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# EPR Signals of Redox Active Copper in EDTA Washed Membranes of the Cyanobacterium Synechococcus 6311

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A signal of $Cu^{2+}$ (g = 2.03) was detected by electron paramagmetic resonance spectroscopy in oxidized membrane preparations of Synechococcus 6311. The membranes were prepared and washed in the presence of EDTA (10mM, pH 8.0) and, hence, were depleted of adventitious copper; the treatment also would remove any membrane-associated soluble redox proteins and other paramagnetic metal ions. 0.1% Triton X-100 facilitated detection of the $Cu^{2+}$ signal which was fully reduced by dithionite or ascorbate plus N,N,N',N'-tetramethyl-p-phenylenediamine, and partially reduced NADPH and NADH, which are known to donate electrons to the terminal oxidase of cyanobacteria via the respiratory chain. Using temperature dependence and power saturation of the EPR copper signal, we conclude that copper is a firmly bound constituent of the terminal oxidase in an environment which is very similar if not identical to other cytochrome c oxidase preparations. 

In addition to oxygenic plant-type photosynthesis, cyanobacteria have been shown to carry out, within a prokaryotic cell, "mitochondrial-type" dark respiration (1-3; for review see Refs. 4,5). Recent spectrophotometric and inhibitor studies have indicated the presence of an $aa_3$-type cytochrome c oxidase in membrane preparations from cyanobacteria (6-11). Cytochrome c oxidase (EC 1.9.3.1) from several sources has been shown to contain redox active $Cu^{2+}$ (12-15), and since the growth of Anacystis nidulans on $Cu^{2+}$ -deficient medium (11) resulted in a marked decrease in the ability of membrane preparations to oxidize exogenous

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Abbreviations used:

NADH, Nicotinamide adenine dinucleotide, reduced form.
NADPH, Nicotinamide adenine dinucleotide phosphate, reduced form.
EDTA, Ethylene diamine tetra acetic acid.
Tes, N-tris (Hydroxymethyl) methyl-2-aminoethane sulfonic acid.
Tris, Tris (hydroxymethyl) aminomethane.
EPR, electron paramagnetic resonance.
DCPIP, 2,6-dichlorophenol indophenol.
TMPD, N,N,N',N'-tetramethyl-p-phenylene diamine.
SMP, Submitochondrial particles

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c-type cytochromes (16), redox active $Cu^{2+}$ was predicted in this species. Here we report on an EPR detectable $Cu^{2+}$ signal in membrane preparations of Synechococcus 6311, which undergoes reduction by physiological electron donors.

The purpose of the study is to determine whether the EPR $Cu^{2+}$ signal is in fact arising from cytochrome c oxidase. Another source of redox active $Cu^{2+}$ in photosynthetic organisms is plastocyanin, and although plastocyanin has been reported to be absent from several cyanobacterial species (17) including Anacystis nidulans (18, S. Sandmann, personal communication), which is believed to be an independent isolate of Synechococcus 6311 (19), the presence of small amounts of plastocyanin in Synechococcus 6311 could not be ruled out. Therefore, to distinguish between these two types of $Cu^{2+}$ protein, the physical parameters of temperature dependence and power saturation of the EPR signal in membrane preparations of Synechococcus 6311 were measured and compared to EPR $Cu^{2+}$ signals arising from cytochrome c oxidase in mitochondria, and from plastocyanin in Gloeobacter violaceus.

## Materials and methods

Synechococcus 6311 was obtained from the American Type Culture Collection (ATCC 27145), and grown on Kratz and Myers medium C as described (20). Spheroplasts were prepared according to Biggins (21). The spheroplasts were disrupted either by passage through a Yeda press (1,800 psi) under nitrogen or by ultrasonic oscillation (Branson sonifier, model 350, microtip setting 3/20 min. on ice). Unbroken cells were removed by centrifugation at 3,000 x g (10 min). Membranes were sedimented at 40,000 x g (60 min), then resuspended in 10mM Tris/HCL, pH 8.0, containing 10 mM EDTA, and sedimented again at 40,000 x g (60 min). The pelleted membranes were resuspended in the same medium to approximately 50 ug chlorophyll/ml and frozen in liquid nitrogen. Thawed samples were centrifuged at 40,000 x g (60 min), the membrane pellet was resuspended to 0.5 mg chlorophyll/ml in 10mM Tes/HCl buffer, pH 7.0, containing 10mM EDTA. Finally the membranes were concentrated by centrifugation in quartz EPR tubes at 40,000 x g (60 min) to give a final chlorophyll concentration of about 2 mg/ml.

Submitochondrial particles (SMP) were prepared from beef heart mitochondria as described (22) and resuspend in 10mM EDTA, 10 mM Tes pH 7.0. Plastocyanin was observed by EPR Spectroscopy in whole cells of Gloeobacter violaceus (Fry, Robinson and Packer unpublished results).

Electron transport components in the membranes were oxidized with 50 mM potassium ferricyanide, or air, and reduced with 10 mM Na dithionite or 10 mM ascorbate plus 25 μM TMPD, or with 30 mM NADPH or NADH in the presence of 1.25 mM horse heart cytochrome c and 5 mM KCN. In these experiments the exogenous cytochrome c served as an electron mediator to the cytochrome oxidase which cannot be directly reduced with NAD(P)H (12). Triton X-100 (0.1 %, v/v) was added to the prepartions prior to freezing in liquid nitrogen. EPR spectra were recorded with a Varian E spectrometer, fitted with an Air Products Heli-Tran liquid

helium transfer system, at 20mW power, 1 millitesla modulation amplitude and 9.15 MHz at 100K. Cytochrome oxidase activity was determined from the KCN sensitive oxidation of horse heart ferrocytochrome c as described previously (11,16).

## Results

### Characterization of EPR detectable $Cu^{2+}$

Membrane preparation of Synechoccus 6311 exhibited a low temperature EPR signal in the g = 2 region characteristic of $Cu^{2+}$ (fig. 1B) which was very similar in line shape to the $Cu^{2+}$ signal arising from typical $aa_3$-type cytochrome c oxidases (fig. 1C) (12-15). The temperature dependence of both signals showed a maximum at 100K (Fig. 2), while the EPR $Cu^{2+}$ signal arising from

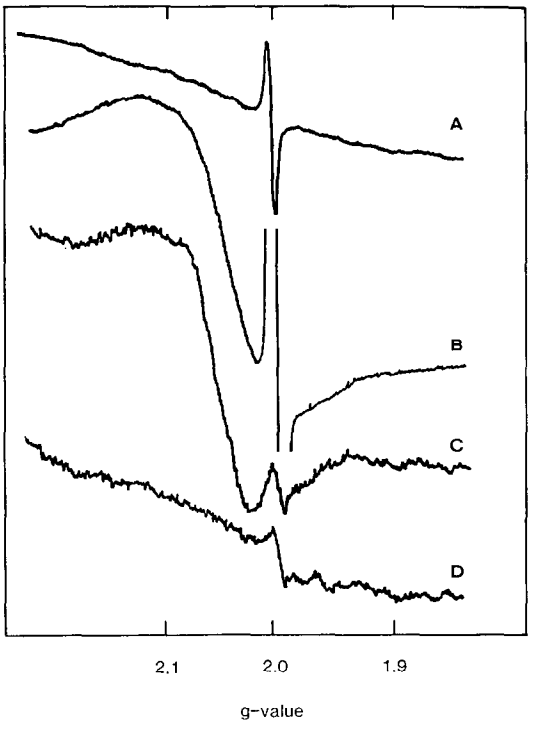


Figure 1:

EPR $Cu^{2+}$ spectra of membrane preparations of Synechococcus 6311 (2.5 mg chlorophyll/ml) in 10 mM Tes 10mM EDTA buffer, pH 7.0. Membranes were prepared by Yeda pressure cell treatment and submitochondrial particles (10 mg protein/ml) were resuspended in 10 mM Tes 10mM EDTA buffer pH 7.0. Spectra were recorded as described in Materials and Methods. 0.1 % (v/v) Triton X-100 had been added prior to freezing.

A. Synechococcus 6311 reduced within 10mM acorbate plus 25µM TMPD in the presence of 5mM KCN.
B. Synechococcus 6311 oxidized with 25mM $K_3Fe(CN)_6$.
C. Submitochondrial particles, oxidized with 25mM $K_3Fe(CN)_6$.
D. Submitochondrial particles, reduced with 10mM acorbate plus 25µM TMPD in the presence of 5mM KCN.

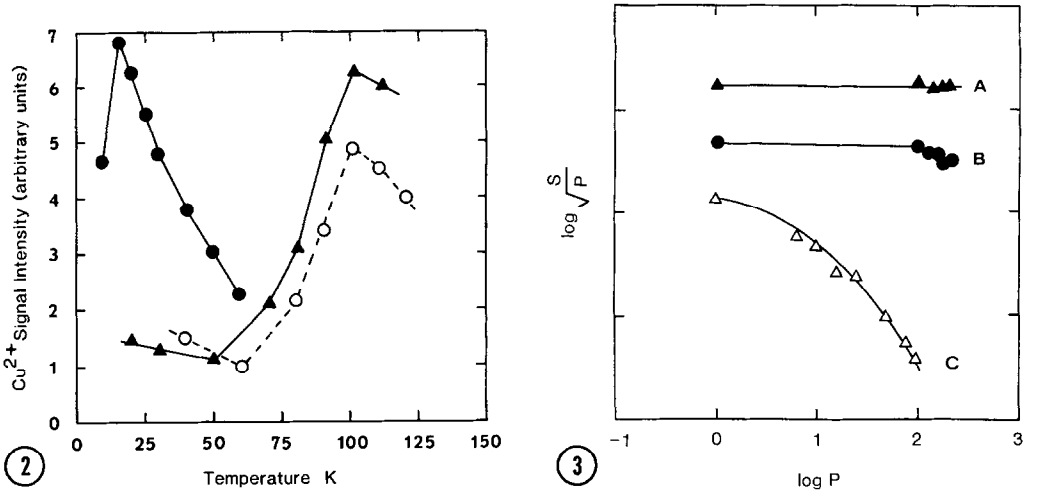


Figure 2:
Temperature dependence of EPR $Cu^{2+}$ signals from Synechococcus 6311 (▲—▲), submitochondrial particles (○--○) and Gloeobacter violaceus (●—●). Spectra were recorded as described in materials and methods.

Figure 3:
Power saturation of oxidized EPR $Cu^{2+}$ signals;

A. Synechococcus 6311 at 100K; B. Submitochondrial particles at 100K; C. Gloeobacter violaceus at 15K.

Gloeobacter violaceus plastocyanin had a temperature optimum around 10-20K (fig. 2) (23,24). The EPR $Cu^{2+}$ signals from Synechococcus and SMP did not saturate with microwave power up to 180 mW (fig. 3A and B), while the EPR $Cu^{2+}$ signal from plastocyanin, observed at lower temperatures, readily saturated with microwave power (Fig. 3) (23,24).

## EPR Detectable $Cu^{2+}$ and Cytochrome c Oxidase Activity

Membranes prepared by ultrasonic oscillation did not exhibit any EPR detectable $Cu^{2+}$ signals in the g = 2 region. Characteristically, cytochrome c oxidase activity in Sonicated or Yeda press preparations correlated with the presence or absence of EPR detectable $Cu^{2+}$ (Table I). The EPR $Cu^{2+}$ signal was enhanced by the addition of 0.1 % (v/v) Triton X-100 or by freeze thaw treatment. It was readily and reversibly reduced (presumably to EPR silent $Cu^{+}$) by artificial reductants such as ascorbate plus TMPD (Fig. 1A) (Cf fig. 1D) and sodium dithionite (Table II).

TABLE I: Cytochrome c oxidase activity and EPR detectable copper in membrane preparation of Synechococcus 6311

| Membrane preparation | Cytochrome c oxidase activity[a] | EPR-detectable $Cu^{2+}$ |
|---|---|---|
| Ultrasonic | not detectable | absent |
| Yeda pressure cell | 160 | present |

[a] nmoles of horse heart ferrocytochrome c oxidized/mg chlorophyll/min

## Physiological Electron Donors and $Cu^{2+}$ Reduction

Yeda press prepared membranes were incubated with NADPH or NADH, which have been shown to act as electron donors to the respiratory chain and, hence, to the cytochrome c oxidase of cyanobacteria (5,7), in the presence of KCN to prevent leakage of electrons from the reduced oxidase. The membrane preparations were supplemented with horse heart cytochrome c to replace any soluble electron mediator(s) lost during the preparation procedure (7). Clearly, in these experiments physiologically reduced cytochrome c is the immediate electron donor to the terminal oxidase; horse heart ferrocytochrome c has been shown to be an excellent reductant to the membrane-bound cytochrome c oxidase of several cyanobacteria (11,16,25). The degree of reduction of the $Cu^{2+}$ EPR signal is presented in Table II, showing the preferential reduction of the cytochrome oxidase by NADPH (10).

TABLE II: Oxidation and reduction of the EPR detectable copper in membrane preparations of Synechococcus 6311 obtained by Yeda Press treatment

| Treatment | $Cu^{2+}$ Signal (% oxidized) |
|---|---|
| none | 100 |
| 25mM $K_3Fe(CN)_6$ | 100 |
| 10mM $Na_2S_2O_4$ | 0 |
| 30mM NADH [a] | 62 |
| 30mM NADPH [a] | 25 |

0.1 % Triton X-100 was added to all samples.

[a] 1.25 mM horse heart cytochrome c and 5 mM KCN were additionally present

## Discussion

The membrane preparations used in this study were washed with 10 mM EDTA to remove adventitious $Cu^{2+}$, together with other membrane-associated paramagnetic metal ions such as manganese (27). The washing treatment would also remove any $Cu^{2+}$-containing and other soluble proteins. Therefore, one of the very few possibilities of intrinsically membrane bound $Cu^{2+}$ in a plastocyanin-free cyanobacerium would be the $aa_3$-type terminal oxidase (5), which is supported by the data presented in this paper. EPR spectra, temperature dependence and power saturation of the $Cu^{2+}$ EPR signal closely resemble EPR spectra published for the $Cu^{2+}$-containing $aa_3$-type cytochrome oxidases from mammalian mitochondria (12,13), yeast mitochondria (14), *Paracoccus denitrificans* (14) and *Thermus thermophilus* (15); they are, however, clearly different from those exhibited by soluble copper proteins such as plastocyanin (23,24,28). Moreover, both EPR detectable $Cu^{2+}$ and cytochrome *c* oxidase activity were absent in parallel from heavily sonicated membranes (Table I) in which the functional integrity of the cytochrome oxidase complex might have been disrupted and lost together with the $Cu^{2+}$. An $aa_3$-type cytochrome oxidase was recently described in terms of optical spectra, photo-action spectra, and differential reactivities towards various *c*-type cytochromes and inhibitors using membrane preparations from several axenic strains of cyanobacteria (see Ref. 5 for review). Similar findings were reported for *Plectonema boryanum* (6,7) and the heterocysts of *Anabaena* 7120 (8). In previous experiments with *A. nidulans* it was shown that the membrane-bound cytochrome $aa_3$ could be physiologically reduced with horse heart ferrocytochrome *c* or reduced pyridine nucleotides, or with ascorbate plus TMPD or DCPIP, and oxidized with moledular oxygen (5,9,11,16); now the same redox behavior has been found for the tightly membrane-bound $Cu^{2+}$ of *Synechococcus* 6311 (Table II). Full reduction of the $Cu^{2+}$ by nucleotides did not occur, which may be due to a bleeding of electrons to other pathways (the oxidized chlorophyll radical was 90% reduced by nucleotides - data not shown) or by a population of $Cu^{2+}$ not reducable by cytochrome *c*, due to damage of the cytochrome *c* oxidase during membrane preparation (26). Appearance of the EPR signal of the oxidized $Cu^{2+}$ was more pronounced

in the presence of 0.1% Triton X-100, probably due to partial solubiliation of the cytochrome c oxidase from the membranes; detergent treatment of the membrane bound oxidase is known to enhance its reactivity towards exogenous c-type cytochromes (11,16).

The isolated membranes used in this study contained both cytoplasmic and intracytoplasmic (thylakoid) membranes; at present there is no satisfactory procedure available to isolate either type of membrane, separated from the other, in a physiologically fully active state (5,29). Therefore, our results do not permit localization of the intrinsic $Cu^{2+}$ on either membrane. However, previous results from work with intact cells or spheroplasts, and isolated membranes, of A. nidulans have indicated an $aa_3$-type terminal oxidase to be present in both cytoplasmic and thylakoid membranes (5,30-32), and preliminary findings with a chlorophyll-free cytoplasmic membrane fraction from the same species showed it to be capable of oxidizing horse heart ferrocytochrome c (V. Molitor, M. Trnka, and G. A. Peschek, unpublished). Moreover, optical and EPR spectra of enriched cytoplasmic membrane fractions of Plectonema boryanum grown in normal and $Cu^{2+}$deficient media recently suggested the possible presence of $Cu^{2+}$-containing $aa_3$ type cytochrome oxidase in the cell membrane of this species (6,7).

From our present findings we conclude the EPR active $Cu^{2+}$ is present in a tightly bound form in the membranes of Synechococcus 6311 where it undergoes physiological redox reactions with respiratory electron donors and acceptors, and is very similar if not identical to the E.C.1.9.3.1 type cytochrome c oxidase which has been described in mammalian and yeast mitochondria (12-14) and bacteria (14,15).

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References

1. Biggins, J. (1969) J. Bacteriol. 99:570-575.
2. Tang, F.L.M., Krogmann, D.W. (1972) Plant Physiol. 49:264-266.

3. Webster, G.C. and Frenkel, A.W. (1953) Plant Physiol. 28:63-69.
4. Stanier, R.Y. and Cohen-Bazire, G. (1977) Ann. Rev. Microbiol. 31, 225-274.
5. Peschek, G.A. (1984) Subcellular Biochemistry 10:83-189.
6. Matthijs, J.C.P. (1984) Ph.D. Thesis, Vrije Universiteit Amsterdam.
7. Matthijs. J.C.P. (1984) In: Adv. Photsynth. Res., Vol. II, pp. 643-646.
8. Houchins, J.P. and Hind, G. (1984) Plant Physiol. 76,456-460.
9. Peschek, G. A. Biochim. Biophys. Acta 635 (1981) 470-475.
10. Peschek, G. A. Biochim. Biophys. Res. Commun. 98 (1981) 72-79.
11. Peschek, G.A., Schmetterer, G., Lauritsch, G., Nitschmann, W.H., Kienzl, P.F. and Muchl, R., (1982) Arch. Microbiol. 131: 261-265.
12. Beinert, H., Griffith, D.E. Warton, D.C. and Sands, R.H. (1962) J. Biol. Chem. 237, 2337-2346.
13. Hoffmann, B.M., Roberts, J.E., Swanson, M., Speck, S.H., and Margoliash, E. (1980) Proc, Natl. Acad. Scie. U.S.A. 77, 1452-1456.
14. Seelig, A., Ludwig, B., Seelig, J., and Schatz, G. (1981) Biochim. Biophys. Acta 636, 162-167.
15. Fee, J.A., Choc, M.G., Findling, K.L., Lorence, R., and Yoshida, T. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 147-151.
16. Kienzl, P.F and Peschek, G.A. (1982) Plant Physiol. 69: 580-584.
17. Sandmann, G. and Böger, P. (1980) Biochim. Biophys. Acta 766, 395-402.
18. Kienzl, P.F. and Peschek, G.A. (1983) FEBS Lett. 162:76-80.
19. Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y. (1979) J. Gen. Microbiol. 111, 1-61.
20. Kratz, W.A., and Myers, J. (1955) Am J. Bot 42 282-287.
21. Biggins, J. (1967) Pl. Physiol. 42:1447-146.
22. Low, H. and Vallin, L. (1963) Biochim. Biophys. Acta 69, 361-374.
23. Sandmann, G., Böger, P., (1980) Pl.Sci.Lett. 17:417-424.
24. Bohner, H., Merkle, H., Kroneck, P., and Böger, P. (1980) Eur.J.Biochem. 105:603-609.
25. Peschek, G.A., Kienzl, P.F., and Schmetterer, G. (1981) FEBS Lett. 131, 11-16.
26. Aasa, R., Albracht, S.P.J., Falk, K.E., Lanne, B. and Vänngard, T. (1976) Biochem. Biophys. Acta 422: 260-272.
27. Cammack, R., Luijk, L.J., Maguire, J.J., Fry, I.V. and Packer, L. (1979) Biochim. Biophys. Acta 548:267-275.
28. Visser, J.W.M., Amsz, J. and Van Gelder, B.F. (1974), Biochim. Biophys. Acta 333 279-287.
29. Omata, T., and Murata, N. (1984) Biochim. Biophys. Acta 766, 395-402.
30. Peschek, G.A., Schmetterer, G., Lauritsch, G., Muchl, R., Kienzl, P.F. and Nitschmann, W.H. (1983) In: Photosynthetic prokaryotes: Cell differentiation and Function, (G.C. Papageorgiou and L. Packer, eds.), pp. 147-162.
31. Peschek, G.A. (1983) J.Bacteriol. 153: 539-542.
32. Peschek, G.A. (1984) Plant Physiol. 75, 968-973.