ELSEVIER

Biochimica et Biophysica Acta 1187 (1994) 369–372

BBA Biochimica et Biophysica Acta

Rapid Report

Immunocytochemical localization of the cytochrome-*c* oxidase in a cyanobacterium, *Synechococcus* PCC7942 (*Anacystis nidulans*)

Günter A. Peschek [a,*], Christian Obinger [a], Debra M. Sherman [b], Louis A. Sherman [b]

[a] *Biophysical Chemistry Group, Institute of Physical Chemistry, University of Vienna, A-1090 Vienna, Austria*
[b] *Department of Biological Sciences, Lilly Hall, Purdue University, West Lafayette, IN 47907, USA*

Received 22 June 1994

Abstract

Immuno-gold labeling and electron microscopy was used to localize the cytochrome-*c* oxidase on thin-sectioned cyanobacterium *Synechococcus* PCC 7942 (*Anacystis nidulans*) which had been grown photoautotrophically to the linear (light-limited) growth phase. The overwhelming fraction of the gold particles was found associated with the plasma membrane. On immunoblots, however, both plasma and thylakoid membranes, freshly isolated and separated, gave the expected cross-reaction though the latter to a significantly lesser extent that the former. Spectrophotometric and polarographic measurements of cytochrome *c* oxidation by extensively purified plasma and thylakoid membranes gave 95 and 3 nmol cytochrome *c* oxidized per min and mg membrane protein for plasma and thylakoid membranes, respectively, thus conforming to the immunocytochemical data. The results will be discussed with respect to the difficulty of obtaining cyanobacterial thylakoid membrane preparations absolutely free of plasma membranes and the quantitatively variable localization of certain bioenergetic key enzymes and electron transport components in both plasma and thylakoid membranes of cyanobacteria.

Keywords: Cytochrome-*c* oxidase; Respiration; Cytoplasmic (plasma) membrane; Intracytoplasmic (thylakoid) membrane; Localization; Immuno-gold labeling; Cyanobacterium; (*Anacystis nidulans* R2); (*Synechococcus* PCC7942)

Cyanobacteria (blue green algae) are the largest and most diversified, ecologically most successful and evolutionarily most important group of not only photosynthetic prokaryotes, but prokaryotes in general. They are unified by the capability of oxygenic, plant-type photosynthesis which had arisen about 3.2 billion years ago as evidenced by geological and microfossil records [1]. Having been the first to introduce bulk amounts of molecular oxygen into a previously anoxic or near-anoxic terrestrial biosphere they naturally render themselves likely candidates for having been the first aerobic respirers as well [2,3]. A typical cyanobacterium comprises two types of bioenergetically competent membrane systems, viz. the chlorophyll-containing intracytoplasmic or thylakoid membranes (ICM) and the cytoplasmic or plasma membrane (CM) which, apart from *Gloeobacter violaceus* [4], does not contain chlorophyll [5]. Initial attempts to separate CM from ICM [6], to localize respiratory electron transport (in particular: the cytochrome oxidase) in CM [7], or to detect *a*-type cytochrome at all in a cyanobacterium [8] remained unsuccessful. Yet, when more refined and reliable methods for the preparation and separation of cyanobacterial CM and ICM became available [9,10], it was clearly shown that (i) in all 25 cyanobacterial strains tested so far the CM does contain respiratory electron transport components (in particular: the cytochrome-*c* oxidase; Refs. [9,11–13]), (ii) ICM contain a dual-functional photosynthetic-respiratory electron transport chain [6,14–16], and (iii) the typical cyanobacterial cytochrome oxidase is of the Cu-containing cytochrome *aa*3-type [17–19] which recently was demonstrated also on a genetic level [20–22]. However, doubts about the intracellular localization of respiratory electron transport in cyanobacteria still remained [23], notably as it became obvious that this localization crucially depends on the growth conditions of the organisms [13,19]. By applying for the first time immuno-gold labeling tech-

* Corresponding author. Fax: +43 1 3104597.

SSDI 0005-2728(94)00120-0

niques to the problem of localizing the cytochrome-*c* oxidase in fully respiration-competent cyanobacterium *Anacystis nidulans*, this paper shows directly that, in accordance with activity measurements on highly purified membranes [13,19], the CM houses by far the major share of this respiratory key enzyme.

Synechococcus PCC7942 was obtained from the Pasteur Culture Collection, Paris, France, grown photoautotrophically in axenic batch cultures (Kratz and Myers medium D, 1.5% (v/v) CO_2 in sterile air, 20–25 $W m^{-2}$ warm white fluorescent light, pH between 7.8 and 8.8) and harvested by centrifugation at room temperature from linearly growing, light-limited cultures when the cell density had reached a packed cell mass of 3.2 μl/ml (equivalent to 0.97 mg dry weight, 0.54 mg protein and 24 μg chlorophyll). Membranes were isolated by French pressure cell extrusion of EDTA/lysozyme-pretreated cells [19], CM and ICM were separated and purified by discontinuous sucrose density gradient centrifugation [9,19], and cytochrome-*c* oxidase activity was measured at 35°C in a dual wavelength spectrophotometer (Shimadzu UV-300) at 550–540 nm, or polarographically with a Clark-type oxygen electrode, using horse ferrocytochrome *c* as a substrate [23,24]. Immunoblotting of membrane proteins with monospecific antibodies raised against subunits I and II of the *Paracoccus denitrificans* enzyme [25] was performed as described [13,26]. Freeze substitution preparation of whole cells of *Synechococcus* and immuno-gold labeling of the cytochrome oxidase were carried out according to published protocols [27,28]; the antibody used (at a dilution of 1:4000) had been raised in rabbits against subunit I of the cytochrome-*c* oxidase from *Anacystis nidulans* in the laboratory and with the help of Dr. M.H. Tadros, Freiburg, Germany [29]. Antibody against *P. denitrificans* cytochrome-*c* oxidase subunit I [25] basically gave the same result though against a less clean background (not shown).

Table 1
Specific activities of horse heart cytochrome *c* oxidation assayed at different stages of membrane purification (recentrifugation; cf Refs. [13,19])

Purification step	Specific rate of cyt *c* oxidation [a]	
	C membrane	IC membrane
(a)	40	12
(b)	65	7
(c)	83	4
(d)	95	3

For details see text. Steps a–c are those shown in Fig. 1 (lanes 2a–c). (a) refers to membranes taken directly from the first (flotation) sucrose density gradient, (b), (c) and (d) refer to membranes purified by one, two and three recentrifugations on a fresh gradient [13,30].
[a] Data shown are expressed as nmol cytochrome *c* oxidized/min per mg membrane protein and are the means of five determinations each performed on a separately isolated and purified batch of membranes, standard deviations ranging within 15% of the corresponding mean. The stoichiometry between O_2 reduced and cytochrome *c* oxidized was 4.17 ± 0.22 as averaged over all respective measurements and the reaction was always fully blocked by 2–3 μM KCN.

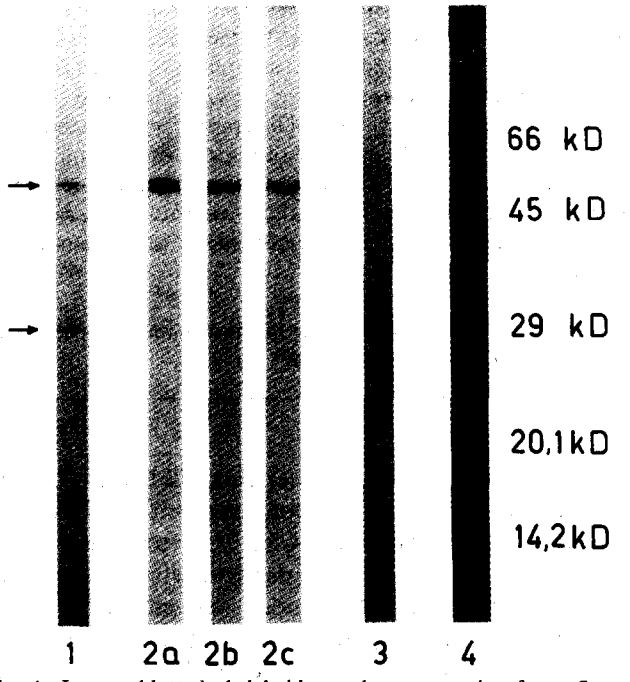

Fig. 1. Immunoblotted thylakoid membrane proteins from *Synechococcus* sp. PCC 7942 during progressive purification of the membranes (a–c) utilizing antibody raised against *Paracoccus denitrificans* cytochrome *c* oxidase subunit I (2a–c) and II (3); a refers to membranes taken directly from flotation gradient without further purification (cf. Ref. [19]). 50 μg protein was applied to each lane. Dilution of antisera was 1:500. Goat anti-rabbit-horseradish peroxidase conjugate served as the second antibody [26]. Antibodies raised against subunits I and II of the enzymes from rat liver mitochondria and *Anacystis nidulans* gave the same immunological response as shown in the figure. Lane 4 represents marker proteins (Sigma MW-SDS-70L kit). Progressively less intensive immunological cross-reaction with increasing purification (lanes 2a–c) reflects increasing removal from the ICM preparations of CM which therefore must contain intrinsically more of the enzyme than does ICM; this corresponds to the change in specific cytochrome-*c* oxidase activities in the respective membrane fraction (Table 1).

Fig. 1 shows immunoblotted cytochrome-*c* oxidase from thylakoid membranes of *Synechococcus* PCC 7942 at different stages of purification (re-centrifugation of the membranes, cf. Ref. [13]). The purer the ICM preparation, the less intense the immunological cross-reaction, pointing to progressive removal of CM, in which the specific cytochrome-*c* oxidase content is inherently higher. Clearly, contamination of (deeply green) ICM with (yellow) CM is much less easily detectable than the reciprocal contamination; the antibody, however, does not care about colors. Similarly, and in accordance with increasing purification (diluting-out the CM-bound cytochrome oxidase from CM-contaminated ICM; Fig. 1), also the specific cytochrome oxidase activity of ICM decreases with increasing purification while the reverse is true of CM

(Table 1; cf. Ref. [13]). Finally, when *Synechococcus* cells were harvested from light-limited cultures (linear growth phase, cf. Ref. [30]) and subjected to freeze substitution preparation and immuno-gold labeling of the cytochrome oxidase, electron micrographs such as shown in Fig. 2 were obtained, clearly showing the highest degree of labeling on the plasma membrane. This result is in marked contrast to earlier claims that no respiratory electron transport activity was catalysed by the CM [7] and that no cytochrome *a* occurred in *Synechococcus* [8]. By use of a similar cytochemical technique it was recently shown that both CM and ICM of the marine N_2-fixing filamentous, yet heterocyst-free cyanobacterium *Trichodesmiun thiebautii* contain aa_3-type cytochrome oxidase, and that the amount of oxidase per cell neatly correlated with the amount of nitrogenase per cell [31], thus implicating a crucial role of respiration for N_2-fixing [32] or otherwise 'stress-exposed' [13,19,29] cyanobacteria. Association of bioenergetically significant enzymes and electron transport components with CM and/or ICM to different degrees depending on the energetic situation of the cell seems

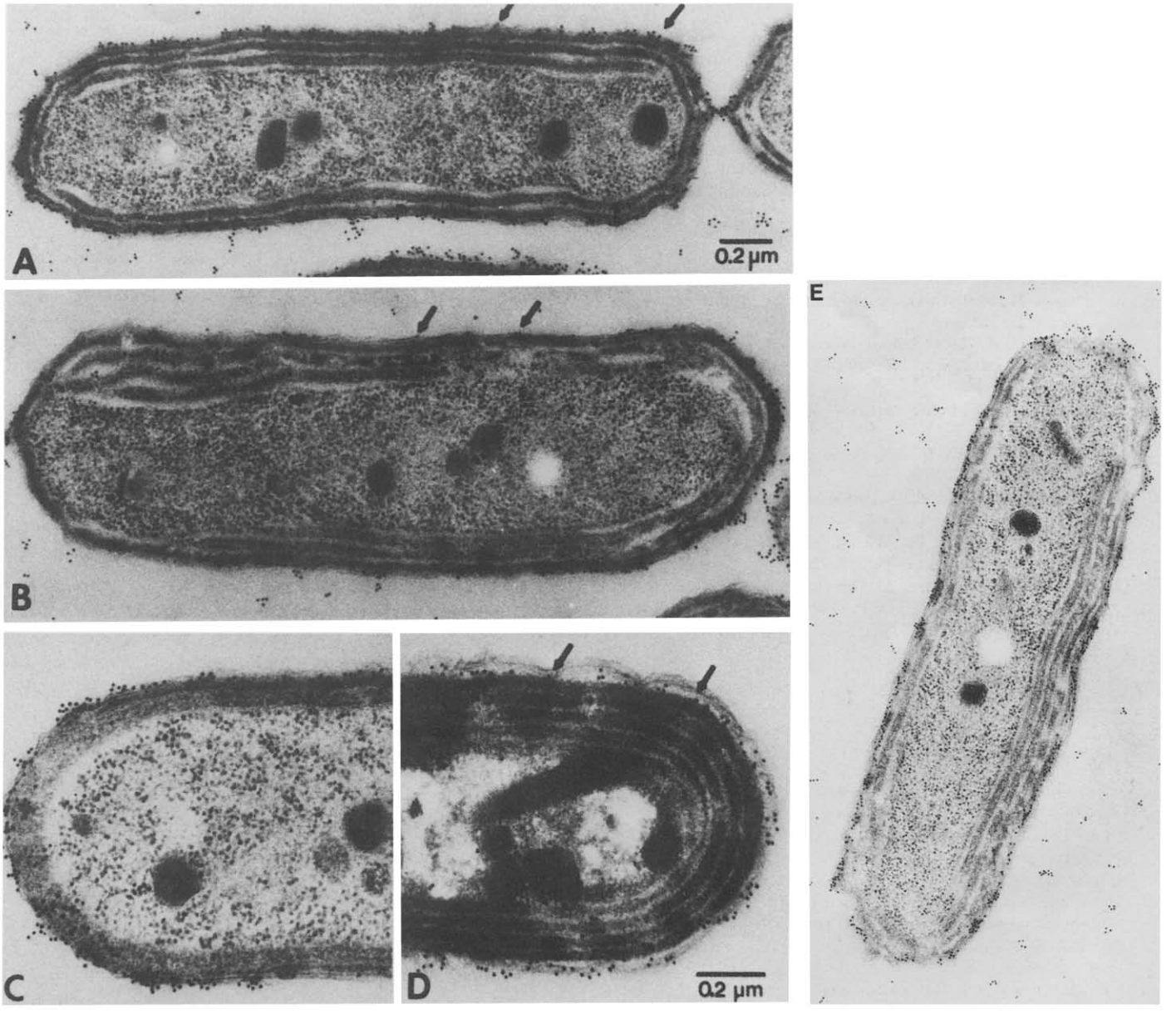

Fig. 2. Immunocytochemistry of intact cells of *Synechococcus* PCC 7942 utilizing antibody raised against cytochrome-*c* oxidase subunit I. (A, B, C, E) The cells were prepared by freeze substitution using ethanol as the organic solvent [27]. Most of the gold particles (10 nm diameter) are localized on the cell envelope which includes the cytoplasmic membrane and the contiguous outermost thylakoid membrane. (D) Cells were prepared by conventional chemical fixation. This procedure often separates the cell wall from the plasma membrane as shown. The arrows identify areas of the plasma membrane that are covered with gold particles. Such distribution indicates that the cytochrome oxidase is mostly confined to the plasma membrane (see text).

to be a more general phenomenon in cyanobacteria, as was recently also shown for F-type and P-type ATPases in three different species [33].

We thank Dr. B. Ludwig, Frankfurt, Germany, and Dr. B. Kadenbach, Marburg, Germany, for the generous gift of antibodies against *Paracoccus* and mitchondrial cytochrome-*c* oxidases and Dr. M.H. Tadros, Freiburg, Germany, for preparing the antibody against *Anacystis* cytochrome-*c* oxidase subunits I and II. We also thank Mr. Otto Kuntner for most skilful technical assistance.

References

[1] Schopf, J.W. and Barghoorn, E.S. (1967) Science 156, 508–512.
[2] Babcock, G.T. and Wikström, M. (1992) Nature 356, 301–308.
[3] Peschek, G.A., Niederhauser, H. and Obinger, C. (1992) EBEC Short Reports 7, 48.
[4] Rippka, R., Waterbury, J. and Cohen-Bazire, G. (1974) Arch. Mikrobiol. 100, 419–436.
[5] Peschek, G.A., Hinterstoissser, B., Wastyn, M., Kuntner, O., Pineau, B., Missbichler, A. and Lang, J. (1989) J. Biol. Chem. 264, 11827–11832.
[6] Peschek, G.A. (1984) Subcell. Biochem. 10, 85–191.
[7] Omata, T. and Murata, N. (1985) Biochim. Biophys. Acta 810, 354–361.
[8] Omata, T. and Murata, N. (1984) Biochim. Biophys. Acta 766, 395–402.
[9] Molitor, V., Trnka, M. and Peschek, G.A. (1987) Curr. Microbiol. 14, 263–268.
[10] Murata, N. and Omata, T. (1988) Methods Enzymol. 167, 245–251.
[11] Wastyn, M., Achatz, A., Molitor, V. and Peschek, G.A. (1988) Biochim. Biophys. Acta 935, 217–224.
[12] Peschek, G.A., Wastyn, M., Molitor, V., Kraushaar, H., Obinger, C. and Matthijs, H.C.P. (1989) in Highlights in Modern Biochemistry, vol. 1 (Kotyk, A., Skoda, J., Paces, V. and Kosta, V., eds.), pp. 893–902, VSP Publishers, Zeist, The Netherlands.
[13] Peschek, G.A., Molitor, V., Trnka, M., Wastyn, M. and Erber, W. (1988) Methods Enzymol. 167, 437–449.
[14] Aoki, M. and Katoh, S. 1982. Biochim. Biophys. Acta 682, 307–314.
[15] Peschek, G.A. (1983) Biochem. J. 210, 269–272.
[16] Sandmann, G. and Malkin, R. (1984) Arch. Biochem. Biophys. 234, 105–111.
[17] Häfele, U., Scherer, S. and Böger, P. (1988) Biochim. Biophys. Acta 934, 186–190.
[18] Peschek, G.A. (1987) in The Cyanobacteria (Fay, P. and Van Baalen, C., eds.), pp. 119–161, Elsevier Science, New York.
[19] Peschek, G.A., Wastyn, M., Trnka, M., Molitor, V., Fry, I.V. and Packer, L. (1989) Biochemistry 28, 3057–3063.
[20] Alge, D. and Peschek, G.A. (1993) Biochem. Mol. Biol. Int. 29, 511–525.
[21] Alge, D., Schmetterer, G. and Peschek, G.A. (1994) Gene 138, 127–132.
[22] Sone, N., Tano, H. and Ishizuka, M. (1993) Biochim. Biophys. Acta 1183, 130–138.
[23] Nicholls, P., Obinger, C., Niederhauser, H. and Peschek, G.A. (1992) Biochim. Biophys. Acta 1098, 184–190.
[24] Molitor, V. and Peschek, G.A. (1986) FEBS Lett. 195, 145–150.
[25] Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77, 196–200.
[26] Trnka, M. and Peschek, G.A. (1986) Biochem. Biophys. Res. Commun. 136, 235–241.
[27] Howard, R.J. and O'Donnell, K.L. (1987) Exp. Mycol. 11, 250–269.
[28] Van Tuinen, E. and Riezman, H. (1987) J. Histochem. 35, 327–333.
[29] Molitor, V., Trnka, M., Erber, W., Steffan, I., Rivière, M.-E., Arrio, B., Springer-Lederer, H. and Peschek, G.A. (1990) Arch. Microbiol. 154, 112–119.
[30] Molitor, V., Erber, W. and Peschek, G.A. (1986) FEBS Lett. 204, 251–256.
[31] Bergman, B., Siddiqui, P.J.A., Carpenter, E.J. and Peschek, G.A. (1993) Appl. Environ. Microbiol. 59, 3239–3244.
[32] Peschek, G.A., Villgratter, K. and Wastyn, M. (1991) Plant Soil 137, 17–24.
[33] Neisser, A., Fromwald, S., Schmatzberger, A. and Peschek, G.A. (1994) Biochem. Biophys. Res. Commun. 200, 884–892.