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IMMUNOLOGICAL IDENTIFICATION OF aa_3-TYPE CYTOCHROME OXIDASE IN MEMBRANE PREPARATIONS OF THE CYANOBACTERIUM ANACYSTIS NIDULANS

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Membranes were isolated from the cyanobacterium Anacystis nidulans by French press extrusion of lysozyme-treated cells. The membranes were solubilized with sodium dodecylsulfate and subjected to denaturing polyacrylamide gel electrophoresis. Separated polypeptides were transferred to nitrocellulose by Western blotting, and incubated with antibodies against aa_3-type cytochrome oxidase of Paracoccus denitrificans; antibodies against subunits I and II, and against the holoenzyme, were used and gave pronounced complementary cross reaction with two of the Anacystis membrane polypeptides corresponding to molecular weights of approximately 55,000 and 32,000, respectively. From this we conclude that an aa_3-type cytochrome oxidase is present in Anacystis nidulans as was previously suggested from spectral evidence (G.A.Peschek, Biochim.Biophys.Acta 635 (1981) 470-475), and that this enzyme is composed of at least two subunits with apparent homology to subunits I and II of the corresponding Paracoccus cytochrome oxidase. © 1986 Academic Press, Inc.

The cyanobacteria or blue-green algae form the largest, and ecologically as well as evolutionarily most important group of photosynthetic prokaryotes (1-3). The outstanding feature of cyanobacteria is their capacity to carry out oxygenic, plant-type photosynthesis and aerobic respiration together in a prokaryotic ("bacterial") cell (3-6). Yet, in marked contrast to photosynthesis (1,2,7-13) there is a severe and almost anachronistic lack of knowledge in the field of cyanobacterial respiration (1,2,6).

Some time ago spectral investigations on crude membrane preparations of A. nidulans (14) and other cyanobacteria (15,16) have indicated the presence of

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Abbreviations used: BSA, bovine serum albumin; EPR, electron paramagnetic resonance; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline (137.0 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 6.4 mM Na_2HPO_4, pH 7.2); PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate.

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cytochrome aa_3 as a functional terminal oxidase (cf.Ref.6 for review). The occurrence of aa_3-type cytochrome in plasma membrane preparations of Plectonema boryanum was suggested from Cu^{2+}-EPR and potentiometric titration (17). Redox-active copper in extensively EDTA-washed membranes of Synechococcus 6311 was characterized by EPR spectrometry as showing features strikingly similar to the Cu of other aa_3-type cytochrome oxidases (18). The presence of cytochrome aa_3 in isolated heterocysts of Anabaena PCC 7120 was inferred from optical spectrophotometry (19). In this paper we want to show that two of the polypeptides separated from solubilized membranes of A. nidulans by SDS-PAGE, after electrophoretic transfer to nitrocellulose, produced a strong and specific immunological cross reaction with antibodies against aa_3-type cytochrome oxidase from Paracoccus denitrificans (20,21). This may be construed as definite evidence for the occurrence of an aa_3-type cytochrome oxidase in A. nidulans as was previously concluded from spectral data (6,14).

MATERIALS AND METHODS

Axenic batch cultures of Anacystis nidulans (Synechococcus sp.), strain 1402-1 (Göttingen, F.R.G.), were grown in modified medium D at 20-25 w/m^2 warm white fluorescent light (as measured with a YSI radiometer, model 65, at the surface of the vessels), 35-38°C, supplied with a stream of 1.5% CO_2 in sterile air as previously described (22). Purity of the cultures was routinely checked under the phase contrast microscope or by streaking a few drops of the culture on Difco Bacto nutrient agar, followed by a four weeks' incubation period at 35°C. The data presented in this report were obtained with cultures free of bacterial and other contaminants. Cells were harvested during the late logarithmic phase of growth (65-70 h after inoculation) by centrifugation at room temperature (4,000 x g, 10 min), washed twice with distilled water, and resuspended in 5 mM NaCl, 2 mM Na_2EDTA, and 10 mM Hepes/NaOH buffer (final pH 7.4). Suspensions were adjusted to a density of 80 µl of packed cells/ml, made up to a concentration of 20% (w/w) sucrose and 0.05% lysozyme, and incubated at 37°C and room light for 2h. The pellet of a subsequent centrifugation at 3,000 x g and room temperature for 10 min (giving a colorless supernatant) was resuspended in the original volume of the Hepes/EDTA/NaCl buffer containing 1 mM PMSF and 0.0075% DNAse I (Sigma), and incubated at room temperature for 15 min. The suspension was passed once through a pre-cooled French pressure cell at 33 MPa (4,790 p.s.i.), which afforded almost 100% cell breakage. After centrifugation at 5,000 x g and 4°C for 10 min to remove intact cells and cell debris, the membranes in the supernatant were sedimented at 175,000 x g (4°C, 45 min), recentrifuged once (to get rid of most of the phycocyanin), and resuspended in 10 mM Hepes/NaOH buffer (pH 7.4) to give a protein concentration of 2-8 mg/ml as determined according to Bradford (23).

Aliquots of the membranes were subjected to SDS-PAGE according to Laemmli (24). Membranes were first precipitated with acetone (1 volume of membrane suspension/5 volumes of acetone) at -20°C for 2h in the dark, pelleted with an Eppendorf centrifuge (10,000 x g; 2 min), air-dried at room temperature, suspended in Laemmli buffer containing 5% (v/v) mercaptoethanol and 3% (w/v)

SDS (protein/SDS ratio = 1/8, w/w), and incubated at 95°C for 3 min. After another Eppendorf centrifugation the partly delipidated, and solubilized, membranes in the supernatant (about 20-30 µl each) were layered on top of the polyacrylamide gels (140 x 160 x 0.75 mm; 12.5% acrylamide) and electrophoresed in a water-cooled BioRad Protean Slab Cell I operated at constant power for 2.5h using 8w for the stacking gel and 15w for the separating gel. Separated polypeptide bands on the gels were stained with Coomassie brilliant blue R250 overnight, then destained for 4h with 40% (v/v) methanol containing 7.5% (v/v) acetic acid. Alternatively the silver stain procedure was applied (25). Marker proteins of the Sigma MW-SDS-70L-Kit were used for calibration of molecular weights.

Part of the unstained SDS polyacrylamide gel slabs containing the separated polypeptides was used for immunological cross-reaction assays which were conducted as follows. The gels were incubated with transfer buffer (25 mM tris/HCl, pH 8.3; 192 mM glycine/20% (v/v) methanol) at room temperature for 1h, including 3 changes of the buffer. Polypeptides were electrophoretically transferred from the gels to nitrocellulose (Schleicher & Schüll, 0.45 µ pore size) in a water-cooled BioRad Trans-Blot Cell using either 75 V and 0.23 A or 90 V and 0.30 A, and a transfer time of 2-2.5h. The nitrocellulose containing transferred polypeptides was either stained with Amido Black 10B or incubated first with 3% BSA/PBS for 2h in order to saturate unspecific binding sites, then with antibodies against subunit I or II of Paracoccus denitrificans cytochrome oxidase, or against the holoenzyme (20), in appropriate dilution with 3% BSA/PBS, overnight at room temperature on a reciprocal shaker. The nitrocellulose was washed free of excess antibodies with 0.05% Tween-20 in PBS (3-times, 15 min each), then with PBS (3-times, 15 min each). Further incubation (90 min) was with goat anti-rabbit-horse raddish peroxidase-conjugate (from Sigma) in suitable dilution with 3% BSA/PBS, followed by a 90-min-wash as above. Color was developed with the 4-chloro-1-naphthol-containing reagent of BioRad. Alternatively, ^{125}I-labeled goat anti-rabbit IgG was used as a second antibody which was detected by autoradiography. Control assays were performed by leaving out either the first or the second antibody; in neither case could a cross-reaction be detected.

Cytochrome oxidase activity of the membranes was measured at room temperature in a Shimadzu UV-300 dual wavelength spectrophotometer as described previously (16,26), using 10 µM ascorbate-reduced and dialysed horse heart cytochrome c (Sigma, Type VI) as a substrate, together with approximately 50 µg of membrane protein/ml. A value of 19.5 $mM^{-1}.cm^{-1}$ was used for the absorption coefficient of reduced cytochrome c at 550-540 nm (27). The protein/chlorophyll ratio (w/w) of isolated and washed membranes was 6-10. Chlorophyll content (28) of intact cells was 2-2.5% (w/w).

RESULTS AND DISCUSSION

Table I illustrates the activity of cytochrome c oxidase in the isolated Anacystis membranes, and its sensitivity toward a few specific inhibitors and activators. This activity pattern compares well with what is known from the mitochondrial cytochrome oxidase (16). Note that the rate of cytochrome c oxidation as measured here on the isolated membranes could more than fully account for maximum uncoupled respiration rates observed with intact cells of A. nidulans (around 150 nmol O_2 taken up per mg dry weight and hour at room temperature). Also note that the isolated membranes used in this study were

TABLE I. Effect of inhibitors and activators on the cytochrome c oxidase activity (nmol/min per mg protein) of membrane preparations of Anacystis nidulans

Inhibitor (mM) or activator[a]								
None	KCN	NaN_3	CO[b]	Na_2S	Salicyl aldoxim	NaCl	Tween-80	N_2[c]
	(0.01)	(1)	(0.86)	(0.01)	(20)	(100)	(3.5% w/v)	
120	0.0	3	5	0.0	4	0.0	260	0.0

[a] Substances added 5 min prior to cyt c (cf. Materials and Methods; also cf. Ref.26)

[b] Suspensions bubbled with CO/O_2 = 9/1 (v/v) for 5 min

[c] Assay performed anaerobically in N_2-flushed Thunberg cuvettes

"crude" membranes in terms of containing, on an area basis, roughly 80-90% thylakoid membrane and 10-20% plasma membrane as was derived from planimetric measurements on electron micrographs of thin-sections of intact A. nidulans (29,30).

Results from SDS-PAGE and immunoblotting with isolated and purified Paracoccus cytochrome oxidase are illustrated in Fig.1; for some unknown reason the molecular weight of subunit II consistently resulted about 6 kDa higher than given in the original publication (20), yet cross-reactions were perfectly reproducible. Fig.2 shows the overall polypeptide pattern upon SDS-PAGE of the Anacystis membranes before (lane 1) and after (lane 2) the Western blotting procedure, as well as the pattern of polypeptides actually transferred to the nitrocellulose (lane 3). It is seen that most of the polypeptides have been transferred under the conditions used, and the same is true of the marker proteins (lanes 4-6). Strictly complementary cross-reaction between two of the separated and transferred Anacystis membrane proteins, and the antibodies against corresponding subunits of Paracoccus cytochrome oxidase is apparent from Fig.3; here, lane 1 stems from antibody against the holoenzyme (two cross-reacted bands), lanes 2 and 3 from antibody against subunit I and lane 4 from antibody against subunit II (one cross-reacted band on each lane). The second antibody was goat anti-rabbit-horse raddish peroxidase conjugate

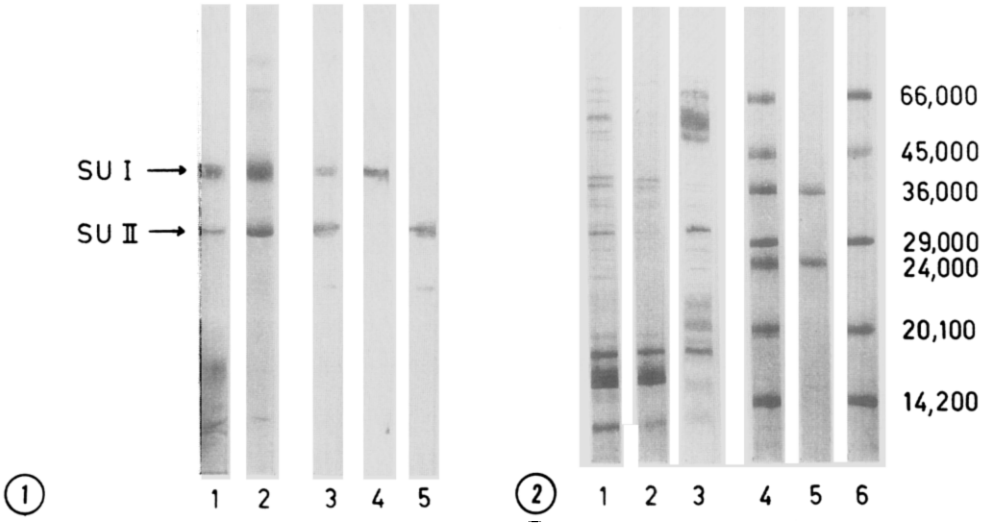

Figure 1: SDS-PAGE (lane 1 and 2) and immunoblotting (lane 3-5) of the cytochrome oxidase from Paracoccus denitrificans. Lane 1, silver stain, 5 µg protein; lane 2, Coomassie blue, 20 µg protein; lane 3-5, antibodies against the holoenzyme (dilution 1:300), subunit I (1:600) and II (1:500), respectively; 1.5 µg protein on each gel. Cf. Materials and Methods, and Ref. 20.

Figure 2: SDS-PAGE and Western blotting of Anacystis membrane proteins (lane 1-3) and marker proteins (lane 4-6). Gels were stained with Coomassie blue before (1,4) and after (2,5) transfer to nitrocellulose. Transferred polypeptides on the nitrocellulose were stained with Amido Black (3,6). 25 µg Anacystis membrane protein.

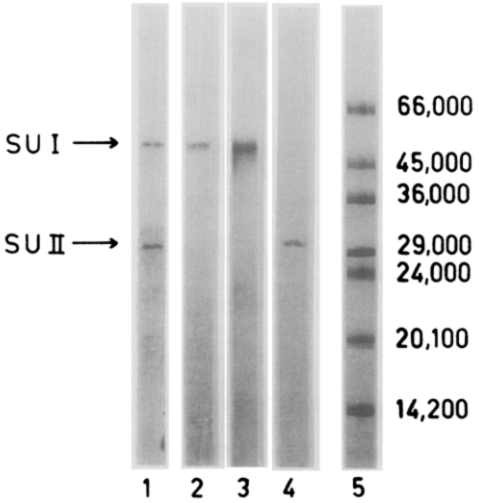

Figure 3: Immunological cross-reaction of separated Anacystis membrane polypeptides on nitrocellulose with antibodies against P. denitrificans cytochrome oxidase (holoenzyme, dilution 1:100, lane 1), subunit I (1:300, lane 2 and 3) and II (1:125, lane 4). Lane 1-4, 75 µg protein; lane 5, marker proteins. The second antibody was goat anti-rabbit-horse raddish peroxidase conjugate (1,2, 4) or ^{125}J-labeled goat anti-rabbit IgG (3). No cross-reacted bands were visible if either the first or the second antibody was omitted.

with lanes 2 and 4 (detected by peroxidase color reaction), and ^{125}J-labeled goat anti-rabbit IgG with lane 3 (detected by autoradiography). Occasionally, minor proteolytic degradation products appeared (Figs.1 and 3) which could not be totally suppressed despite storage of the membranes at -25°C and use of the protease inhibitor PMSF.

CONCLUDING REMARKS

The results described in the previous section appear to leave little doubt that Anacystis nidulans is endowed with an aa_3-type cytochrome oxidase subunits I and II of which, though markedly larger (viz. approximately 55 and 32 kD, respectively), behave immunologically similar to the corresponding subunits of the enzyme from Paracoccus denitrificans (approximately 45 and 28 kD, respectively; Refs. 20,21). As we took the utmost care to avoid contamination of our cultures (cf. Materials and Methods) these results cannot have been pretended by organisms other than A. nidulans itself. Previous failure to observe the immunological cross reaction (Ludwig and Peschek, unpublished; cf.Ref.6) may be attributed to unfavorable assay conditions. On the other hand, photodissociation and action spectra had suggested that cytochrome aa_3 might indeed function as a competent cytochrome c oxidase in A. nidulans (14,16). In the light of these and the present findings, the reported lack of a-type cytochromes in A. nidulans (31) must be viewed with caution since we were able to reproduce every detail of the data discussed here by using the very strain of A. nidulans that had been claimed to be devoid of cytochrome aa_3 (M.Trnka, V.Molitor and G.A.Peschek, unpublished observations). Reasons for the discrepancy remain unclear. Some of the present results have been communicated at the International Workshop on the Bioenergetics of Blue-Green Algae (Cyanobacteria) in Chios, Greece, September 1985, and the Fifth International Symposium on Photosynthetic Prokaryotes in Grindelwald, Switzerland (32).

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REFERENCES

1. Stanier, R.Y. and Cohen-Bazire, G. (1977) Ann. Rev. Microbiol. 31, 225-274.
2. Carr, N.G. and Whitton, B.A., eds. (1982) The Biology of Cyanobacteria, 2nd ed., Blackwell, Oxford.
3. Broda, E. (1975) The Evolution of the Bioenergetic Processes, Pergamon Press, Oxford.
4. Broda, E. and Peschek, G.A. (1979) J. Theor. Biol. 81, 201-212.
5. Peschek, G.A. (1981) Photosynthetica 15, 543-554.
6. Peschek, G.A. (1984) Subcell. Biochem. 10, 85-191.
7. Krogmann, D.W. (1977) In: Photosynthesis I. Photosynthetic electron transport and photophosphorylation. Encyclopedia of Plant Physiol. New Series, Vol. 5 (A. Trebst and M. Avron, eds.), Springer, Berlin, pp. 625-636.
8. Sandmann, G. and Böger, P. (1980) Plant Sci. Lett. 17, 417-424.
9. Hirano, M., Satoh, K. and Katoh, S. (1980) Photosynthesis Res. 1, 149-162.
10. Aoki, M. and Katoh, S. (1982) Biochim. Biophys. Acta 682, 307-314.
11. Nanba, M. and Katoh, S. (1985) Biochim. Biophys. Acta 808, 39-45.
12. Peschek, G.A. and Schmetterer, G. (1982) Biochem. Biophys. Res.Commun. 108, 1188-1195.
13. Peschek, G.A. (1983) Biochem. J. 210, 269-272.
14. Peschek, G.A. (1981) Biochim. Biophys. Acta 635, 470-475.
15. Peschek, G.A. (1981) Biochem. Biophys. Res. Commun. 98, 72-79.
16. Peschek, G.A., Schmetterer, G., Lauritsch, G., Nitschmann, W.H., Kienzl, P.F. and Muchl, R. (1982) Arch. Microbiol. 131, 261-265.
17. Matthijs, H.C.P. (1984) Ph.D. Thesis, Vrije Universiteit Amsterdam.
18. Fry, I.V., Peschek, G.A., Huflejt, M. and Packer, L. (1985) Biochem. Biophys. Res. Commun. 129, 109-116.
19. Houchins, J.P. and Hind, G. (1984) Plant Physiol. 76, 456-460.
20. Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 196-200.
21. Ludwig, B. (1980) Biochim. Biophys. Acta 594, 177-189.
22. Nitschmann, W.H. and Peschek, G.A. (1985) Arch. Microbiol. 141, 330-336.
23. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
24. Laemmli, U.K. (1970) Nature 227, 680-685.
25. Merril, C.R., Goldmann, D., Sedmann, S.A. and Ebert, M.H. (1981) Science 211, 1437-1438.
26. Molitor, V. and Peschek, G.A. (1986) FEBS Lett. 195, 145-150.
27. Yonetani, T. (1965) J. Biol. Chem. 236, 1680-1688.
28. Mackinney, G. (1941) J. Biol. Chem. 140, 315-322.
29. Allen, M.M. (1968) J. Bacteriol. 96, 836-841.
30. Schmetterer, G., Peschek, G.A. and Sleytr, U.B. (1983) Protoplasma 115, 202-207.
31. Omata, T. and Murata, N. (1984) Biochim. Biophys. Acta 766, 395-402.
32. Trnka, M., Molitor, V., Kuntner, O. and Peschek, G.A. (1985) In: Abstracts of the 5th International Symposium on Photosynthetic Prokaryotes (H. Zuber, R. Brunisholz, R. Bürgi, G. Frank, P. Füglistaller and W. Sidler, eds.), p. 246.