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Cytochrome aa_3 from heterocysts of the cyanobacterium *Anabaena variabilis*: isolation and spectral characterization

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Cytochrome *c* oxidase (ferrocytochrome-*c*: oxygen oxidoreductase, EC 1.9.3.1) has been prepared from heterocysts of *Anabaena variabilis*. The enzyme was solubilized with desoxycholate (0.2%) and octyl-β-D-thioglucoside (1%) followed by purification on two anion-exchange columns in the presence of genapol x-080. Purification factors of 145 with regard to protein and of 1373 with regard to chlorophyll were obtained. The γ-peak of the oxidized enzyme is at 420 nm. The difference spectra (oxidized / reduced) show absorption peaks at 440, 517 and 604 nm. The CO compound of the reduced enzyme exhibits maxima at 435 and 586 nm. The absorption characteristics including CO-difference spectra together with a high cyanide sensitivity ($K_i = 0.5$ μM) indicate that the oxidase is of the aa_3 type.

Introduction

In the last few years different types of cytochrome-*c* oxidases have been purified and characterized from various bacterial sources [1,2]. Little is known on cyanobacterial cytochrome-*c* oxidase(s) although they are of special interest from an evolutionary point [3], and with respect to the interaction of photosynthetic and respiratory electron transport (for review see Ref. 4). This lack of knowledge is due to serious obstacles concerning the purification of cytochrome-*c* oxidase(s) from cyanobacteria: the enzyme is present in comparatively low concentration, respiration rates are very low and cannot be stimulated by substrates [5], high contents of photosynthetic pigments (chlorophylls and carotenoids) make the spectral characterization very difficult, and, finally, the enzyme proved to be highly unstable during purification.

In 1953, Webster and Frenkel [6] interpreted the KCN sensitivity of cyanobacterial respiration in terms of an aa_3-type terminal oxidase. The first partial photometric characterization was reported by Peschek [7] who showed partial difference spectra and CO-difference spectra from acetone-extracted membranes of *Anacystis nidulans* containing cytochrome-*c* oxidase. An aa_3 cytochrome-*c* oxidase in *A. nidulans* was suggested, but the occurrence of other terminal oxidases could not be excluded. Immunoblotting experiments, using an antibody against *Paracoccus* cytochrome aa_3 [8], provided further evidence for a terminal cytochrome aa_3 oxidase in *A. nidulans*. Heterocysts of *Anabaena variabilis* are known to have high respiration rates with regard to protein and chlorophyll. Houchins and Hind [9] could record a broad peak at 603 nm in the difference spectra of heterocystous membranes prepared from *A. variabilis*, apparently containing cytochrome aa_3.

Abbreviations: PMSF, phenylmethylsulphonyl fluoride; PMS, phenazine methosulfate.

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In this paper for the first time absolute and difference spectra of a partially purified cyanobacterial cytochrome-*c* oxidase are reported and interpreted in terms of an aa_3-type cytochrome-*c* oxidase.

Materials and Methods

Preparation of heterocystous membranes

Axenic *A. variabilis* was grown in batch cultures according to Ref. 10. The inoculation was equivalent to 0.2 μg chlorophyll per ml and cultures were grown for 26 h with 5% CO_2. Cells were washed twice with buffer A containing 50 mM Tris-HCl, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.5). Approx. 100 g of fresh wet cells were resuspended in 200 ml buffer A and passed through a French pressure cell (Aminco, model J4-3339) at 500 psi. Intact heterocysts were collected by centrifugation for 7 min at $1400 \times g$. Washed heterocysts (three times, 7 min, $1400 \times g$) were broken by passing them three times through the French pressure cell at 1800 psi. Membranes of heterocysts were centrifuged for 45 min at $180\,000 \times g$.

Preparation of cytochrome-c oxidase

Membranes of heterocysts were resuspended in buffer B (Tris-HCl 40 mM, EDTA 1 mM (pH 7.3)) to a protein content of 2–4 mg/ml. This membrane suspension was mixed with detergent stock solutions (10% detergent) to include final concentration of both 1% octyl-β-D-thioglucoside (Calbiochem, Frankfurt, F.R.G.) and 0.2% desoxycholate (Sigma, Deisenhofen, F.R.G.). The suspension was incubated at 4°C for 2 h without stirring and centrifuged at $450\,000 \times g$ for 90 min. The supernatant was gently agitated for 1 h with Bio-Beads (1 g Bio-Beads per 0.02 g desoxycholate). Bio-Beads were removed by filtration and genapol x-080 (Calbiochem, Frankfurt F.R.G.) was added to a final concentration of 0.1%.

The genapol x-080-containing solution was applied to a FPLC-Mono Q HR 10/10 column (Pharmacia), pre-equilibrated with buffer C (40 mM Bistris, 1 mM EDTA, 0.1% genapol x-080 (pH 6.3)). After washing with buffer C (until the absorbance at 280 nm was less than 0.1), the column was developed with a NaCl gradient (0–0.35 M). Fractions (4 ml) were collected and assayed for cytochrome-*c* oxidase. Fractions with high cytochrome-*c* oxidase activity were pooled and desalted by Pd-10 columns (Pharmacia), equilibrated with buffer C. Subsequently, the concentration of genapol x-080 was raised up to 0.2%. After loading on a FPLC-Mono Q HR 5/5 column, preequilibrated with buffer C, proteins were eluted with a NaCl gradient (0–0.35 M, 1 ml fractions). Spectra were recorded after desalting on Pd-10 columns. FPLC column chromatography was performed at room temperature. The preparation, starting from the solubilized enzyme including spectroscopic characterization, was performed within 12 h.

Analytical methods

Protein was estimated according to Ref. 11 using the Bio-Rad assay, chlorophyll according to Ref. 12. Cytochrome-*c* oxidase activity was measured by oxidation of horseheart cytochrome-*c* (Sigma, F.R.G.) following the absorbance change at 550 nm ($\varepsilon = 21\ mM^{-1} \cdot cm^{-1}$), either in a double-beam spectrophotometer (Perkin Elmer, model 124) or by the dual-wavelength mode (550–540 nm, $\varepsilon = 19.5\ mM^{-1} \cdot cm^{-1}$) using a Shimadzu UV-300 spectrophotometer, which was also used to record the spectra. The assay (1 ml) contained 40 mM Tris-HCl, 1 mM EDTA, 0.05% desoxycholate, 0.05% genapol x-080, 10 μM ferrocytochrome *c* (pH 7.5). SDS-polyacrylamide gel electrophoresis was performed according to Ref. 13 in 15% polyacrylamide gels. Prior to electrophoresis, samples were concentrated and dialysed with Ultra Thimbles UH 100/25 (Schleicher & Schüll, Dassel, F.R.G.).

Results and Discussion

Purification

Optimum solubilization of cytochrome-*c* oxidase was achieved by the combined application of desoxycholate and octyl-β-D-thioglucoside. For purification factors during solubilization and purification procedure see Table I. For anionic-exchange chromatography the ionic detergent desoxycholate was replaced by genapol x-080 without any loss of activity. A typical elution pattern of the Mono Q HR 10/10 column is shown in Fig.

TABLE I

PURIFICATION OF CYTOCHROME-*c* OXIDASE

Purification step	Enzyme Act. [a]	Chlorophyll Conc. ($\mu g \cdot ml^{-1}$)	Protein Conc. ($\mu g \cdot ml^{-1}$)	Spec. Act. [b]	Yield (%)
Heterocystous membranes	1670	180	1980	841	100
Solubilized enzyme	1670	60	490	3440	100
Mono Q HR 10/10	1180	n.d. [c]	n.d. [c]	n.d. [c]	64
Mono Q HR 5/5	7300	0.6	60	121700	41

[a] nmol ferrocytochrome-*c* oxidized per ml per min.
[b] nmol ferrocytochrome-*c* oxidized per min per mg protein.
[c] n.d., not determined.

1A. Note, that an enormous contribution of the 280 nm absorbance was due to pigments like chlorophyll and carotenoids. Cytochrome-*c* oxidase eluted at a NaCl concentration of 180–190 mM, the main absorbance peaks at 280 nm showed up at lower salt concentrations. A typical elution pattern of the second anion-exchange column, Mono Q HR 5/5, is shown in Fig. 1B. To purify of further chlorophyll during this chromatographic step, the genapol x-080 concentration of the fraction applied to this column was increased to 0.2%. After the purification procedure, the specific activity of cytochrome-*c* oxidase increased from 841 in the membrane fraction to 121 700 in the pooled fraction of the second ion-exchange chromatography, with a yield of 41% of cytochrome-*c* oxidase activity. The purification procedure described removed the chlorophyll content down to 0.1% of the starting material. The preparation has not yet been purified to homogeneity. The SDS-gel (Fig. 2) shows bands at 19–23, 35, 42–46 and 67 kDa which cannot yet be assigned to cytochrome-*c* oxidase.

Spectral properties

Typical absorption spectra of the partially purified cytochrome-*c* oxidase are given in Fig. 3. The absorption peak at 665 nm was due to a small

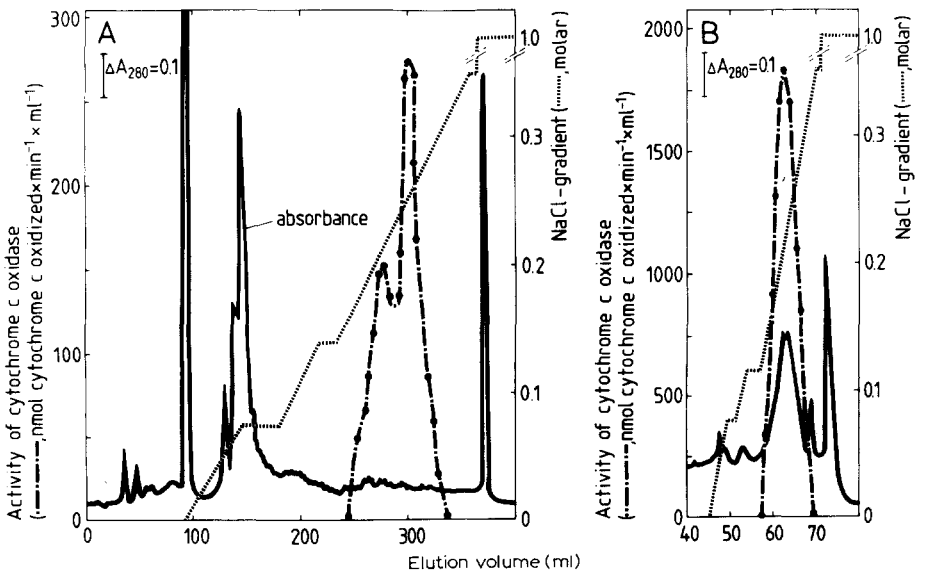

Fig. 1. Elution patterns of *Anabaena* cytochrome-*c* oxidase from anion-exchange columns. (A) Mono Q HR 10/10, flow rate 4 $ml \cdot min^{-1}$ and (B) Mono Q HR 5/5, flow rate 2 $ml \cdot min^{-1}$. Details given in Materials and Methods.

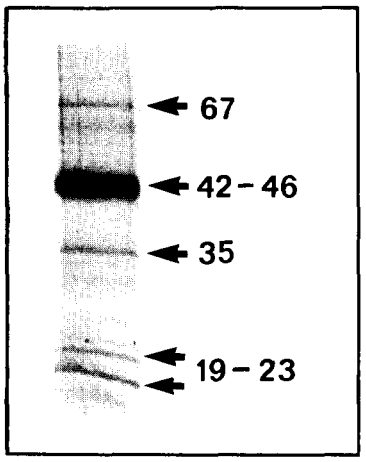

Fig. 2. SDS-polyacrylamide gel electrophoresis of *Anabaena* cytochrome-*c* oxidase after Coomassie blue staining. Molecular weights were estimated according to a standard curve with low-molecular-weight markers (Pharmacia, Uppsala, Sweden).

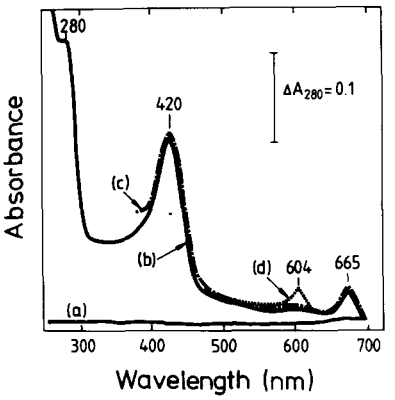

Fig. 3. Absorbance spectra of *Anabaena* cytochrome-*c* oxidase, recorded from a partially purified cytochrome-*c* oxidase, containing an enzyme activity of 6045 nmol ferrocytochrome *c* oxidized per min per ml (equivalent to 100700 nmol ferrocytochrome-*c* oxidized per min per mg protein). (a) baseline, (b) oxidized enzyme (as prepared), (c) enzyme reduced with a few grains of ascorbate, (d) reduced with ascorbate plus 20 μM PMS.

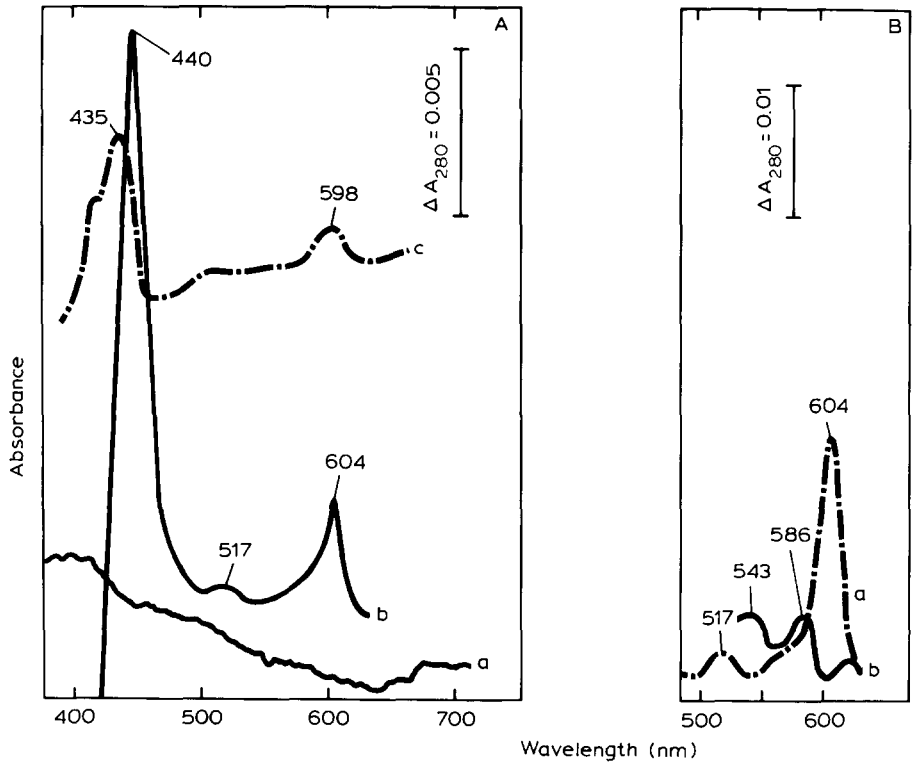

Fig. 4. Difference spectra of *Anabaena* cytochrome-*c* oxidase. (A) Baseline (a), ascorbate-reduced minus oxidized (b), and (ascorbate-reduced plus CO) minus (ascorbate-reduced) (c). (B) Ascorbate/PMS-reduced minus oxidized (a), and (ascorbate/PMS-reduced plus CO) minus (ascorbate/PMS-reduced) (b).

contamination with chlorophyll, while the absorption maximum at 420 nm is typical for the Soret band of oxidized cytochrome aa_3 [2]. In agreement with Houchins and Hind [9], ascorbate could only partially reduce the enzyme – the spectrum showing a small shift of the Soret band to longer wavelengths. Complete reduction was obtained by addition of ascorbate plus phenazine methosulfate (PMS), revealing a characteristic absorption maximum at 604 nm, indicative of heme *a*. A complete reduction by sodium dithionite showing the Soret band could not be performed due to a color change of the genapol-containing sample after dithionite addition.

The difference spectrum of reduced cytochrome-*c* oxidase (Fig. 4) was characterized by its α-, β- and γ-peaks at 604, 517 and 440 nm. Note that the α-maximum of the ascorbate-reduced enzyme raised after the addition of PMS from an absorbance of 0.0043 to 0.015, indicating that only 29% of the oxidase could be reduced by ascorbate.

Fig. 4 also presents the CO-difference spectrum, (ascorbate-reduced plus CO) minus (ascorbate-reduced) with peaks at 435 nm and 598 nm and a shoulder at 417 nm. The spectrum (ascorbate/PMS-reduced plus CO) minus (ascorbate/PMS-reduced) revealed a broad band at 543 nm and a peak at 586 nm. The γ-peak at 435 nm together with the α-peak at 598 nm (ascorbate-reduced) and at 586 nm (ascorbate /PMS-reduced) are characteristic for heme a_3 liganded with CO [2,7]. The shoulder at 417 nm and the additional peak at 543 nm indicate the instability of heme aa_3 [14].

Using the results reported in this paper, the comparison of spectral properties of mammalian [15] and bacterial cytochrome-*c* oxidases [1,2] and the sensitivity of the oxidase against cyanide ($K_i = 0.5$ μM [16]) allows for the reliable conclusion that the cytochrome-*c* oxidase of heterocysts of *A. variabilis* is of the aa_3 type.

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