Vol. 98, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1981 Pages 72-79

# SPECTRAL PROPERTIES OF A CYANOBACTERIAL CYTOCHROME c OXIDASE: EVIDENCE FOR CYTOCHROME a.$a_3$

Günter A. Peschek

Institute of Physical Chemistry
The University of Vienna
Währingerstraße 42
A-1090 Vienna, Austria

Received November 12,1980

## SUMMARY

Membranes isolated from Nostoc sp. strain Mac oxidised NAD(P)H and horse heart ferrocytochrome c in dark reactions inhibited by KCN, $NaN_3$, CO, and by anaerobiosis. Reduced minus oxidised difference spectra revealed peaks at 603 and 445 nm which shifted to 590 and 430 nm, respectively, in reduced plus CO minus reduced spectra. In presence of suitable electron mediators the pigment could be reduced also with NAD(P)H or ascorbate; KCN prevented this reduction. Photoaction spectra of CO-inhibited membranes showed peaks at 590 and 430 nm. From the results it is concluded that cytochrome a.$a_3$ is a functional respiratory oxidase in Nostoc sp. strain Mac.

Despite several reports on dark oxidation of cytochrome c in cyanobacteria (1-5) the nature of the terminal oxidase has not yet revealed itself to any investigator. Nor could respiratory functions of other electron transport components ever be convincingly demonstrated (6). Certain results, however, have been taken to indicate that photosynthetic and respiratory electron transport might share a common pathway (7-9) and that both perhaps are exclusively situated on the thylakoids (10): Plastoquinone in Anabaena cylindrica (11) and Anacystis nidulans (12), and cytochrome $c_{553}$ and plastocyanin in Anabaena variabilis (5), besides their well-established roles in photosynthesis (13) may also serve in respiration under certain circumstances; reductive (presumably respiratory) sites were detected ultracytochemically in the dark on the thylakoids, but not on the plasmamembrane of Nostoc sphericum (10); and

0006-291X/81/010072-08$01.00/0

on density gradients of cell-free extracts the cytochrome oxidase activity was mostly associated with the chlorophyll-containing fraction (3-5,14). By contrast, some respiratory function of the plasmamembrane of Anacystis nidulans was recently inferred from studies on tetranitro blue tetrazolium and tellurite reduction (14), and respiratory electron flow in Anacystis was characterized more closely (12,14-16). This investigation presents evidence for the occurrence of cytochrome $a.a_3$ as a functional respiratory oxidase in Nostoc sp. strain Mac.

## MATERIALS AND METHODS

Nostoc sp. strain Mac originally isolated from the coralloid roots of the cycad Macrozamia lucida (17) was a generous gift from Dr.C.van Baalen (18). It was grown axenically at 39°C under the conditions previously described for A.nidulans (19). Purity of the cultures was always checked carefully; only cultures found to be free of bacterial contaminants were processed further. Cells were harvested in the early stationary phase (30-40 µg Chl/ml), washed twice with 25 mM Na,K-phosphate buffer, pH 7.8, resuspended in the same medium supplemented with 0.3 M mannitol and 1 mM dithiothreitol[1], and finally ruptured in a pre-cooled French press at 20.000 psi. The resulting suspension was immediately centrifuged at 3.000 g and 4°C for 20 min to remove unbroken cells (about 20%) and cell debris. The supernatant was centrifuged at 144.000 g and 4°C for 1 h, the pellet was re-suspended in fresh breakage medium and centrifuged as before. The final pellet was suspended in DTT-free breakage medium, again centrifuged as before to remove any phycobilins still adhering to the membranes, suspended in fresh DTT-free breakage medium brought to pH 7.4 with a few drops of 0.1 N HCl, and immediately used, after appropriate dilution with the same medium, for recording the spectra.

Difference spectra were taken at 4°C in a fully computerized dual wavelength spectrophotometer model 557 of Perkin Elmer, using thermostatted 1 cm cuvettes. Baselines were at first recorded and stored in the computer. On displaying the difference spectrum the respective baseline was then automatically subtracted. Oxidation of the membranes by aeration gave qualitatively similar effects like oxidation by $H_2O_2$ or ferricyanide. The latter reagents, however, were found to amplify and sharpen the peaks. Owing to the high chlorophyll content of the membranes (roughly 0.1 mg Chl/mg protein) it was not possible to use native (green) membranes in the Soret region of the spectra; thus, for this purpose the membranes were briefly extracted ("de-chlorophyllized") three times with -25°C cold 80% aqueous acetone, each treatment followed by centrifugation at 40.000 g and -20°C for 20 min. The white membranes thus obtained did not catalyse electron transport from NAD(P)H to $O_2$ any more, and oxidase activity (with reduced horse heart cytochrome as electron donor) as well as cytochrome $a.a_3$ content (as judged spectrophotometrically from the relative heights of the peak at 603 nm) were reduced

[1] Abbreviations used: DCPIP, 2,6-dichlorophenol indophenol; DTT, dithiothreitol; PMS, phenazine methosulfate; TMPD, N,N,N',N'-tetramethyl-p-phenylene diamine

by about 40%. Yet chemical reduction and oxidation of the cytochrome still were possible in the white particles, and unpredictable absorption changes of chlorophyll did not interfere any longer; moreover, the de-chlorophyllized membranes could be used at much higher protein concentrations (see Fig.4). Extraction at temperatures higher than -20°C, however, led to dramatic loss of the cytochrome.

Photoaction spectra were recorded on de-chlorophyllized membranes suspended in 25 mM Na,K-phosphate buffer, pH 7.4, supplemented with 0.3 M mannitol and 2 mM $MgCl_2$. After gassing the membranes with a mixture of 90% CO and 10% $O_2$ for 5 min in the dark the assay compartment (see below) was quickly sealed. A solution of 10 mM Na-ascorbate, pH 7.4, 0.1 mM TMPD and 0.01 mM horse heart cytochrome c (final concentrations) was injected after another 5 min (ensuring completion of the CO reaction) to start respiratory oxygen uptake which was then followed with a Clark type oxygen electrode as previously described (15,16). The diluted membrane suspension (2.5 ml; 1.6 mg protein/ml) was contained in a thermostatted (35°C) glass chamber which could be illuminated from one side with an Oriel 1000 W Xenon lamp equipped with a monochromator. Increments of down to 5 nm were used for monochromatic illumination. Final light intensity at each wavelength was adjusted to 25 $W/m^2$ by use of Kodak Wratten neutral gray filters as measured with a YSI radiometer, type 65. Alternatively, an appropriate set of interference filters purchased from Schott AG., Mainz, FRG, with half band widths of approx. 10 nm was also used. A new batch of membranes was employed at each wavelength, and in each case the uninhibited rates of oxygen uptake in suspensions gassed with air, and with 10% $O_2$ in $N_2$, respectively, were also measured.

All chemicals used were of the purest grade available. Chlorophyll and protein were determined as previously described (19).

## RESULTS AND DISCUSSION

Figs. 1-3 show difference spectra obtained between 500 and 660 nm with native (green) membrane fragments essentially free of any adhering phycobilin material. Owing to the high chlorophyll content of the membranes and, more particularly, to irregular absorption changes of the chlorophyll upon addition of strong reductants and/or oxidants (see the Materials and Methods section) the spectra could not be extended into regions of high specific chlorophyll absorption even in very dilute suspensions.

The pronounced peaks around 560 nm are due to cytochromes b-559 and b-564, respectively, both of which are known to participate in the photosynthetic electron transport of cyanobacteria (20); analogous b-type cytochromes are found in chloroplasts (21). As expected cytochrome b-564 was reducible by dithionite only (Figs. 1A and 2A) while cytochrome b-559 was also reduced by ascorbate (Figs. 1B and 2B). More interesting,

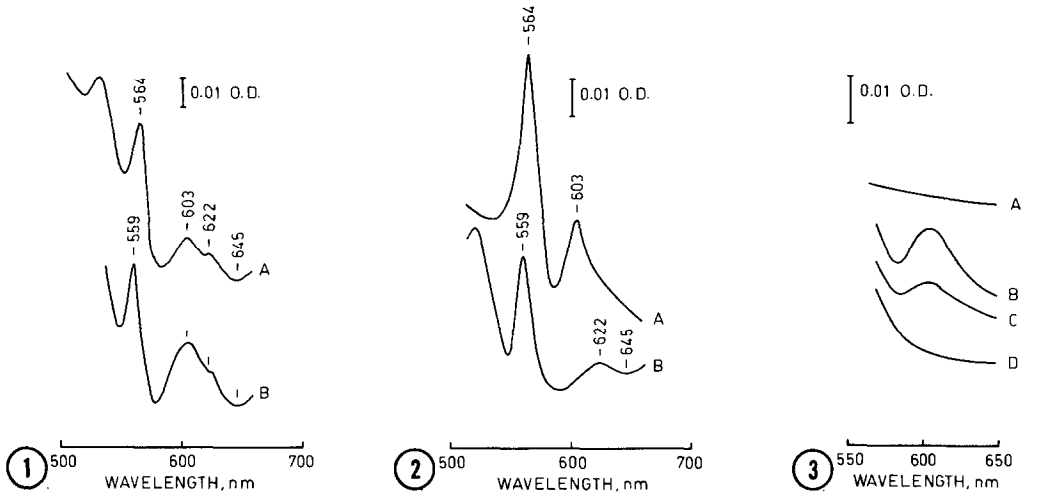



Fig.1 A: Dithionite-reduced minus (0.1% $H_2O_2$)-oxidised, B: ascorbate plus PMS-reduced minus ferricyanide-oxidised difference spectra of green Nostoc membranes (0.5 mg protein/ml.

Fig.2 A: Dithionite-reduced minus ascorbate-reduced, B: ascorbate-reduced minus ferricyanide-oxidised difference spectra of green Nostoc membranes (0.6 mg protein/ml).

Fig.3 A: Anaerobic minus aerobic difference spectra of green Nostoc membranes (0.6 mg protein per ml). Each of the cuvettes originally contained 0.01 mM horse heart ferricytochrome c prepared by ferricyanide-oxidation and subsequent dialysis of commercially available horse heart cytochrome c. A: control spectrum; B: sample cuvette reduced with 10 mM ascorbate plus 0.1 mM TMPD or DCPIP; C: sample cuvette reduced with 3 mM NADPH; D: 3 mM NADPH added to sample cuvette after 5 min pre-incubation with 0.05 mM KCN.

however, was the conspicuous peak at 603 nm seen in ascorbate plus PMS-reduced minus oxidised (Fig. 1B), in dithionite-reduced minus oxidised (Fig. 1A), and in dithionite-reduced minus ascorbate-reduced (Fig. 2A) difference spectra. Ascorbate alone did not produce the reduced $\alpha$-band at 603 nm (Fig. 2B). On the other hand also physiological electron donors, viz. NADPH and NADH, as well as ascorbate in presence of TMPD or DCPIP reduced the cytochrome (Fig. 3) provided a catalytic amount of horse heart cytochrome c was added. Apparently, exogenous cytochrome c substituted for endogenous cytochrome c which must have been removed from the membranes during isolation (vid., no c-type cytochrome traceable in Figs. 1-4) but which, in vivo, acts as a respiratory intermediate for electrons originating from NAD(P)H or ascorbate. Pre-incubation of the membranes with KCN prevented formation of the peak at 603 nm either in presence of NAD(P)H or ascorbate plus TMPD or DCPIP (Fig. 3D). These

Table I.

Respiratory oxygen uptake and oxidation of horse heart cytochrome c by Nostoc membranes. Rates given as nmol/min.mg protein). 13-27 mg of membrane protein/ml.

| Assay Suspension | Oxygen uptake with ultimate electron donors[a] | | | Cytochrome c oxidation[b] |
|---|---|---|---|---|
| | Ascorbate | NADPH | NADH | |
| Complete[c] | 93 (44)[d] | 33 (o) | 16 (o) | 360 |
| + 0.01 mM KCN | 90 0.1 | 9 | 7 | 3 |
| + 3 mM $NaN_3$ | 32 (14) | 22 | 11 | 13 |
| + 0.76 mM CO | 9 (5) | 11 | 7 | 5 |
| - TMPD | 90 (46) | - | - | - |
| - Cytochrome c | 16 (9) | 7 | 5 | - |
| Anaerobic | - | - | - | 0 |

[a] Values corrected for the small oxygen uptake in absence of donors.

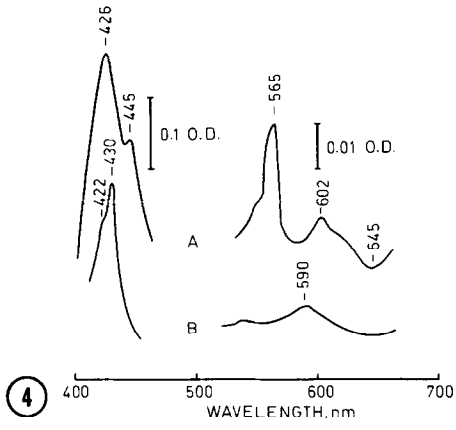
[b] Oxidation of ascorbate-reduced and dialysed horse heart cytochrome c (0.01 mM) was followed at 550 minus 540 nm in a Perkin Elmer 557 dual wavelength spectrophotometer using a differential absorption coefficient of 19.7 $mM^{-1}cm^{-1}$.

[c] Complete assay suspensions for oxygen measurements contained, besides membranes, 0.01 mM cytochrome c (+0.1 mM TMPD or DCPIP) together with 10 mM Na-ascorbate or 3 mM NADPH or 3 mM NADH.

[d] Values in brackets refer to de-chlorophyllized membranes.

results strongly indicated that the pigment absorbing at 603 nm might be a respiratory cytochrome c oxidase in its reduced form.

Rates and inhibitor sensitivities of cytochrome c oxidation and of respiratory $O_2$ uptake supported by NADPH, NADH and ascorbate as the ultimate electron donors (cf.Ref. 16) are given in Table I. Again the donor function of NAD(P)H and ascorbate was markedly enhanced by exogenous cytochrome c. Oxidation of cytochrome c was strictly aerobic and similarly sensitive to the inhibitors as was $O_2$ uptake (Table I). Note the virtually complete inhibition of the cytochrome c oxidase reaction by 0.01 mM KCN as opposed to considerable residual $O_2$ uptake with NAD(P)H as substrates (Table I); also dependence on exogenous cytochrome c was not absolute in case of NAD(P)H. Thus electrons from



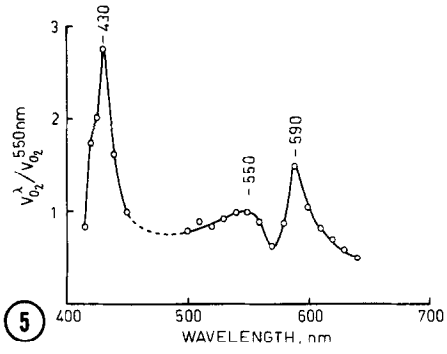




Fig.4 A: Dithionite-reduced minus (0.1% $H_2O_2$)-oxidised difference spectrum of de-chlorophyllized (white) Nostoc membranes, 12 mg protein/ml.
B: Both sample and reference cuvettes containing white Nostoc membranes were reduced with dithionite; then CO was slowly bubbled through the sample cuvette for 5 min and the spectrum recorded; 17 mg protein/ml.

Fig.5 Photoaction spectrum of CO-inhibited oxygen uptake by the de-chlorophyllized Nostoc membranes in presence of horse heart cytochrome c. For details see the Methods section.

flavoprotein-linked substrates may be "drained off" the respiratory chain at sites before the terminal oxidase or even before cytochrome c. A similar conclusion has recently been drawn from respiratory studies on isolated membranes of hydrogenase-induced Anacystis nidulans (16).

Corroborating evidence for the 603 nm redox pigment to be a terminal oxidase in Nostoc sp. was obtained from CO difference spectra (Fig. 4) and from photoaction spectra (Fig. 5). These spectra were recorded on de-chlorophyllized membranes prepared by extraction with aqueous acetone at low temperature as described in the Methods section. A prominent $\gamma$-peak around 425 nm (corresponding $\alpha$-peaks at 559-564 nm) in the dithionite-reduced minus oxidised difference spectrum (Fig. 4A) was apparently due to one or both of the photosynthetic b-type cytochromes mentioned above (cf.Figs. 1 and 2). These peaks were no longer observed in dithionite-reduced plus CO minus dithionite-reduced spectra (Fig. 4B). On the other hand, the peaks at 445 and 602 nm in the reduced minus

oxidised spectrum clearly shifted to 430 and 590 nm, respectively, in the CO difference spectrum (cf.Figs. 4A and B).

It is suggested that the autoxidable membrane-bound pigment of Nostoc showing reduced $\alpha$-bands at 602-604 and 590 nm and $\gamma$-bands at 445 and 430 nm in absence and in presence of CO, respectively, might be best described as cytochrome a.$a_3$. Spectral features, reactivity with horse heart cytochrome c, and inhibitor sensitivity (cf.Ref.14) are strikingly reminiscent of mammalian cytochrome c oxidase. The similarity is further stressed by the photoaction spectrum of respiratory $O_2$ uptake by CO-inhibited Nostoc membranes showing distinct peaks at 590 and 430 nm (Fig. 5) which obviously coincide with the corresponding $\alpha$- and $\gamma$-peaks, respectively, of the CO-complexed oxidase (Fig. 4B).

## CONCLUDING REMARKS

Cytochrome a.$a_3$ has not been described so far in cyanobacteria, nor could any other cyanobacterial cytochrome ever be convincingly shown to be involved in respiratory electron flow (6). By identifying and characterizing the terminal oxidase of Nostoc sp. strain Mac as a cytochrome a.$a_3$ the present report moreover appears to be the first to clearly demonstrate, at the same time, the respiratory function of a cyanobacterial cytochrome. Minor features of the spectra described here without further discussion might hint at the possible occurrence of still other types of oxidases in the Nostoc membranes: Small shoulders seen in CO-reduced and photoaction spectra (Figs. 4 and 5) might be traced to cytochrome o (22); similarly, the shoulder at 622 nm together with the trough at 645 nm in some of the spectra (Figs. 1, 2B, 4A) might be ascribed to cytochrome d (23). However, the experimental facts known at present do not permit to draw substantial conclusions. Work is in progress to further characterize chemical nature, kinetic properties and localization of the membrane-bound terminal oxidase(s) of this and other

cyanobacterial species. Preliminary results have been presented at the 5th International Congress on Photosynthesis, September 7-13, 1980, Kallithea (Kassandra), Greece.

ACKNOWLEDGEMENTS

Progress in the early phases of this study was facilitated by stimulating discussions with Drs. W.Lockau and G.Hauske, Regensburg, FRG. Valuable cooperation of Dr.G.Schmetterer, Vienna, Austria, is gratefully acknowledged. Thanks are also due to Dr.I.Schuster, Sandoz Research Institute, Vienna, Austria, for kind permission to use equipment of her laboratory. This investigation was supported by financial aid from the Hochschuljubiläumsstiftung der Stadt Wien.

REFERENCES

1. Webster,G.C. and Frenkel,A.W. (1953) Plant Physiol. 28, 63-69
2. Biggins,J. (1969) J.Bacteriol. 99, 570-575
3. Leach,C.K. and Carr,N.G. (1970) J.Gen.Microbiol. 64, 55-70
4. Tang,F.L.M. and Krogmann,D.W. (1972) Plant Physiol. 49, 264-266
5. Lockau,W. (1980) Arch.Microbiol. (in press)
6. Stanier,R.Y. and Cohen-Bazire,G. (1977) Ann.Rev.Microbiol. 31, 225-274
7. Jones,L.W. and Myers,J. (1963) Nature 199, 670-672
8. Hoch,G., Owens,O.H. and Kok,B. (1963) Arch.Biochem.Biophys. 101, 171-180
9. Crofts,A.R. and Wood,P.M. (1978) Curr.Top.Bioenerg. 7, 175-244
10. Bisalputra,T., Brown,D.L. and Weier,T.E. (1969) J.Ultrastruct. Res. 27, 182-197
11. Eisbrenner,G. and Bothe,H. (1980) Arch.Microbiol. 123, 37-45
12. Peschek,G.A. (1980) Biochem.J. 186, 515-523
13. Krogmann,D.W. (1973) in The Biology of Blue-Green Algae (Carr,N.G. and Whitton,B.A., eds.), pp. 80-98, Blackwell, Oxford
14. Peschek,G.A., Schmetterer,G., Lockau,W. and Sleytr,U.B. (1980) In: Proc. 5th Int.Cong. Photosynthesis (in press)
15. Peschek,G.A. (1979) Biochim.Biophys.Acta 548, 203-215
16. Peschek,G.A. (1980) Arch.Microbiol. 125, 123-131
17. Bowyer,J.W. and Skerman,V.B.D. (1968) J.Gen.Microbiol. 54, 299-306
18. Bottomley,P.J. and Van Baalen,C. (1978) J.Bacteriol. 135, 888-894
19. Peschek,G.A. (1978) Arch.Microbiol. 119, 313-322
20. Stewart,A.C. and Bendall,D.S. (1980) Biochem.J. 188, 351-361
21. Rich,P.R. and Bendall,D.S. (1980) Biochim.Biophys.Acta 591, 153-161
22. Daniel,R.M. (1970) Biochim.Biophys.Acta 216, 328-341
23. Castor,L.N. and Chance,B. (1959) J.Biol.Chem. 234, 1587-1592