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IMMUNOLOGICAL AND SPECTRAL CHARACTERIZATION OF PARTLY PURIFIED CYTOCHROME OXIDASE FROM THE CYANOBACTERIUM SYNECHOCYSTIS 6714

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SUMMARY: Membranes were isolated by French pressure cell extrusion of lysozyme-preincubated cells of the cyanobacterium Synechocystis 6714 after growth in the presence of 0.4 M NaCl for 4 days. These cells showed up to 6-fold respiratory activity (oxygen uptake) when compared to control cells. Separation of plasma and thylakoid membranes revealed that the major part of cytochrome c oxidase was associated with the latter. Immunoblotting of sodium dodecylsulfate polyacrylamide gel electrophorized membranes with antisera raised against subunit I, subunit II, and the holoenzyme of the aa_3-type cytochrome oxidase from Paracoccus denitrificans gave specific and complementary cross-reactions at apparent molecular weights of about 25 and 17-18 kDa, respectively. Crude membranes were solubilized also with n-octyl glucoside, and the cytochrome oxidase was separated from the extract by affinity chromatography using immobilized cytochrome c from Saccharomyces cerevisiae. The enzyme was eluted with KCl/octyl glucoside. Dialysed and concentrated enzyme solution, which was free of b- and c-type cytochromes, gave reduced α- and γ-peaks around 603 and 443 nm, respectively. Upon treatment of the sample with carbon monoxide the peaks were found at 593 and 433 nm, respectively. Photodissociation spectra of the CO-complexed enzyme were in full agreement with cytochrome aa_3 being a functional cytochrome oxidase in Synechocystis 6714.

Cyanobacteria (blue-green algae) are phototrophic prokaryotes uniquely endowed with mechanisms for oxygenic, plant-type photosynthesis and aerobic respiration (1,2). In marked contrast to photosynthesis, cyanobacterial respiration has not been receiving proper attention up to recent years (3,4). First indication that the cytochrome oxidase of cyanobacteria might be an aa_3-type enzyme was provided by spectrophotometric studies on crude membrane preparations (5-7). Only quite recently, however, could these findings be substantiated using refined membrane preparation and separation procedures (8,9) and immunological techniques (9,10). Thus with Anacystis nidulans it was found that the SDS-

Abbreviations: CM, cytoplasmic (plasma) membrane; ICM, intracytoplasmic (thylakoid) membranes; cyt, cytochrome, pmf, protonmotive force; EPR, electron paramagnetic resonance; HEPES, 4-(2-hydroxyethyl-)1-piperazine-ethanesulfonate; PMS, phenazine methosulfate; TMPD, N,N,N',N'-tetramethyl-p-phenylene diamine.

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solubilized cytochrome oxidase consisted of (at least) two subunits homologous to the corresponding polypeptides of the aa_3-type P. denitrificans cytochrome oxidase as evidenced by immunological cross-reactivity (9-11), and that the oxidase was predominantly situated in the plasma membrane (9,12), especially after prolonged growth in the presence of elevated concentrations of NaCl which led to a pronounced increase of cytochrome oxidase levels (12). During the present investigation we noted a similar phenomenon with Synechocystis 6714, another unicellular cyanobacterium but belonging to a different typological subgroup (13). Contrary to Anacystis, however, isolated thylakoid membranes from Synechocystis always showed higher cytochrome oxidase activity than plasma membranes. Thus we simply used crude membranes from NaCl-grown Synechocystis to partly purify the cytochrome oxidase through cytochrome c affinity chromatography of n-octyl glucoside extracts. Spectral, immunological and reactivity properties of the solubilized and/or partly purified enzyme clearly conformed to an aa_3-type cytochrome oxidase (14,15).

MATERIALS AND METHODS

Synechocystis 6714 (Pasteur Culture Collection of Cyanobacteria, Paris, France) was grown photoautotrophically at 34°C in medium BG-11 (13) supplemented with 1 mM Na_2CO_3 and 10 mM $NaHCO_3$ and gassed with 1.5% CO_2 in sterile air in axenic batch cultures illuminated with 15-20 w/m^2 warm white fluorescent light (as measured with a YSI Radiometer, model 65, at the surface of the vessel). NaCl (0.4 M final concentration) was added to the culture as a concentrated solution in sterile BG-11 (cf. Fig.1). Usually, the cyanobacteria were harvested by centrifugation at room temperature after 4-5 days of growth in the presence of 0.4 M NaCl, washed free of the salt with 10 mM HEPES/NaOH buffer (pH 7.4), and immediately used for the experiments.

Membranes were prepared by French pressure cell extrusion of lysozyme-pretreated cells (8,9). The protein/chlorophyll ratio of crude membranes was around 20. CM and ICM were separated by discontinuous sucrose density gradient centrifugation (8,9). Isolated and purified CM was devoid of spectroscopically detectable chlorophyll. Protein and chlorophyll were measured according to Refs. 16 or 17 (depenending on detergent concentration) and 18, respectively. Crude membranes were solubilized with various concentrations of Triton X-100 or n-octyl glucoside (cf. Fig. 2) at 4°C for 30 min, followed by centrifugation at 175,000 g (1h) in a Beckman ultracentrifuge, model L5-50.

Affinity chromatography of the Synechocystis cytochrome oxidase present in 1% n-octyl glucoside extracts was performed on Saccharomyces cerevisiae cytochrome c (type VIII, Sigma) covalently coupled to activated thiol-Sepharose 4B (Pharmacia) according to the methods devised by Azzi et al. (19-22) except that Triton X-100 was replaced by octyl glucoside in all purification steps. Finally, fractions eluting at 120-150 mM KCl (0.1-0.5% octylglucoside) contained spectrally pure cytochrome aa_3, without contaminating b- or c-type, or other, cytochromes. Pooled fractions were dialysed against 10 mM K-phosphate buffer (pH 6.8) containing 0.1% octyl glucoside, concentrated on DEAE Biogel (22), and immediately used for the assays without further purification. The yield, based on heme a, was around 10% only, which is lower than the yield given for the same procedure

applied to other bacterial aa_3-type cytochrome oxidases (e.g. cf. Ref.21) At the same time the activity was highly unstable, only 20-30% of the activity measured on crude membranes at the earliest time possible (i.e. 7-10 h after harvest of the cells) being left after further 24 h storage of the membranes or extracts at 4°C in the dark.

Oxidation of ascorbate-reduced and dialysed horse heart or yeast cytochrome c (both of which exhibited roughly equal affinity to the Synechocystis oxidase), as well as reduction of the partly oxidized cytochromes (cf. Fig.3), was measured with a Shimadzu UV-300 dual-wavelength spectrophotometer at room temperature using $\Delta\varepsilon$ (red - ox) = 19.5/mM.cm at 550-540 nm (23). The spectrophotometer was equipped with a cross-illumination device; 20 w/m^2 monochromatic light (at the site of cuvettes) was provided by a 1,000 w Oriel Xenon Lamp through light pipes and appropriate interference filters (Schott AG, Mainz, FRG; half-band width approximately 10-12 nm). The cuvettes used contained 0.4 ml sample solution or suspension (1 cm light path). Room temperature difference spectra were recorded in the same apparatus at 1 nm slit width, using a Shimadzu Sapcom computer for baseline correction and data storage. Photodissociation spectra were determined according to Appleby (24).

Oxygen uptake was measured polarographically at room temperature (approximately 23-26°C) using a YSI Oxygen Monitor, model 53, the sample compartment holding 2.5 ml, and recorded with a high-sensitivity Servogor S recorder (Goerz, Vienna, Austria). The basic reaction mixture contained, in 10 mM K-phosphate buffer (final pH 6.8), 0.5 mM TMPD, 3 mM Na-ascorbate, 1 mM Na-EDTA, 1 mg protein, and varying amounts of horse heart cytochrome c (cf. Fig.4). All activities were determined in the presence of 0.1% octyl glucoside.

Sodium dodecyl sulfate poolyacrylamide gel electrophoresis (25) of the crude membranes and Western blotting (26,27) was performed as previously described for Anacystis membranes (10), using polyclonal antibodies against subunits I and II, and the holoenzyme, of the aa_3-type cytochrome oxidase from Paracoccus denitrificans (15,28) and goat anti-rabbit IgG-horse radish peroxidase conjugate as a second antibody; cross-reacted bands were made visible through hydrogen peroxide/4-chloro-1-naphthol color reaction (10).

All data given in figures and tables of this paper are mean values of at least five measurements on samples obtained from different batches of independently harvested cultures, standard deviations being within 15 to 20% for each series of determinations.

RESULTS AND DISCUSSION

Fig.1 shows the effect of NaCl (0.4 M final concentration, added to actively growing cultures) on growth and cyt c oxidase activity in isolated CM and ICM of Synechocystis 6714. As compared to Anacystis (12), the lag phase needed for adaptation to the high NaCl medium (29) is shorter, and the NaCl-induced increase in cyt c oxidase activity is mostly due to the ICM (up to 8-fold stimulation) while the CM almost lacks any activity when the cells are grown in normal medium BG-11 (< 30 mM Na^+) but incorporates cyt oxidase up to an activity level of 30 nmol/min per mg protein only during 3-4 days of growth in the high NaCl medium. This indicates that, in contrast to Anacystis (12,30), in Synechocystis most of the trans-CM pmf necessary for active Na^+ extrusion is provided through a CM-bound H^+-translocating ATPase powered by ATP from coupled respiratory electron transport in the ICM, with CM-bound electron transport per se playing a supplementary role only (30).

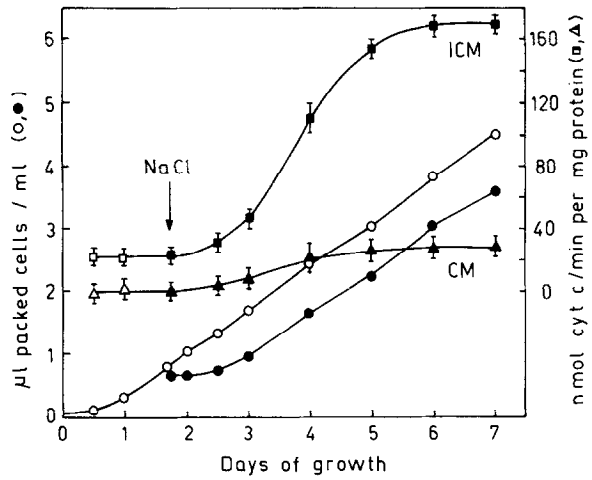

Fig.1. Photoautotrophic growth of Synechocystis 6714 in the absence (o-o) and presence (●-●) of 0.4 M NaCl, and oxidation of horse heart cyt c by isolated and purified CM and ICM preparations. Cyt c from Saccharomyces cerevisiae was as active as that from horse heart (not shown; for details cf. Materials and Methods). Replacing NaCl by KCl gave the same results as obtained with control cells (not shown). Note that seemingly linear instead of "logarithmic" growth resulted from increasing light limitation (self--shadowing of the cells) with progressive growth at rather low light intensities (cf. Refs. 12 and 29).

Membranes from cells grown in the presence of 0.4 M KCl behaved like those from normal, i.e. low-NaCl cells (cf. Ref.12).

Attempts to solubilize the Synechocystis membranes with Triton X-100 which, at 2-3% (w/v) afforded 100% solubilzation, gave extracts yet with greatly diminished cyt oxidase activity while n-octyl glucoside, whose solubilizing power was less, yielded extracts with much higher activity (Fig.2). Therefore 1% octyl glucoside was chosen to solubilize the (crude)

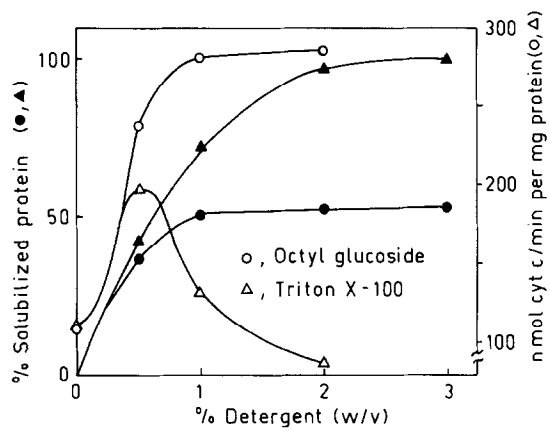

Fig.2. Effect of increasing concentrations of Triton X-100 and n-octyl glucoside on the extent of membrane solubilization (●, ▲) and on the activity of the solubilized cyt c oxidase (○, △). Crude membranes (2 mg protein/ml) were solubilized at 4°C for 30 min throughout. Oxidation of horse heart ferrocytochrome c (20 µM) was followed by dual wavelength spectrophotometry at room temperature (cf. Materials and Methods).

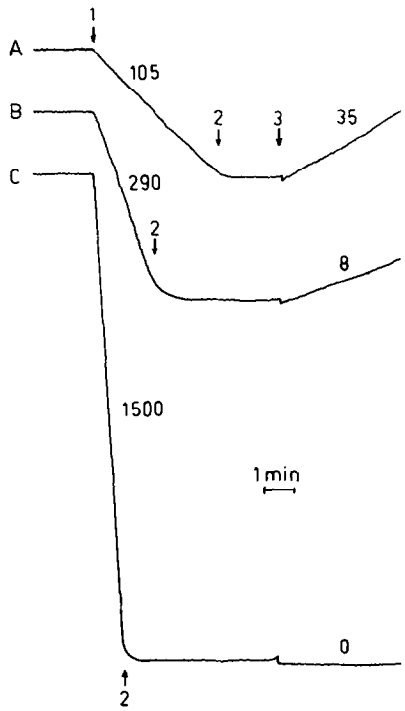

Fig.3. Spectrophotometric recorder traces of cyt c oxidation (downward deflection) and reduction (upward deflection) by crude membranes (A), 1% octyl glucoside extracts (B) and partly purified cyt oxidase in the presence of 0.1% octyl glucoside (C). Numbers adjacent to the traces give nmol cyt c/min per mg protein. 25 µM horse heart (or yeast) cyt c (80% reduced) was present initially. - Additions: (1), 30 (A and B) or 15 (C) mg protein/ml; (2), 3 µM KCN; (3) 3 mM NADH (the rate of reduction was saturated at this concentration; 3 mM NADPH gave roughly the same effect, not shown). Measurements taken at room temperature.

membranes of Synechocystis, and also in all further purification steps (20-22) Triton X-100 was replaced by octyl glucoside (cf. Materials and Methods). Fig.3 describes the oxidation and reduction of horse heart (or yeast) cyt c by crude membranes (A), octyl glucoside extracts (B) and cyt c affinity chromatographed enzyme (C). Membrane solubilization and cyt oxidase purification resulted in up to 3- and 15-fold increased cyt c oxidase activity (per mg protein) when proceeding from crude membranes to solubilized membranes and the partly purified oxidase, respectively, while in the same order the ratio of cyt c oxidase/reductase activities increased from 3 to 36 and infinity, showing that the partly purified cyt c oxidase was free of reductase activity (Fig.3). Cyt c oxidase activities in crude membranes and partly partly purified enzyme preparations in the absence and presence of some inhibitors are seen from Table 1. Note that the inhibitory effect of the copper-chelating salicyl aldoxime (7) indicates involvement of redox-active copper in the oxidase function as was recently demonstrated for Anacystis using EPR spectrometry (31). High affinity

Table 1 Cyt oxidase activity of crude Synechocystis membranes and partly purified enzyme preparations in the presence and absence of typical inhibitors. - Spectrophotometric assays conducted at room temperature (20 µM horse heart or yeast ferrocytochrome c initially present; for details cf. Materials and Methods). Standard deviations of measurements on at least 5 separate preparations are given.

Addition	Final concentration	nmol ferrocytochrome c oxidized per min and mg protein	
		Crude membranes[a]	Purified enzyme[b]
None	-	110 ± 10	1,450 ± 90
Carbon monoxide[c]	-	18 ± 2	75 ± 6
KCN	3 µM	0.0	0.0
NaN_3	1 mM	21 ± 3	105 ± 8
Salicyl aldoxime	10 mM	9 ± 1	90 ± 7
KCl	100 mM	5 ± 3	810 ± 50
N_2[d]	-	0.0	0.0

[a] 20-35 µg protein/ml

[b] 7-15 µg protein/ml (in the presence of 0.1% octyl glucoside)

[c] Samples were sparged with 90% (v/v) CO in air for 5 min; other inhibitors were added 1 min prior to the reduced cyt c

[d] Assays conducted anaerobically in Thunberg cuvettes repeatedly evacuated and flushed with oxygen-free nitrogen

toward eukaryotic cyt c and inhibition of the oxidase by very low concentrations of cyanide (and by carbon monoxide) clearly point to a "mammalian-type" enzyme (cf. Ref.7).

The cyt oxidase activity of crude membranes, octyl glucoside extracts, and partly purified enzyme preparations was measured by both dual wavelength spectrophotometry (oxidation of horse heart or yeast cyt c) and polarography (oxygen uptake in the presence of ascorbate-reduced TMPD and cyt c). Dependence of these reactions on the concentrations of cyt c and of neutral salt is shown in Fig.4 A and B, respectively. It can be derived that, in vitro, the oxidase is saturated by 20 µM cyt c only, and that the electron equivalent stoichiometry between cyt c oxidation and O_2 reduction is roughly 4; no hydrogen peroxide was produced during the reaction as the rate of

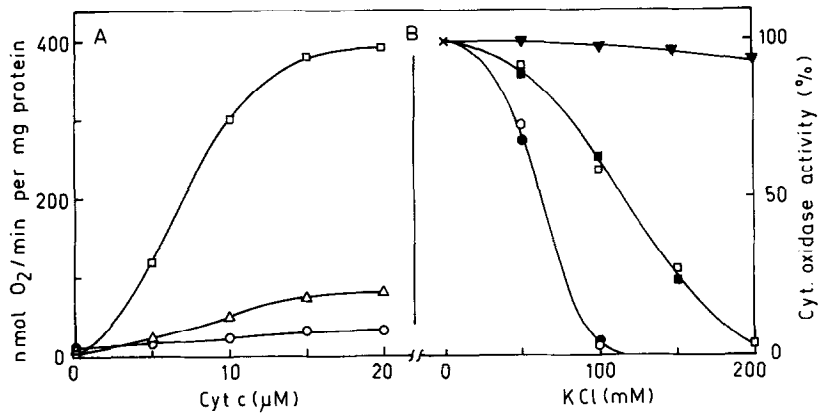

Fig.4. Dependence of cyt oxidase activity in crude membranes (○), octyl glucoside-solubilized membranes (△), and partly purified enzyme preparations in the presence of 0.1% octyl glucoside (□) on the concentration of horse heart (or yeast) cyt c (A, polarographic assay) or KCl (B, polarographic (○, □) and spectrophotometric (●, ■) assays with 20 μM horse heart or yeast cyt c). ▼-▼, TMPD/ascorbate-mediated oxygen uptake without added cyt c. Measurements taken at room temperature. For details cf. Materials and Methods.

oxygen consumption was not diminished by the addition of catalase (results not shown). Similar to other cyt c oxidase systems (21,32,33) elevated ionic strength effectively prevented the interaction between reduced cyt c and the oxidase, both in crude membranes and in a partly purified form (Fig.4B); with membraneous cyt oxidase, however, the inhibition was more pronounced than with the partly purified enzyme, probably due to increased importance of electrostatic interaction in the case of membrane vesicles compared to simple detergent micelles. In the pure ascorbate-TMPD-oxidase system the ionic strength was almost without any influence on the rate of the oxidase reaction (Fig.4B; cf. Ref.21).

Solubilization of crude membranes with 3% SDS followed by SDS-PAGE (25) and Western blotting (26,27), and incubating the nitrocellulose (containing transferred polypeptides) with antisera raised against subunits I and II and the holoenzyme of *Paracoccus denitrificans* aa_3-type cyt oxidase (15,28) according to the procedure previously published for *Anacystis* membranes (10) resulted in two specifically and complementarily cross-reacting protein bands at apparent molecular weights of about 25 and 17-18 kDa, respectively, ascribed to subunits I and II of the *Synechocystis* aa_3-type cyt oxidase which obviously shows a great deal of homology to the corresponding subunits of the *P. denitrificans* enzyme (15,28). Similar results have recently been obtained with another unicellular cyanobacterium, *Anacystis nidulans* (9,10); in this case, however, apparent molecular weights of subunits I and II were found to be 48.5-55 and 32-38.5 kDa, respectively (extreme values from 23 independent preparations), i.e. significantly higher at any rate than those

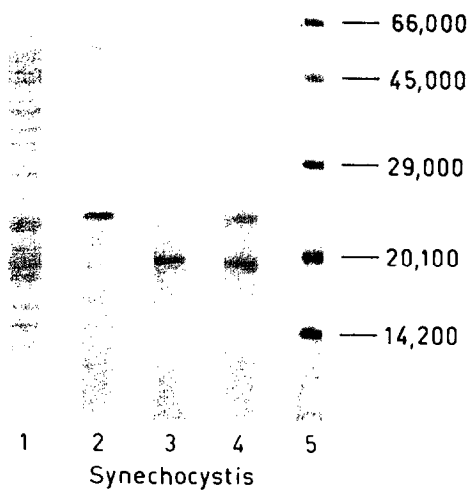

Fig.5 SDS-PAGE (lane 1), and immunoblotting (lanes 2-4) of Synechocystis membrane proteins using antisera raised against P. denitrificans cyt oxidase holoenzyme (dilution 1:300, lane 4), subunit I (1:300, lane 2) and subunit II (1:100, lane 3). Between 50 and 75 µg SDS-solubilized membrane protein was applied to each lane. Separated polypeptides were stained with Coomassie brilliant blue (lane 1), Western-blotted and immunologically cross-reacted protein bands were made visible with GAR-HRP as a second antibody followed by 4-chloro-1-naphthol/hydrogen peroxide color reaction. Lane 5, marker proteins (Sigma MW-SDS-70L-Kit). For experimental details cf. Ref.10.

determined here on Synechocystis membranes (mean values from 5 independent preparations; cf. Materials and Methods). Yet, in either species the highly specific and with respect to subunits I and II and the holoenzyme strictly complementary cross-reaction indicates the presence of a cyt c oxidase closely homologous to the cyt aa_3-type enzyme from P. denitrificans.

Reduced-minus-oxidized difference spectra determined on the partly purified enzyme (Fig.6A) revealed a conspicuous α-peak at 603 nm together with a γ-peak at 443 nm, which is clear indication of an a-type cytochrome (14). Moreover, since either peak shifted to shorter wavelengths by about 10 nm in dithionite-reduced plus CO minus dithionite-reduced spectra (Fig.6A, curve c) the presence of cyt a_3 is inferred. Photoreversibility of the cyt oxidase/CO complex was seen from the fact that, upon cross-illumination with 593 nm light corresponding to the reduced α-peak of the CO-complexed oxidase, the γ-peak of the reduced CO compound at 433 nm disappeared while, at the same time, the reduced γ-peak of the uninhibited enzyme at 443 nm reappeared (24). This, in addition to the results from immunological cross-reaction experiments (Fig.5) may be regarded as most conclusive evidence for the occurrence of an aa_3-type cytochrome as a functional terminal oxidase in the cyanobacterium Synechocystis 6714.

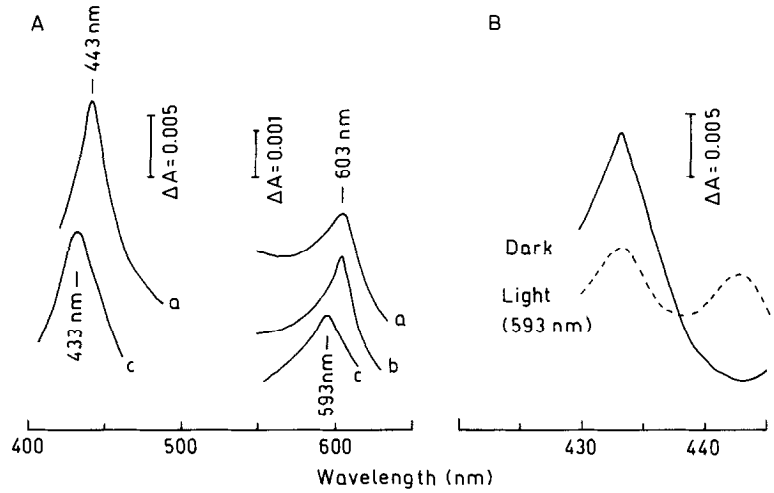

Fig.6 Reduced-minus-oxidized difference spectra (A) and photodissociation spectrum (B) of partly purified cyt oxidase preparations from Synechocystis in the presence of 1% octyl glucoside. (a), dithionite-reduced minus aerated; (b), ascorbate-plus-PMS-reduced minus ferricyanide-oxidized; (c) dithionite-reduced plus CO minus dithionite-reduced. - For photodissociation of the cyt oxidase/CO complex (B) cross-illumination with 680 and 563 nm, used for control, was without effect (not shown). Cuvettes contained 400 µl sample (0.7-2.2 mg protein/ml). Spectra taken at room temperature. For details cf. Materials and Methods.

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