Volume 213, number 1, 107–112 FEB 04441 March 1987

# A simple, one-step purification of cytochrome *b* from the $bc_1$ complexes of bacteria

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Received 8 December 1986

Cytochrome *b*; Triton X-114; *(Paracoccus denitrificans, Rhodopseudomonas sphaeroides)*

## 1. INTRODUCTION

The *b* cytochromes of the ubiquinol-cytochrome *c* oxidoreductase complex (cytochrome $bc_1$ complex) undergo unique oxidation-reduction reactions as they participate in electron transfer and energy transduction in this segment of the respiratory chain. These reactions are consistent with a protonmotive Q cycle pathway of electron transfer through the $bc_1$ complex [1–3]. In this model cytochrome *b* functions as a transmembrane circuit for recycling of electrons, and may also provide a proton conductance channel to and from ubiquinone redox sites located in hydrophobic domains on this protein [4].

While biophysical techniques have provided information on the thermodynamic [5] and electronic [6,7] properties of the heme centers of cytochrome *b*, little information is available on the structure of the protein. The sequence of the gene coding for cytochrome *b* has been elucidated from several eukaryotic [8–12] and prokaryotic sources [13], and it may soon be possible to manipulate this protein by molecular genetic methods. However, a prerequisite for understanding the function of cytochrome *b* on a mechanistic level is the isolation of the protein in pure form. To date, the extreme hydrophobicity of this protein has hindered its isolation. While methods have been reported for purification of cytochrome *b* (e.g. see [14]) these require numerous steps, result in significant loss of the protein, and yield apparently denatured cytochrome.

Some bacteria produce respiratory complexes which are similar to those of mitochondria, as indicated by their redox prosthetic groups and response to inhibitors of respiration, but which are simpler in polypeptide subunit composition than their mitochondrial counterparts. Hence, a two-subunit cytochrome *c* oxidase complex [15] and a three-subunit cytochrome $bc_1$ complex [16] have been isolated from *Paracoccus denitrificans*, and a cytochrome $bc_1$ complex containing four subunits has been isolated from *Rhodopseudomonas sphaeroides* [17,18].

An attraction of the bacterial respiratory chain complexes is that they offer an opportunity to manipulate genetically cytochrome *b*, which is currently intractable to such manipulation in eukaryotes, where the gene for this protein is located in the mitochondria. In order to gain full advantage of applying current molecular genetic methods to the study of cytochrome *b*, it is useful

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*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/87/$3.50 

to have a simple method for isolation of this protein in a homogeneous form.

We describe here a one-step method for the purification of cytochrome *b* from the $bc_1$ complexes of bacteria. This purification can be accomplished in 30 min, and is based upon the temperature-dependent phase separation of the detergent, Triton X-114 (Triton X is a Rhome and Haas Company trade name). The procedure has been applied to the $bc_1$ complexes of *P. denitrificans* and *R. sphaeroides*, and may be generally applicable to the simple subunit complexes of bacterial respiratory complexes.

## 2. EXPERIMENTAL

The cytochrome $bc_1$ complexes of *P. denitrificans* and *R. sphaeroides* were purified by a procedure developed in this laboratory [16,18]. Triton X-114 was obtained from Sigma and precondensed three times to remove hydrophilic molecules [19]. The concentration of Triton X-114 following condensation was determined from its absorbance at 274 nm, using an extinction coefficient of $E_{0.1\%} = 2.32$.

Protein was measured by a modified Lowry procedure [20] and standardized against serum albumin. SDS-polyacrylamide gel electrophoresis was performed on 12.5% slab gels [21]. Absorption spectra were measured with an Aminco DW-2a UV/Vis spectrophotometer with the sample cuvette thermostatted at 0°C, and were recorded with a Nicolet 2090 digital oscilloscope, which was calibrated for wavelength and interfaced to the spectrophotometer as described [22].

The $bc_1$ complexes were suspended in 50 mM Tris-HCl, pH 7.5, at 1.0–1.5 mg/ml protein. NaCl and Triton X-114 were then added to concentrations of 50–150 mM and 0.5–1.5% (v/v), respectively, and the mixtures were allowed to mix on a rocker table for 1 h at 0°C. The solutions were visibly clear at 0°C, and were allowed to reach the cloud point slowly by insulation of the centrifuge tubes in a foam pad. The slow transition of the cloud point enhanced the separation, presumably by promoting formation of the microscopic phase.

At the cloud point the solutions became turbid, and were incubated at 30°C for 3 min. The samples were then overlaid on a 2-fold volume of a cushion of 6% (w/v) sucrose, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.06% Triton X-114 in a conical centrifuge tube and centrifuged for 5 min at 1200 rpm (300 × *g*) at 25°C in a Beckmann TJ-6 clinical centrifuge equipped with a swinging bucket rotor.

The separation procedure is shown schematically in fig.1. During centrifugation, the detergent phase sedimented through the sucrose cushion, while the aqueous phase remained above the cushion. The aqueous phase and sucrose cushion were then removed, and the two separated phases analyzed as described below. At the appropriate detergent and salt concentrations, the initial phase

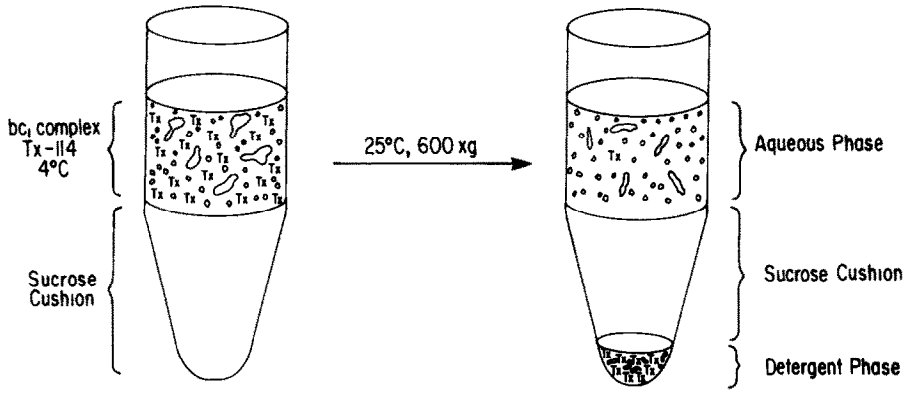


Fig.1. Schematic representation of the purification of cytochrome *b* from bacterial $bc_1$ complexes by phase separation with Triton X-114.

separation is nearly quantitative. However, an additional wash of each phase may be necessary to yield a clean preparation.

## 3. RESULTS AND DISCUSSION

The purification of cytochrome *b* by temperature-dependent phase separation into Triton X-114 is based on the method of Bordier [19]. The Triton X series of nonionic detergents undergo a temperature-dependent, microscopic phase separation which results in the formation of two distinct phases; an aqueous phase which is depleted in detergent, and a detergent-enriched phase. The temperature at which the phase separation occurs, termed the 'cloud point', depends on the number of oxyethylene groups of the detergent [23]. Triton X-114 reaches a cloud point at 20°C [24]. Upon phase separation, hydrophilic proteins partition preferentially into the aqueous phase, while hydrophobic, integral membrane proteins partition preferentially into the detergent phase [19].

The $bc_1$ complex from *P. denitrificans* consists of three subunits; a 62 kDa protein which is cytochrome $c_1$, a 39 kDa protein which is cytochrome *b*, and a 20 kDa protein which is the iron-sulfur protein [16]. Upon phase separation with Triton X-114, cytochrome $c_1$ and the iron-

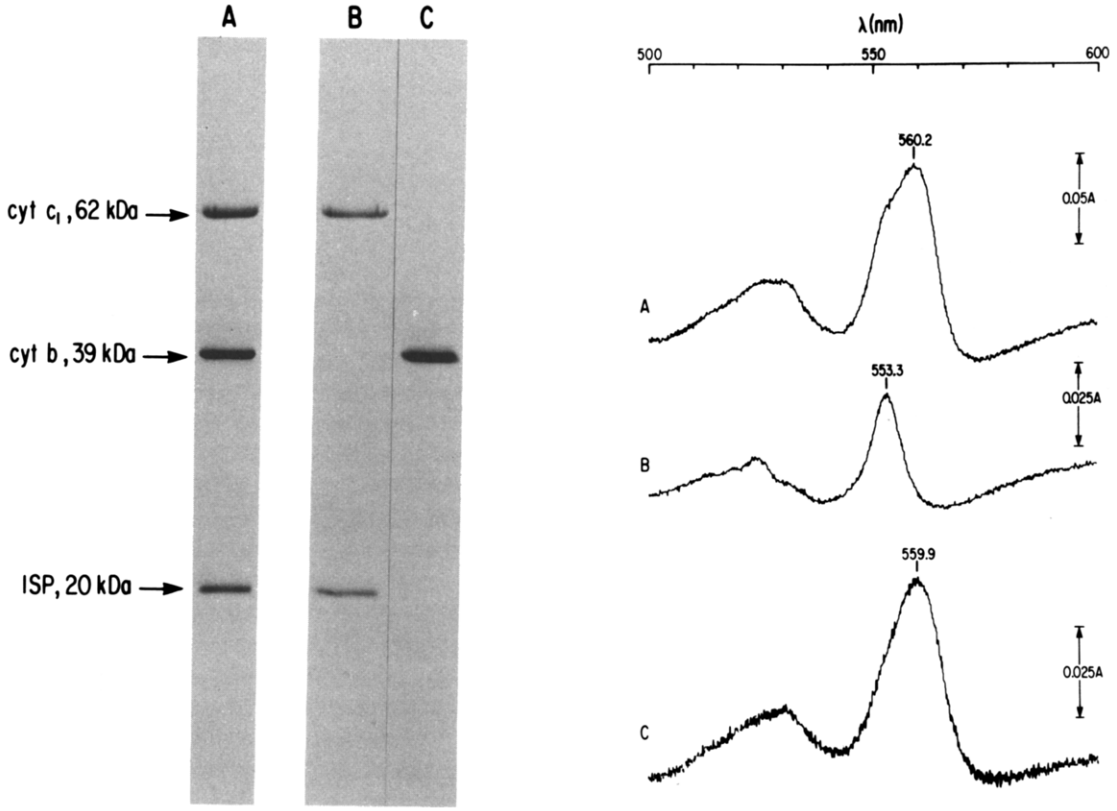


Fig.2. Purification of cytochrome *b* from the $bc_1$ complex of *P. denitrificans*. (Left) SDS-PAGE; lanes: (A) purified $bc_1$ complex from *P. denitrificans* [16]; (B) aqueous phase following phase separation, containing cytochrome $c_1$ and the Rieske iron-sulfur protein; (C) detergent phase following phase separation, containing cytochrome *b*. (Right) Absorption difference spectra of the dithionite-reduced minus ferricyanide-oxidized cytochrome $bc_1$ complex from *P. denitrificans* (A) and of the aqueous (B) and detergent phases (C) following phase separation with Triton X-114. Spectra were taken at 0°C. The protein concentration of the purified $bc_1$ complex was 0.32 mg/ml.

sulfur protein partition into the aqueous phase, and cytochrome *b* partitions into the detergent phase. The detergent phase appears as a cherry red droplet due to the presence therein of cytochrome *b*. The aqueous phase appears a faint pink due to the presence therein of cytochrome $c_1$.

The $bc_1$ complex from *R. sphaeroides* consists of four subunits; a 44 kDa protein which is cytochrome *b*, a 32 kDa protein which is cytochrome $c_1$, a 21 kDa protein which is the iron-sulfur protein [antibodies raised against the iron-sulfur protein of *P. denitrificans* cross-react with this polypeptide in the complex of the photosynthetic bacterium and with the iron-sulfur protein of the beef heart and yeast $bc_1$ complexes (Yang, X. and Trumpower, B., unpublished)], and a 13 kDa polypeptide of unknown function. In a manner analogous to *P. denitrificans*, cytochrome $c_1$ and the iron-sulfur protein partition into the aqueous phase, and cytochrome *b* partitions into the detergent phase. The 13 kDa polypeptide also partitions into the aqueous phase at lower detergent concentrations (0.5%). At detergent concentrations low enough to partition quantitatively the 13 kDa subunit into the aqueous phase, partitioning of cytochrome *b* into the detergent phase is incomplete. However, quantitative recovery of cytochrome *b* can be accomplished by washing the aqueous phase twice with 0.5% Triton X-114.

The concentrations of Triton X-114 and salt in-

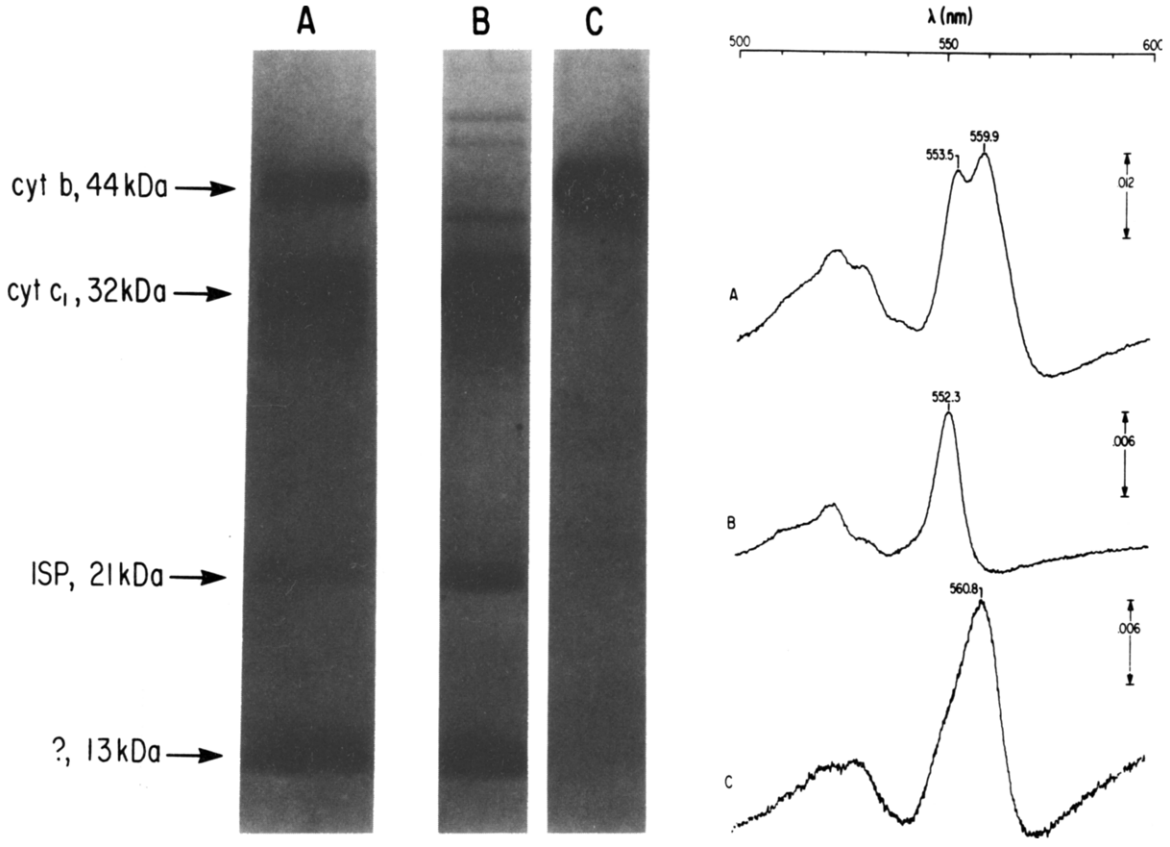


Fig.3. Purification of cytochrome *b* from the $bc_1$ complex of *R. sphaeroides*. (Left) SDS-PAGE; lanes: (A) purified $bc_1$ complex from *R. sphaeroides* [18]; (B) aqueous phase following phase separation, containing cytochrome $c_1$, the Rieske iron-sulfur protein, and the 10 kDa subunit; (C) detergent phase following phase separation, containing cytochrome *b*. (Right) Absorption difference spectra of the dithionite-reduced minus ferricyanide-oxidized cytochrome $bc_1$ complex from *R. sphaeroides* (A) and of the aqueous (B) and detergent phases (C) following phase separation with Triton X-114. Spectra were taken at 0°C. The protein concentration of the purified $bc_1$ complex was 0.09 mg/ml.

fluence the efficiency of separation of the respiratory complexes. Some polypeptides have a tendency to partition into both phases. In these instances we found that it is necessary to manipulate the detergent and salt concentrations to achieve separation. At higher salt concentrations it may also be necessary to increase the specific gravity of the cushion by adding a few crystals of sucrose. Dilution of the aqueous phase is observed with each separation due to diffusion. It is thus necessary to remove visually the aqueous phase from the cushion.

We also attempted to use this method to purify the cytochromes *b* from yeast and beef heart mitochondria, which contain larger numbers of peptides than their bacterial counterparts [18]. With these species we found that some of the polypeptides of the cytochrome $bc_1$ complexes resist clean partitioning into a single phase. This may indicate stronger structural associations in the mitochondrial complexes. However, with further experimentation, it may be possible to achieve a comparable purification of mitochondrial cytochrome *b*.

The biochemical and biophysical properties of the three-subunit $bc_1$ complex from *P. denitrificans* have been described elsewhere [16]. As can be seen in the left panel of fig.2 the Triton X-114-purified cytochrome *b* is homogeneous by electrophoretic criteria, and the resolution of cytochrome *b* from the iron-sulfur protein and cytochrome $c_1$ subunits is quantitative.

Absorption difference spectra of the purified $bc_1$ complex from *P. denitrificans*, and of the aqueous and detergent phases are shown in the right panel of fig.2. Spectra were recorded at 0°C, since samples at room temperature are above the cloud point. The spectra indicate that the cytochrome is not grossly denatured. Indeed, the low-potential heme may be present in the oxidized form, since it was apparent in spectra taken immediately following reduction with dithionite, but disappeared in spectra of the same sample taken minutes later.

Phase separation of the purified $bc_1$ complex from *R. sphaeroides* yields results analogous to those of *P. denitrificans*, as shown in fig.3. Again, the electrophoresis gels and absorption spectra demonstrate that the separation is nearly quantitative, although two detergent extractions were required in this instance.

Finally, it should be noted that treatment of bacterial $bc_1$ complexes with Triton X-114 is also an effective method for purification of cytochrome $c_1$ and the iron-sulfur protein. These polypeptides are present in an essentially detergent-free aqueous phase following treatment with Triton X-114, and an additional gel filtration step is all that should be required for their separation.

The simplicity of this method, and the quantitative recoveries obtained, should make this a useful procedure when it is necessary to analyze small amounts of sample, such as in confirming sequence changes introduced into these proteins by site-specific mutagenesis. In addition, this method may be applicable to other bacterial species which produce cytochrome $bc_1$ complexes having subunit compositions similar to those examined here. And finally, the observation that the cytochrome *b* is initially, albeit transiently, recovered as two spectrally distinct forms, indicates that this procedure might be extended to obtain resolution and reconstitution of a biologically active form of this integral membrane protein.

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