGCPRH6, using pGB3CSAL as a template. The resulting
product was digested by Aat II and Sal I and used to replace the Aat II-Sal I fragment of pGB3CSAL leading to plasmid pGB10CH6. Finally the Bgl II-EcoR I fragment of pGB10CH6 was cloned back into pGB1 to yield the complete $fbc$ operon carrying a his-tag sequence fused to the end of the $fbcC$ coding frame. All PCR products were verified by DNA sequencing. The two mutant $fbc$ operons, carrying the his-tag, were subcloned into pRK415 vector. These constructs were introduced in $E. coli$ strain S17-1 (14) and the property of this $E. coli$ strain to conjugate with $Rb. sphaeroides$ was used to transfer the recombinant plasmids into BC17, a $Rb. sphaeroides$ strain carrying a deletion of the endogenous $fbc$ operon (13). The $Rb. sphaeroides$ strains carrying the his-tagged cyt $b$ or cyt $c_1$ were respectively designated BH6 and CH6.

**Purification of the his-tagged $bc_1$-complex.** $Rb. sphaeroides$ cells were collected by 10 min centrifugation at 14000×g, washed once with 50 mM MOPS, pH 7.00, 100 mM KCl (buffer A) and resuspended in buffer A (about 1/150 of the initial culture volume). DNase I and RNase A were added to the suspension at final concentrations of 100 µg/ml and 10 µg/ml, respectively. The cells were disrupted in a French press at 12,800 psi. Unbroken cells were removed by centrifugation at 30,000×g for 30 min. To collect the chromatophores, the supernatant was further centrifuged at 100,000×g for 90 min. The pellet was resuspended in buffer A with 25% glycerol. The final concentration was adjusted to $\Delta A_{562-577 \text{nm}} = 0.6$ and the membrane suspension was stored at -80°C until used. Solubilization of the membranes and binding of the his-tagged $bc_1$ complex to the Ni-NTA resin (QIAGEN, Inc., Valencia, CA) were carried out simultaneously at 4°C as described below. All following buffers contain 0.2 mM PMSF and 0.1 mM ε-aminocaproic acid as protease inhibitors.
One volume of chromatophores ($\Delta A_{562-577\text{nm}} = 0.6$) (usually 10 mL) was diluted with 0.3 volumes (3 mL) of buffer $B$ (50 mM MOPS pH 7.8, 100 mM NaCl, 1 mM MgSO$_4$, 20% (w/v) glycerol and 5mM histidine) and 0.7 volumes (7 mL) of Ni-NTA resin equilibrated in buffer $B$ (approximately a 1:1 mixture). While slowly stirring this mixture, a solution of 10% (w/v) dodecyl maltoside (DM) (Sigma) was added drop by drop to a final concentration of 0.66 mg DM/mg protein. The membranes were incubated with slow stirring at 4°C for 40 minutes. Subsequent steps were also performed at 4°C. The mixture was transferred to an empty, glass Kontes flex-column (Fischer Scientific) and the 3.5 mL Ni-NTA resin was packed while eluting. The column was washed with about 20 column volumes (70 mL) of buffer $C$ (50 mM MOPS pH=7.8, 100 mM NaCl, 1 mM MgSO$_4$, 0.01 % DM), until the eluant was clear ($\Delta A_{280\text{nm}} < 0.01$). To decrease the background of unspecific binding, an additional washing with 2 column volumes of buffer $C$ containing 5 mM histidine was performed. The purified bc$_1$ complex was eluted from the column with buffer $C$, containing 200 mM histidine and 15 µg/mL phosphatidyl choline (Avanti Polar-Lipids, Inc., Alabaster, AL). The use of histidine in place of the recommended imidazole was essential for the preparation of an active complex (see Results). The preparation was further concentrated using CentrisartI (Sartorius, Edgewood, NY) or Centriplus (Amicon, Inc., Beverley, MA) concentrators with a cut-off of 100,000 Da. When necessary, the histidine was removed by overnight dialysis against 50 mM MOPS pH 7.0, 100 mM NaCl, 1 mM MgSO$_4$, 20% (w/v) glycerol, containing 0.01% DM and 15 µg/mL phosphatidyl choline. When dialysis was not performed, the purified complex was mixed with glycerol (20% (w/v)) prior to freezing. Purified bc$_1$-complex was frozen in liquid nitrogen and stored at -80°C until used.
Estimation of the total molecular weight. The molecular weight of the bc\textsubscript{1}-complex was estimated using an 1.5 cm x 30 cm Ultrogel AcA 34 column (Biosepra, Marlborough, MA) equilibrated with 40 mM Na-phosphate buffer pH 7.0, 50 mM NaCl and 0.02% DM at a flow rate of 0.3 mL/min at 4 °C. A calibration curve was made using β-amylase (2 mg), alcohol dehydrogenase (2 mg), bovine albumin (5 mg) and carbonic anhydrase (2 mg) as molecular weight markers (Sigma). The elution volumes for horse cyt c (2 mg) and thyroglobulin (4 mg) were also determined although they were not used for the calibration curve as they are outside of the linear range specified by the manufacturer for the Ultrogel AcA34. Nevertheless we noticed that the elution volumes of these two polypeptides fitted well the calibration curve. The bc\textsubscript{1}-complex was diluted in the running buffer to a 20 µM concentration and 500 µL samples were loaded on the column.

Optical redox titrations. Optical spectra were obtained using single-beam spectrophotometer, built in the laboratory (15) and interfaced to a PC-compatible computer for control and storage of the data using a program written in-house (Crofts & Guergova-Kuras, unpublished). Absorbance spectra of chromatophore preparations or purified bc\textsubscript{1} complexes were recorded approximately every 10 mV. The potential was modified by addition of either dithionite (sodium hydrosulfite) or potassium ferricyanide, followed by a 3 to 5 minutes equilibration time. For the b-type cytochromes, the titration was performed over a range from -200 to 250 mV. The titration curves were obtained from the absorbance spectra by abstracting values at 561 nm and 569 nm. The curves were fitted with either 2 or 3 Nernst components using the built-in non-linear square fitting algorithm of the data-analysis program Origin 4.0 (Microcal Software, Inc., Northampton, MA). The titration curves for cyt c\textsubscript{1} were obtained as the difference of 554 nm and 548 nm from
absorption spectra taken in the redox region of 0 to 400 mV and were fitted to Nernst components using the same fitting algorithm.

*Flash-induced kinetics.* The flash-induced absorbance changes of the $bc_1$-complex and the reaction center were monitored on a single-beam spectrophotometer in a redox cuvette as previously described (16). Kinetics of the reaction center were followed at 542 nm, of the combined cyt $c_1$ and $c_2$ at 551 nm minus 542 nm, the cyt $b_H$ changes were monitored as the difference between 561 nm and 569 nm(13,15,16).

*EPR measurements.* EPR spectra were obtained using a Bruker 220D X-band spectrometer equipped with a liquid-helium system Bruker ESP-300. The conditions used were: microwave frequency 9.45 GHz, microwave power 2 mW, modulation amplitude 12.5 G, time constant 0.3 s, temperature 8 K.

*Miscellaneous.*

SDS-PAGE analysis was carried out in a SDS-12.5% polyacrylamide gel (SDS-polyacrylamide gel system, Gibco BRL Life Technologies, Gaithersburg, MD) with a running buffer containing 49 mM Tris, 384 mM glycine, and 0.1% (w/v) SDS, pH 8.5). Coomassie blue staining was used for detection of the polypeptides.

Protein determinations were made according to the method of Lowry modified by Peterson (17) using bovine serum albumin as a standard. (Sigma, protein assay kit).

The concentrations of cyt $b$ and cyt $c_1$ in the purified complex were measured from reduced minus oxidized difference spectra as described by Vanneste (18):

\[
[c_{c_1}] = (\Delta A_{553-540}) \cdot 5.365 \cdot 10^{-2} - (\Delta A_{562-577}) \cdot 9.564 \cdot 10^{-3} \text{ mM;}
\]

\[
[c_b] = (\Delta A_{562-577}) \cdot 3.539 \cdot 10^{-2} - (\Delta A_{553-540}) \cdot 1.713 \cdot 10^{-3} \text{ mM.}
\]
Ubiquinol:cytochrome c oxidoreductase activity was assayed using 25 µM horse cyt c as an electron acceptor and 100 µM decylubiquinol (Sigma) as an electron donor (19). The reaction was initiated by adding decylubiquinol to the reaction mixture containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.01% DM, 15 µg/mL phosphatidyl choline, 25 µM horse cyt c and 30 nM bc\textsubscript{1} complex. The reduction of horse cyt c was monitored at 549 nm and an extinction coefficient of 22 000 M\textsuperscript{-1}.cm\textsuperscript{-1} was used (20).

Extraction and HPLC analysis of the quinone content of the purified complex was performed as in Kroger (21).

**RESULTS**

*Purification of bc\textsubscript{1} complex.* Existing procedures for purifying bc\textsubscript{1}-complex from *Rb. sphaeroides* are usually time consuming. In order to simplify this process we have constructed two new strains of *Rb. sphaeroides* in which either cyt b or cyt c\textsubscript{1} have been extended at their C-terminal end by a his-tag. The affinity of the his-tag for divalent metal ions was used to bind the engineered complexes to a Ni\textsuperscript{2+}-nitrilotriacetic acid commercial resin and allow an easy “one-step” recovery of the bc\textsubscript{1}-complex. The construction of the two recombinant genes is described in detail in the Materials and Methods section and summarized on Fig. 1. The sequences of the recombinant proteins are shown on Fig. 2. In both cases they encompass eleven additional amino-acids at their carboxy terminal end. This extension contains both a factor Xa cleavage site and a hexahistidine sequence. The purpose of the factor Xa site was to provide a way to remove in vitro the additional oligopeptide in case it interfered with the properties of the recombinant enzymes. Since there was no deleterious effect due to the additional oligopeptide (see below), this feature was not used in
this study. Introduced into *Rb. sphaeroides*, the recombinant cyt *b* and cyt *c*₁ led, respectively, to strains BH6 and CH6. Both mutant strains grew photosynthetically at the same rate as the wild-type with a generation time of 8 hours. Both strains expressed the *bc₁* complex constitutively. The level of accumulation of *bc₁* complex in both strains was similar to that observed in *Rb. sphaeroides* BC17C (13), a strain where the deletion of the genomic *fbc* operon was complemented *in trans* by an extrachromosomal *fbc* operon cloned into the pRK415 vector.

For most purifications, the starting material was a chromatophore suspension prepared from bacteria grown anaerobically under light. The same procedure was also used successfully to isolate the complex from membrane fragments from bacteria grown under aerobic conditions, in which respiratory mode of growth was used. An important parameter for both the yield of recovery and the activity of the purified enzyme was the concentration of the *bc₁*-complex during the solubilization/binding step. Since the amount of the resin is estimated from a binding capacity of 7 mg *bc₁*-complex/mL resin, which is in the range specified by the manufacturer, the concentration of the *bc₁*-complex will determine the ratio of the resin volume to the total volume. Using a simple trial and error method we estimated that the optimal ratio is at 17% (by vol.). The amount of detergent was then estimated to give a final ratio of 0.66 mg DM/mg total protein. In the following steps the detergent concentration was decreased to 0.01% (w/v) DM since high concentrations of detergent during the column washing lead to the loss of the [Fe-S] protein and the subunit IV. A similar effect was observed when the elution step was performed in the absence of phosphatidyl choline; apart from preliminary characterization, we did not investigate this further.
An important modification of the protocols supplied by the manufacturer for purification of his-tagged proteins using the Ni-nitrilotriacetic acid resin was to replace imidazole with histidine. This substitution allowed us to increase the activity of the preparations from 5 mol cyt $c.s^{-1}.(mol$ cyt $c_1)^{-1}$ for preparations obtained using imidazole to more than 110 mol cyt $c.s^{-1}.(mol$ cyt $c_1)^{-1}$ for preparations obtained using histidine (see Table 2). A probable reason for the low activity of the $bc_1$-complex in the presence of imidazole will be discussed later in the text.

Polypeptide composition and aggregation state. Polypeptide composition of the purified complex was determined by SDS-PAGE using Coomassie staining and the results are shown on Fig. 3. Four bands are present and were assigned as previously to cyt $b$, cyt $c_1$, the [Fe-S] protein and subunit IV (22,23). The gel shows clearly the slower electrophoretic mobility of the his-tagged cyt $b$ and his-tagged cyt $c_1$, as compared to their native counterparts. The apparent molecular weight of the subunits as estimated from their migration were 40.8 kDa for the his-tagged cyt $b$, 40.1 kDa for cyt $b$, 34.1 kDa for the his-tagged cyt $c_1$ and 32.6 kDa for cyt $c_1$. The molecular weight of the [Fe-S] protein was estimated as 19 kDa and of subunit IV as 14 kDa. When the solubilization/binding step was not performed under the optimal resin volume/total volume ratio, minor contamination with high molecular weight were observed (lane 3). The apparent molecular weight of the $bc_1$-complex was determined by gel filtration on Ultrogel AcA 34 in the presence of 0.02% DM. Fig. 4 shows a symmetrical elution peak which corresponds to a particle with a molecular weight of 260 kDa. Taking into account the molecular weight of the detergent micelles, this value corresponds to the presence of two copies of each of the four subunits of the $bc_1$-complex and therefore to a dimeric state of the enzyme in our preparations.
Spectral properties of the prosthetic groups. The visible spectra of the purified \( bc_1 \)-complex after reduction by ascorbate and sodium dithionite are shown on Fig. 5. Ascorbate reduces selectively cyt \( c_1 \) whereas dithionite reduces all of the cytochromes. The purified \( bc_1 \)-complex in air-equilibrated preparations appeared to be fully oxidized since additional oxidation using ferricyanide prior to reduction with ascorbate did not increase the contribution of cyt \( c_1 \). The spectra of cyt \( b_H \) and \( b_L \) in the region of the \( \alpha \) and \( \beta \)-bands obtained from redox titrations are shown on the inset. We have compared the spectra of cyt \( b \) before and after purification from the CH6 strain. The results for BH6 strain are similar and are not shown. The peak of the \( \alpha \)-band of cyt \( b_H \) is at 560 nm, the same position observed in strains of \( Rb. sphaeroides \) carrying the WT complex (16,22,24). This indicates that the presence of the additional histidines does not modify the spectra of cyt \( b_H \). After purification, the spectrum of cyt \( b_H \) remained unchanged which is in contrast with previous observations of a blue shift of this peak after isolation of the \( Rb. sphaeroides \) complex by standard chromatography techniques (22). The characteristic split \( \alpha \)-band of cyt \( b_L \) was clearly seen in the chromatophores of both mutants with peaks at 558.5 and 565.5 nm, similar to those seen in the WT strain. However, the purification procedure caused a sharpening of the longer wavelength peak and a less pronounced splitting (compare traces c and d). The reason for this effect is not clear, but it could be attributed to a difference in polarity of the environments of the cyt \( b_L \) heme in the membrane and in the detergent. The optical spectra of cyt \( c_1 \) in chromatophores and the purified complex from the BH6 and CH6 strains were identical to the WT with the \( \alpha \)-band peak at 552 nm (not shown). The ratio between cyt \( b \) and \( c_1 \) in the purified \( bc_1 \)-complex was 1.9 whether it was calculated from reduced-oxidized difference spectra.
of the purified preparation or, under denaturing conditions, from heme assays with alkaline pyridine. Similar data have been reported for the wild-type bc1-complex (22,23,25). The EPR spectra of the dithionite-reduced Rieske center from bc1-complexes isolated from both the BH6 and the CH6 strains were similar to the WT (26) with g values of 1.76, 1.90 and 2.02.

3.4. Potentiometric titrations. The midpoint potentials of the cytochromes in both chromatophores and purified complexes are listed in Table 3. The presence of a histidine tag on either of the subunits of the complex (cyt b or cyt c1) had little effect on the E_{m,7} values of all three hemes as compared to the WT. Titrations of cyt c1 in preparations obtained using imidazole in the purification procedure provided an explanation for the low activity of these preparations (see above). We observed that while 30% of cyt c1 retained a mid-point potential comparable to the value found for highly active bc1 complex (250-270 mV), 70% showed a lower mid-point potential of 146±6 mV. It has been shown that imidazole can displace the methionine axial heme iron ligand of cyt c (27) and that this type of ligand substitution is accompanied by a decrease in the mid-point potential of the heme (28). We suggest therefore that a similar ligand substitution occurs in cyt c1 of the bc1-complex when imidazole is used in the purification procedure.

The cyt b-150 component has recently been ascribed to the formation of a bi-radical semiquinone:ferroheme complex (Q-.cyt bh+) at the Q_{i}-site (29). In titrations of isolated bc1-complex reported in the literature, the results have been mixed, with a cyt b-150 component found in some preparations, but not in others, likely reflecting the presence or absence of ubiquinone in the purified complex (30, 31). The titrations of the purified complexes from both BH6 and CH6 strains contained the cyt b-150 component (Table 3), indicating that the Q_{i} site in our preparations can equilibrate with a quinone/quinol molecule. This would suggest that probably one of the 3-4
quinone molecules per purified $bc_1$-complex which were estimated from HPLC measurements in complexes purified from both BH6 and CH6 strain, is bound at the Qi site. This was also supported by redox titrations of the purified complexes, performed in presence of antimycin, an inhibitor of the Qi site, where no cyt $b$-150 component was observed (not shown).

*Flash-induced kinetics of the electron transfer in the $bc_1$-complex.* The overall turnover of the modified $bc_1$-complexes, as measured by flash-induced kinetics is essentially the same as in the WT, both when the quinone pool is oxidized (200 mV) or partly reduced (100 mV). The partial reactions of cyt $b_H$ reduction and re-reduction of cyt $c_{total}$ were measured in presence of saturating concentrations of antimycin and the half-times estimated for these reactions indicated that the electron transfer properties of the $bc_1$-complex were not modified by the presence of the his-tag (not shown).

**DISCUSSION**

Our purification protocol combines a biochemical approach where a non-ionic detergent is used for solubilizing membranes from *Rb. sphaeroides* with a molecular biology approach which extend one of the subunits of the complex with an affinity tag, allowing us to isolate the $bc_1$-complex with a high-specificity. The affinity tag, six additional histidines at the C-terminus of either cyt $b$ or cyt $c_1$, via a highly specific binding to a Ni-nitrilotriacetic acid resin allows us to recover the $bc_1$-complex from membrane preparations using a fast and simple ‘one-step’ purification procedure. Our results indicate that the his-tag does not modify the properties of the complex *in situ* as shown by the spectral, redox and electron transfer properties of the $bc_1$-complex. Moreover, none of the
modifications introduced in the original plasmid (13) in order to construct the his-tagged strains modified the functional properties of the $bc_1$-complex as seen in chromatophores. Basically, the BH6 and CH6 strains appear undistinguishable from their wild-type parent.

Our purification procedure yields an active enzyme containing four polypeptides and all four redox cofactors in their stoichiometric ratios. Gel filtration experiments indicate that the $bc_1$-complex is in a dimeric state. The quinol:cyt c oxidoreductase specific activity of the purified $bc_1$-complex was in the range of 110 mol cyt $c$.s$^{-1}$.(mol cyt $c_1$)$^{-1}$, with some preparations showing activities as high as 160 mol cyt $c$.s$^{-1}$.(mol cyt $c_1$)$^{-1}$. These values are similar to those obtained by Ljungdahl et al. (23) and double those of preparations reported by Yu and Yu (25), both procedures using conventional purification methods. The two most significant improvements that our procedure has over the previous methods are the simplification of the purification protocol and the very important increase in the yield of recovery. A mid-scale preparation starting from chromatophore membranes obtained from 6 L of culture, and yielding 60 mg of purified $bc_1$-complex takes only 4 hours to perform. Moreover our average yield of recovery is greater than 50%, at least 4 times higher than the previously reported values (22, 23). We have also successfully applied this purification method to isolate $bc_1$-complex from mutant strains of *Rb. sphaeroides* bearing the his-tag and additional mutations in the *fbc* operon (unpublished results).

**ACKNOWLEDGEMENTS:** This work was supported by NIH Grant GM35438.

**REFERENCES.**


Table 1. List of primers and linkers used to construct the mutant strains.

The mutagenic mismatches are indicated in lowercase. The relevant restriction sites are shown in bold. These primers extend the protein C-terminal end with the sequence: IEGRGHHHHHHH. The motif IEGR corresponds to a factor Xa cleavage site but has not been used in this study. The HHHHHHH motif corresponds to the his-tag sequence.

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<th>Primers</th>
<th>Relevant properties of mutagenic primers</th>
<th>Sequence</th>
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<td>BSAL</td>
<td>Mutagenic primer for introducing a Sal I site into the fbcB gene</td>
<td>5' AAA ACG GTC GTC GCA <strong>Gtc gAc</strong> GGG AAA GGA CAG TGA CGA 3'</td>
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<td>BPRDOWN</td>
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<td>5' GAT CGG GAC GAA CTT CAT CCC GTG GCA GGC TGC 3'</td>
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<tr>
<td>BGFBCPRUP</td>
<td>Bgl II restriction site.</td>
<td>5' GCG CTA <strong>AG ATC TAC</strong> TTC TGG CTG CTC GCG 3'</td>
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<td>BGBPRH6</td>
<td>Mutagenic primer used to introduce the his-tag at the end of cyt b.⁸</td>
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<tr>
<td>C1PRUP</td>
<td></td>
<td>5' GAC GGC CAC GAC GCC AGC GTC CAT 3'</td>
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<tr>
<td>C1SALDOWN</td>
<td>Mutagenic primer for</td>
<td>5' CAT CGA <strong>TCC CGG GGA</strong> <strong>gTC gaC</strong></td>
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introducing a Sal I site into the fbcC gene (contains a Xma I site).

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<th>BGCPRH6</th>
<th>Mutagenic primer used to introduce the his-tag at the end of cyt c. a</th>
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<td>5' tcc ttt ccc <strong>GTC GAC</strong> tta gtg atg gtg atg gtg gtg ccc gcc ccc ctc aat GAC GTT CGT CTT CTT CTT GCC CTT 3'</td>
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<th>HIND</th>
<th>Hind III-Eco47 III-Bgl II sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5' <strong>AGCTTGGAGCGCTACA</strong> 3'</td>
</tr>
<tr>
<td></td>
<td>3' <strong>ACCTCGCGATGTCTAG</strong> 5'</td>
</tr>
</tbody>
</table>
Table 2. Purification of \( bc_1 \)-complex from BH6 and CH6.

Total protein and cyt \( b \) concentrations were determined as described in Materials and Methods. The oxidoreductase activity is expressed in \( \text{mol cyt } c.s^{-1}.(\text{mol cyt } c_1)^{-1} \). BH6 chromatophores were obtained from 2 L of culture, yielding 5 g of wet cells, and CH6 chromatophores from 1 L culture, yielding 3 g wet cells.

<table>
<thead>
<tr>
<th></th>
<th>Total protein (mg)</th>
<th>Total cyt ( b ) (nmol)</th>
<th>Oxido-reductase activity (s(^{-1}))</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatophores (BH6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+DM</td>
<td>471</td>
<td>249</td>
<td>198</td>
<td>100</td>
</tr>
<tr>
<td>Ni-NTA column eluate</td>
<td>12.5</td>
<td>212</td>
<td>118</td>
<td>85</td>
</tr>
<tr>
<td>Chromatophores (CH6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+DM</td>
<td>227</td>
<td>144</td>
<td>191</td>
<td>100</td>
</tr>
<tr>
<td>Ni-NTA column eluate</td>
<td>5.8</td>
<td>104</td>
<td>114</td>
<td>72</td>
</tr>
</tbody>
</table>
Table 3. Mid-point potentials of the cytochromes from the $b_{c_1}$-complex.

All the redox titrations were made in 50 mM Mops pH 7, 100 mM KCl, and in the presence of the following redox mediators: N,N,N’,N’-tetramethyl-p-phenylenediamine (10 µM); 2,3,5,6-tetramethyl-p-phenylenediamine (10 µM); 1,2-naphtoquinone (20 µM); 1,4-naphtoquinone (20 µM); phenazine methosulfate (10 µM); pyocyanine (1 µM); 2-hydroxy-1,4-naphtoquinone (20 µM) and Fe(III) EDTA (100 µM). For the chromatophores 1 µM gramicidin and 25 µM valinomycin were also added. $^a$The fraction of total cyt $b_{H}$ titrating with $E_m \sim 150$ mV in the absence of antimycin (cyt $b_{150}/$ cyt $b_{H}$), expressed as a percentage. This value reflects the interaction between quinol and cyt $b_{H}$ at the Q$_i$ site to form a biradical Q$^-.cyt b_{H}$ complex (29), and gives an indirect estimate of the normal functionality of the site, and the presence of quinone in the isolated complex (see also text).

<table>
<thead>
<tr>
<th></th>
<th>$E_m$ cyt $b_{H}$ (mV)</th>
<th>$E_m$ cyt $b_L$ (mV)</th>
<th>$E_m$ cyt $b_{150}$ (mV)</th>
<th>$^a$cyt $b_{150}$/cyt $b_{H}$ (%)</th>
<th>$E_m$ cyt $c_{1}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatophores</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from wild type</td>
<td>42±3</td>
<td>-88±6</td>
<td>156±8</td>
<td>25</td>
<td>n.d</td>
</tr>
<tr>
<td>Chromatophores from the</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>strain with the His-tagged cyt $b$</td>
<td>44±2</td>
<td>-77±5</td>
<td>163±5</td>
<td>34</td>
<td>n.d</td>
</tr>
<tr>
<td>Isolated $b_{c_1}$-complex from the</td>
<td>30±3</td>
<td>-78±7</td>
<td>169±10</td>
<td>23</td>
<td>273±4</td>
</tr>
<tr>
<td>strain with the His-tagged cyt $b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromatophores from the</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>strain with the His-tagged cyt $c_1$</td>
<td>43±2</td>
<td>-72±5</td>
<td>158±3</td>
<td>37</td>
<td>n.d</td>
</tr>
<tr>
<td>Table: Isolated $bc_1$-complex from the strain with the His-tagged cyt $c_1$</td>
<td>48±3</td>
<td>-61±5</td>
<td>165±5</td>
<td>22</td>
<td>251±4</td>
</tr>
</tbody>
</table>
**Figure Legends.**

Fig. 1 Construction of pGB11BH6 and pGB11CH6. Plasmid maps summarize the strategy for the extension of the cyt \( b \) or cyt \( c_1 \) with a his-tag sequence. Details of the vector constructions are given in the text. The sequence of *Rb. sphaeroides* containing the \( fbc \) operon is shown with a black box. The position of the open reading frames (ORF) encoding the Rieske ISP protein (\( fbcF \)), cyt \( b \) (\( fbcB \)) and cyt \( c_1 \) (\( fbcC \)) are shown with open arrows. Sequences of the plasmid vectors are shown with either a single line (pUC 9 vector) or with a double line (pT7T319U vector). Restriction sites are indicated only when they are unique in the plasmid.

Fig. 2 DNA and corresponding aminoacid sequences of the wild-type and recombinant proteins. Only the carboxy-terminal part of the proteins is shown. A) WT cyt \( b \), B) recombinant cyt \( b \), C) WT cyt \( c_1 \) and D) recombinant cyt \( c_1 \). Letters in italic represent the mutated/inserted sequence. Underlined bases show the Sal I restriction site. The black boxes show the his-tag sequence. This sequence contains also the factor Xa proteolytic site (IEGR) fused to the hexahistidine motif.

Fig. 3 SDS-PAGE analysis of purified \( bc_1 \)-complex. Lane 1 – complex purified from the BH6 strain, lane 2 – molecular weight protein ladder (BenchMark Protein Ladder, GibcoBRL), lane 3 – complex purified from the CH6 strain. Samples of 25 \( \mu \)g of protein were used to load the purified \( bc_1 \)-complexes.

Fig. 4 Gel filtration of purified \( bc_1 \)-complex from BH6 strain on Ultrogel AcA 34 (1.5 cm x 30 cm) column. Proteins were detected by the absorption at 280 nm. The inset shows the calibration
curve of the molecular mass vs. $V_e/V_0$. The void volume ($V_0$) was determined by the elution of Blue Dextran. Standards shown as (o), include thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa).

Fig. 5 Absorption spectra of $bc_1$-complex purified from the CH6 strain. (-----) dithionite reduced, (---) ascorbate reduced, (-----) spectrum of cyt $b$ obtained as a difference between the dithionite-reduced and ascorbate-reduced spectra. Inset: spectra deconvoluted from redox titrations showing the $\alpha$-band region of cyt $b_H$ and cyt $b_L$ in chromatophores (solid line) and in the purified $bc_1$-complex (dashed line). Spectra (a) and (b) show cyt $b_H$, spectra (c) and (d) show cyt $b_L$.
Introduction of a unique Sal I site at the 3' end of the fbcB ORF using PCR mutagenesis.

Introduction of the histag sequence at the 3' end of the fbcB ORF using PCR mutagenesis.

Introduction of the histag sequence at the 3' end of the fbcC ORF using PCR mutagenesis.

Reconstruction of the whole fbc operon bearing the histag sequence.

Reconstruction of the whole fbc operon bearing the histag sequence.

Introduction of the histag sequence at the 3' end of the fbcC ORF using PCR mutagenesis.
Fig. 2.

A) cyt b sequence

\[ \begin{align*}
\text{TVVAE} \\
\text{ACG GTG GCA GAG TAA GGG}
\end{align*} \]

B) cyt b sequence and histag sequence

\[ \begin{align*}
\text{TVVAEIEGRHHHHH} \\
\text{ACG GTG GCA GAG ATT GAG GGG CAC CAC CAT CAC TAA GTG GAC GGG}
\end{align*} \]

C) cyt c1 sequence

\[ \begin{align*}
\text{KTNV} \\
\text{AAG AAG ACG AAG GTG TGA TCC}
\end{align*} \]

D) cyt c1 sequence and histag sequence

\[ \begin{align*}
\text{KTNVIIEGRHHHHH} \\
\text{AAG AAG ACG AAG GTG ATT GAG GGG CAC CAC CAT CAC TAA GTG GAC TCC}
\end{align*} \]
Fig. 3.
Fig. 4.

![Graph showing absorbance at 280 nm against elution volume, ml. The graph includes a peak at V_e and an inset showing apparent M, kDa against V_e/V_o. The inset highlights the bc1 complex.](image)