# Fractionation of cytochromes of phototrophically grown *Chloroflexus aurantiacus*. Is there a cytochrome *bc* complex among them?

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Received 10 October 2001; revised 10 December 2001; accepted 19 December 2001

First published online 16 January 2002

Edited by Richard Cogdell

**Abstract The cytochrome-containing membrane complexes of the phototrophically grown green non-sulfur bacterium *Chloroflexus aurantiacus* were fractionated by anion exchange chromatography. Three cytochrome *b* and four cytochrome *c* peaks were observed. None of the separated complexes met the features of the cytochrome *bc* complex. Two main cytochrome *b*-containing complexes were further purified: a dimer of identical subunits with unknown function and a succinate:quinone oxidoreductase containing three subunit species. Two novel multisubunit complexes, similar to each other, with two heme *c*-bearing subunits were also purified. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Cytochrome; *Chloroflexus aurantiacus*

## 1. Introduction

The cytochrome *bc* ($bc_1$, $b_6f$, and others) complex is a component of photosynthetic and respiratory electron-transporting chains. It transfers electrons from the quinol reduced on the N-side of the membrane of mitochondria, chloroplasts, or bacteria to a mobile electron transporter on the P-side of the membrane and simultaneously transfers two protons across the membrane per electron. Bacterial *bc* complexes contain three essential subunits bearing cofactors: diheme cytochrome *b*, FeS Rieske factor, and a *c*-type cytochrome; additional subunits without cofactors may also exist (see [1] for a review). The complex is widely distributed among bacteria, yet there are oxygen-respiring species which lack it, e.g. *Escherichia coli* [2], or do not express it in usual conditions, e.g. *Bacillus subtilis* [3]. In such cases quinol is oxidized by a terminal quinoloxidase instead of the team of *bc* complex and cytochrome oxidase. However, in photosynthetic chains the *bc* complex is assumed to be an obligate component [1] since there must be a membrane device transferring electrons from quinol to the donor side of photosystem I or the bacterial photoreaction center. Indeed, the *bc* complex has been found by direct isolation or genetically in four of five classes of photosynthetic bacteria: purple bacteria [4,5], cyanobacteria [6], heliobacteria [7], and green sulfur bacteria [8] (see also [9] for a recent review on the evolution of the *bc* complex).

Green non-sulfur bacteria represent the fifth and evolutionarily lowest class of bacteria possessing chlorophyll-based photosynthesis [10,11], and *Chloroflexus aurantiacus* [12] is the most studied species among them. The established components of the *C. aurantiacus* cyclic e-transporting chain are: photosynthetic reaction center [13,14]; menaquinone, as the sole bacterium quinone [15]; four-heme cytochrome $c_{554}$, the direct e-donor to reaction center [16,17]; and presumably the copper protein auracyanin [18,19]. The last is assumed to be a soluble mobile e-carrier at the periplasmic side of the bacterium membrane since *C. aurantiacus* does not contain soluble cytochromes [20]. Some indirect indications in favor of the presence of a *bc* complex in *C. aurantiacus* were found when the bacterial cytochromes and iron–sulfur proteins were investigated in situ [21,22]. However, the assumed complex was not isolated.

In this study membrane cytochrome-containing complexes of *C. aurantiacus* were fractionated. Several complexes containing either cytochrome(s) *b* or *c* were found, some novel ones among them, but none of them fitted the common features of the *bc* complex.

## 2. Materials and methods

*C. aurantiacus* strain B3 from the collection of phototrophic bacteria of Moscow State University was grown in 1 l screw-capped bottles illuminated by two pairs of 100 W incandescent lamps (20 000 lux on each side) at 56°C in a medium described earlier [23]. The cells were grown to a density of 2.5–3 g wet weight/l in 12 h cycles, a portion of the culture served as inoculum for the next cycle. The cells were sedimented at $2000\times g$ for 10 min, washed twice in 5 mM Tris–HCl (pH 8.0), suspended in a mixture giving finally 50 mM Tris–HCl (pH 8.0), 0.5 mg/ml lysozyme, 4 mM EDTA, and 10% glycerol and frozen at −12°C. The suspension was thawed, heated to 30°C, incubated for 30 min, and mixed with two volumes of a cold buffer containing 5 mM $MgSO_4$ and 0.03 mg/ml DNase I. The mixture was incubated on ice for another 10 min then unbroken cells were sedimented at $2000\times g$ for 15 min. Membranes were sedimented at $20\,000\times g$ for 30 min and washed twice in 10 mM Tris–HCl, 1 mM EDTA (pH 8.1).

The membranes were suspended in a buffer containing 50 mM Tris–HCl, 1% Triton X-100, pH 8.1 (buffer A) at an optical density of 1.0 at 865 nm and incubated at 20°C for 1 h. Three ml of the mixture was placed into six centrifugal tubes each containing three 1 ml layers of 10%, 15%, and 60% sucrose in buffer A (the two lower layers were without Triton X-100) and centrifuged at 45 000 rpm in a Ti.60 bucket rotor for 1 h at 20°C. The upper and 10% sucrose layers were collected by syringe as solubilized material. The 15% sucrose layer and the upper part of the 60% sucrose layer were collected as unsolubilized colored material. An almost colorless gelatinous pellet consisting evidently of the cell wall fragments was discarded.

Solubilized material was applied to an TSK DEAE-650 (S) (Merck) column ($1\times 2$ cm). The column was then washed with 5 ml of buffer A and absorbed material was eluted with a 20 ml gradient of 0–120 mM

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0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)02236-6

$(NH_4)_2SO_4$ in buffer A stabilized by a backward 10–0% gradient of glycerol. Forty 0.5 ml fractions were collected and the resting sorbed material was pushed out by 200 mM ammonium sulfate in buffer A.

Equal 0.2 ml aliquots of each fraction were placed into two microcuvettes and to each cuvette 0.3 ml of a mixture containing 0.133 M NaOH and 33% pyridine was added. To one cuvette 1–2 mg of dithionite was added and 5 μl of 0.2 M ferricyanide to the other. Difference spectra from 530 to 630 nm were recorded at Hitachi 557 spectrophotometer. Concentrations of hemes *b* and *c* were calculated by the approach of Berry and Trumpower [24] simplified to the measurement of optical absorption at 550 and 556 nm over the line drawn between minima at 535–540 and 566–575 nm. Corresponding extinction coefficients for pyridine hemochromes *c* (25.5 and 9.5 $mM^{-1}$) and *b* (14.2 and 28.5 $mM^{-1}$) were calculated from the curves plotted on the base of Table 1 of the indicated paper [24].

To isolate individual cytochrome complexes a sufficient amount of the membranes was solubilized at the indicated conditions and unsolubilized material was sedimented in a fixed-angle rotor at $144\,000\times g$ for 1 h. The supernatant liquid was applied on an TSK DEAE-650 (S) column (2×2 cm). Absorbed material was eluted by 50 ml of a 0–120 mM $(NH_4)_2SO_4$ gradient in buffer A. Unabsorbed material, which contained some amount of cytochrome *b*, was diluted twofold with water, titrated with 25 mM Tris base to pH 8.3, and applied to the same column equilibrated with 25 mM Tris–HCl, pH 8.3, Triton X-100 0.5%. No cytochrome was detected in the new unabsorbed fraction. Absorbed material was eluted by a 0–20 mM $(NH_4)_2SO_4$ gradient in the indicated buffer. Fractions with maximal content of each cytochrome were rechromatographed on the 1×2 cm TSK DEAE-650 (S) column using gradients of proper ranges of $(NH_4)_2SO_4$ concentration. The resulting preparations were condensed on a Centriprep 30 concentrator (Amicon) and further purified on a Sephacryl S-300 (Pharmacia) column (1.5×96 cm) equilibrated with 20 mM Tris–HCl (pH 8.1), 0.1% Triton X-100. 12–17% Acrylamide gradient SDS–PAGE was done according to Laemmli [25]. Samples were mixed with equal volumes of a buffer containing 200 mM Tris–HCl (pH 7.0), 2% SDS, 100 mM dithiothreitol, and 8 M urea. Gels were stained for cytochrome *c* [26] or, after 1 h fixing in 30% ethanol, for protein by colloidal Coomassie G-250 [27]. Blue native PAGE [28] in 5–20% acrylamide gradient gels was also used.

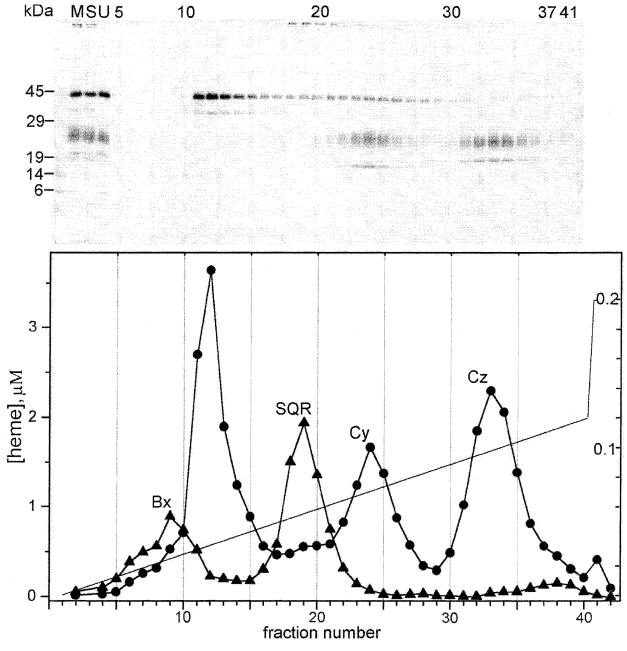


Fig. 1. Fractionation of *C. aurantiacus* cytochromes on the TSK-DEAE column. Top: SDS–PAGE stained for cytochromes. Lanes: M, membranes; S, solubilized material; U, unsolubilized material. To each slot approximately 15 pmol of total cytochrome was applied. To the next slots 4 μl of fractions 5–37 and 41 mixed with an equal volume of the solubilizing buffer was applied. Bottom: Plot of the cytochrome concentration vs. fraction number. Triangles, cytochrome *b*; circles, cytochrome *c*.

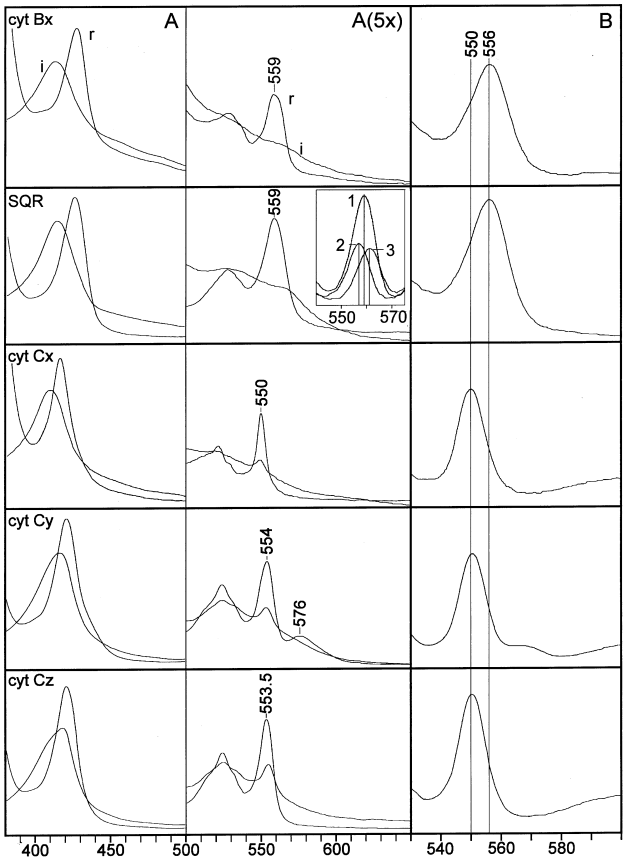


Fig. 2. A: Optical spectra of the separated cytochromes as obtained (i) and dithionite-reduced (r). Inset: Reduced minus oxidized differential spectra of the succinate:quinone oxidoreductase: (1) dithionite–ferricyanide; (2) dithionite–menaquinol (MK-4 freshly reduced [32]); (3) menaquinol–ferricyanide. B: Dithionite-reduced minus ferricyanide-oxidized differential pyridine hemochrome spectra of the cytochromes.

## 3. Results

At the applied conditions about 80% of total cytochrome content were solubilized. The cytochromes with heme *c*-bearing bands at 34 and 19 kDa were solubilized completely, while the unsolubilized fraction was enriched in cytochromes *c* displaying bands of 43, 25, and 21 kDa (Fig. 1, lanes S and U). Heme *b* in the unsolubilized fraction was on the threshold of detection.

Three cytochrome *b*- and three cytochrome *c*-containing peaks can be seen in the plot in Fig. 1. The first massive cytochrome *c* peak displayed a heme-staining band at 43 kDa in SDS–PAGE that corresponds to the four-heme cytochrome $c_{554}$ [16]. This cytochrome was not further investigated here. A minor chromatographic peak with a heme-staining band at 34 kDa moved slightly behind the maximum of the cytochrome $c_{554}$ massive peak and so could not be seen as an individual peak in the plot. This cytochrome was predominant in aerobic [22,29] and scanty in phototrophic cultures [22], as confirmed here. The cytochrome was twice rechromatographed on the small TSK-DEAE column and as a result was freed from cytochrome $c_{554}$ but not from other proteins (Figs. 3 and 4). The pyridine hemochrome analysis showed that this fraction contained only cytochrome *c* (Fig. 2). It was preliminarily designated here cytochrome $c_x$. Although

this cytochrome remains to be purified from contaminants, for the purpose of the present investigation it is sufficient to know that the fraction contains only one cytochrome species.

The next two cytochrome *c* peaks, designated $c_y$ and $c_z$, contained similar multisubunit complexes. They both moved as large complexes of 400 kDa in blue native PAGE and contained two heme *c*-bearing subunits: a diffuse band at 25 kDa and sharp ones at 19 and 21 kDa in $c_y$ and $c_z$, respectively (Figs. 3 and 4). They displayed the same major subunits at 95, 38, 37, and 18 kDa and differed in the set of minor subunits. Both cytochromes were partially reduced in the obtained preparations. Optical spectra of the dithionite-reduced cytochromes $c_y$ and $c_z$ showed α-bands at 554 and 553.5 nm, respectively (Fig. 2). The spectrum of the first complex had an additional small maximum of unclear nature at 576 nm. An additional maximum was also seen at 568 nm in the red-ox pyridine hemochrome spectrum. The complexes also did not meet the features of the *bc* complex. These novel cytochrome complexes deserve further detailed study.

Two cytochrome *b* fractions, first in the unabsorbed material and second eluted at the beginning of the salt gradient, proved to be the same protein. They both displayed a 27 kDa subunit although the first preparation had two additional faint bands of impurities (Fig. 3). In blue native PAGE one diffuse band about 60 kDa was displayed. So this cytochrome *b*, designated $b_x$, can be considered a dimer of identical subunits.

The second cytochrome *b* peak coincided with the peak of succinate dehydrogenase activity (not shown). The purified enzyme displayed two hemes *b*, the first was reduced by succinate or by menaquinol (α-peak at 561 nm) and the second by dithionite (α-peak at 557 nm) (Fig. 2, inset). The complex contained three subunit species of 70, 27 and 23 kDa (Fig. 3). By these characteristics the complex was similar to succinate:menaquinone oxidoreductase of *B. subtilis* [30]. Bands at

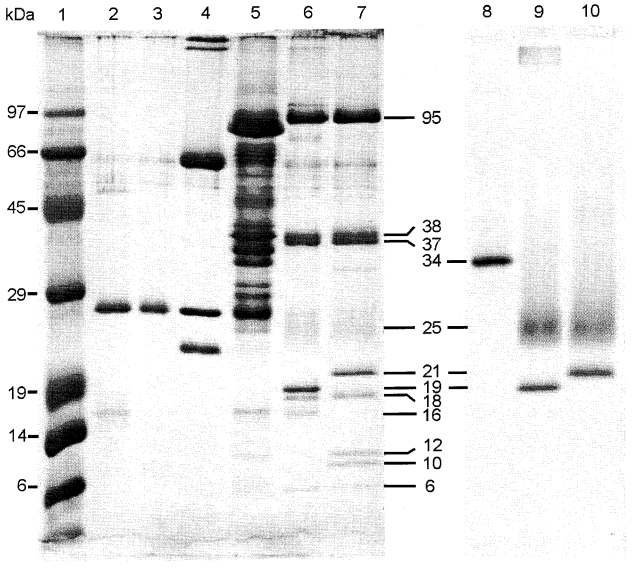


Fig. 3. SDS–PAGE of the purified complexes. Lanes: 1, protein standards; 2, $b_x$ from the unabsorbed material after the first DEAE column; 3, $b_x$ from the first cytochrome *b* peak; 4, succinate:quinone reductase; 5, 8, final preparation of $c_x$; 6, 9, $c_y$; 7, 10, $c_z$. Approximately 10 pmol $b_x$ and $c_x$, and 20 pmol succinate:quinone reductase, $c_y$, and $c_z$ were applied. The left half of the gel with lines 1–7 was stained for protein and the right one for heme *c*.

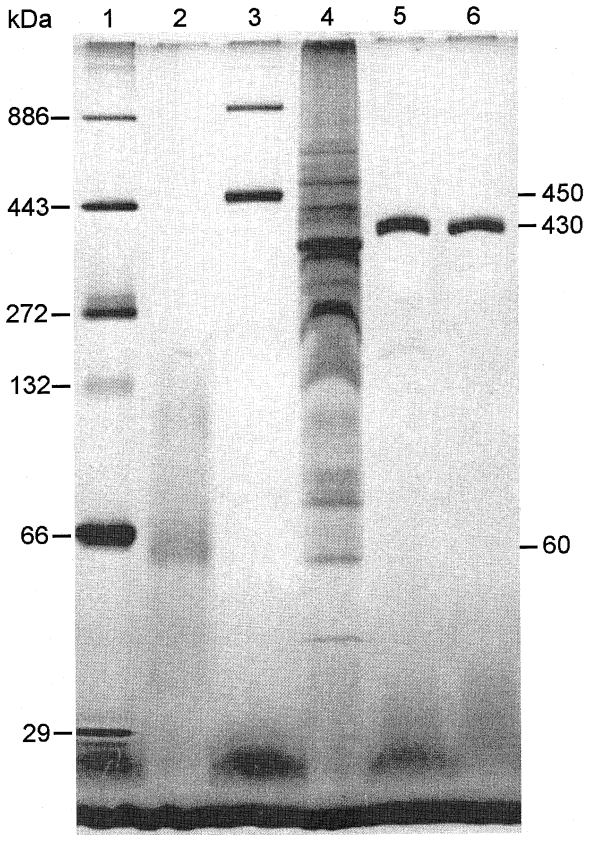


Fig. 4. Blue native PAGE. Lanes: 1, protein standards; 2, $b_x$ (~10 pmol); 3, succinate:quinone reductase; 4, final preparation of $c_x$; 5, $c_y$; 6, $c_z$ (all ~5 pmol). The gel was stained by Coomassie G-250.

the top of the gel were aggregates with undissociated heme *b* as can be seen in Fig. 1 at the lines M, S, and 18–21. In blue native PAGE the complex was at the position of 450 kDa (Fig. 4), so it should be considered a tetramer of the three-subunit subcomplexes. A slight band at 900 kDa was evidently an artificial octamer. More detailed characterization of the enzyme is now in progress and will be published elsewhere.

A small cytochrome *b* peak was detected in the descending half of the cytochrome $c_z$ peak (Fig. 1). This fraction was rechromatographed and enriched in heme *b* content to about 50%. No additional heme-staining bands were detected in this preparation except two bands belonging to cytochrome $c_z$ (not shown). So this small fraction was formed by some cytochrome *b* only.

## 4. Discussion

All the cytochromes *c* visualized by the heme-staining bands were attributed to certain entities and none of them met the features of the cytochrome *bc* complex. The same is also true for the cytochromes *b*. The possibility that some cytochrome *b*-containing complex was hidden in the 'shadow' of the massive cytochrome *b* fractions could not to be excluded, but such a complex would not contain a cytochrome *c*. Four hemes *b* have been found by titration of redox changes in the membranes of phototrophically grown *C. aurantiacus* [21]. The pair of hemes *b* that were most prominent and equal in con-

tent with mid-point potentials −70 and +65 mV have been assumed to be low and high potential hemes *b* of a $bc_1$ complex. But as shown here the most abundant cytochrome *b*-containing complex of *C. aurantiacus* is the succinate:menaquinone oxidoreductase with low and high potential hemes like other succinate:quinone reductases of type B [31]. Thus the results of the present study strongly argue against the existence of a *bc* complex in *C. aurantiacus*.

The absence of a *bc* cytochrome complex in *C. aurantiacus* puts the question, important from an evolutionary point of view, what device works instead. Indeed, there must be one that oxidizes menaquinol, reduces reaction center-bound cytochrome $c_{554}$ via a mobile e-transporter, and transfers at least one proton per electron through the membrane to save energy for ATP synthesis. Further investigations are to shed light at the problem. Possibly one of the observed novel complexes plays the role of the *bc* complex.

*Acknowledgements:* The author would like to thank Dr. B.N. Ivanov, Institute of Basic Biological Problems, Pushchino and Dr. R.E. Blankenship, Arizona State University for critical reading of the manuscript and helpful comments.

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