Incubation of obligately photoautotrophic and aerobic cyanobacterium Anacystis nidulans (Synechococcus sp. PCC 6301) in the light in the presence of the photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea and equilibrated with approximately 1% (v/v) O₂ in N₂ (10 μM O₂ in solution) led to a decrease of the heme A content of isolated cytoplasmic membranes and to the appearance of heme O. The latter was not seen in membranes from fully aerated cells (>210 μM dissolved O₂). Non-covalently bound hemes extracted from the membranes were identified by reversed phase high performance liquid chromatography. Heme A and O contents of the membranes changed in a reversible fashion solely depending on the ambient oxygen regime. Both hemes A and O combine with the same apoprotein as suggested by immunoblotting. CO/reduced-minus-reduced optical difference spectra, photoaction spectra of CO-inhibited O₂ uptake by the membranes, and pyridine hemochromogen spectra pointed to either heme belonging to a functional form of the terminal oxidase. The NADH:O₂ oxidoreductase reaction catalyzed by membranes from both high O₂ and low O₂ cells was strictly dependent on the addition of catalytic amounts of cytochrome c, fully inhibited by 1.2 μM KCN, and insensitive to 5 μM 2-n-heptyl-4-hydroxyquinoline-N-oxide. O₂ uptake by the membranes was effectively catalyzed by N,N,N',N'-tetramethyl-p-phenylenediamine but not 2-methylnaphthoquinol or plastoquinol-1 as artificial substrates. Therefore we conclude that the cyanobacterial respiratory oxidase, irrespective of the type of heme in its O₂-reducing center, is a cytochrome c rather than a quinol oxidase.

Cyanobacteria are phototrophic prokaryotes uniquely capable of oxygenic (plant type) photosynthesis and aerobic (cytochrome oxidase based) respiration (Fay and Van Baalen, 1987; Bryant, 1994). Their immediate ancestors are generally thought to be responsible for the first bulk amounts of oxygen gas in a previously anaerobic biosphere and atmosphere (Barghoorn and Schopf, 1966; Broda, 1975). Consequently, they must have been (among) the first to cope with poisonous free oxygen (Morris, 1975; Babcock and Wikström, 1992; Peschek, 1992). Most efficiently and significantly from the viewpoints of ecology and evolution in the long run this happened by transforming pre-existing photosynthetic mechanisms into those of aerobic respiration, finally reducing oxygen back to water (“conversion hypothesis”; see Broda (1975) and Broda and Peschek (1979)), involving a heme-type respiratory oxidase as the crucial component. Interestingly, it has not been until fairly recently that the cyanobacterial respiratory oxidase was established to be an aa₃-type enzyme very similar to the mitochondrial or Paracoccus denitrificans enzyme (Peschek, 1981; Peschek et al., 1988, 1989a, 1989b; Alge and Peschek, 1993; Alge et al., 1994). In most cyanobacteria the cytochrome c oxidase was localized in both (chlorophyll-free) cytoplasmic (CM)² and green thylakoid membranes, relative shares of the enzyme (and of the other respiratory electron transport components altogether) in either membrane critically depending on growth conditions (Peschek et al., 1988, 1989a, 1989b, 1994a; Molitor et al., 1990). The most widely investigated species Anacystis nidulans was repeatedly, but in contrast to initial reports (Omata and Murata, 1984, 1985), shown to contain much more cytochrome c oxidase (per mg of membrane protein) in cytoplasmic than in thylakoid membranes (Peschek et al., 1994a, 1994b). It was also shown that growth in salt-stressed conditions can still markedly enhance the content of the cytochrome c oxidase in the cytoplasmic membrane (particularly in the CM-II fraction; see Molitor et al., 1990, Nicholls et al. (1992), and Peschek et al. (1994a)). Direct analytical determination of cytochrome a (heme A) in cyanobacterial membranes was previously achieved with membranes isolated from Anabaena variabilis using the laborious classical techniques of column chromatography and alkaline pyridine ferrohemochrome derivation (Wastyn et al., 1988). A major obstacle to successful heme extraction and HPLC identification (Svensson et al., 1993; Lübben and Morand, 1994) from cyanobacteria has been the tremendous amount of photosynthetic pigments (viz. chlorophyll and phycobilins) in these cells. In the present work we overcome this problem by using chlorophyll-free cytoplasmic membranes (CM-II fraction; see Nicholls et al. (1992), Peschek et al. (1989c), and Hinterstoesser et al. (1993)) from A. nidulans, which contain high levels of respiratory pigments, in particular of cytochrome c oxidase (Peschek et al., 1988, 1989a, 1989b). A-, B-, and O-type hemes could be clearly identified in these membranes, proportions of hemes A and O reversibly depending on the oxygen concentration present in the incubation medium. Our results are in agreement with a biosynthetic sequence of heme B → heme O → heme A (2-vinyl-8-methyl-/2-hydroxyethyl-farnesyl-8-methyl-2-hydroxyethyl-farnesyl-8-formyl/iron porphyrin) whereby some oxygenase enzyme appears to participate in the last step. Direct involvement of O₂ in the biosynthesis of chlorophyll b (formyl group) from chlorophyll a (methyl group) was recently confirmed.

² The abbreviations used are: CM, cytoplasmic membrane; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; HPLC, high performance liquid chromatography.

² M. Lübben and G. A. Peschek, unpublished data.
Heme Promiscuity in Cyanobacterial Cytochrome c Oxidase

EXPERIMENTAL PROCEDURES

Materials and Growth Conditions—A. nidulans (Synecococcus sp. strain PCC 6301) was obtained from the Pasteur culture collection (courtesy of Mme. Rosi Rippka-Hermand) and grown axenically and photautotrophically in the presence of 0.5 M NaCl at 40°C and 20 wattsm⁻² warm white fluorescent light as measured with a YSI radiometer, model 65, at the surface of the growth vessels as described (Mölitor et al., 1986, 1990; Peschek et al., 1994a). Actively growing cultures were sparged with 1.5% (v/v) CO₂ in air, steady-state concentrations of O₂ in the suspensions amounting to 30–35% (v/v) oxygen saturation (150–175% air saturation) corresponding to a steady-state O₂ concentration in the liquid medium of between 310 and 360 μM. When cell concentrations had reached approximately 3 μl of packed cell mass/ml (light-limited, linearly growing cultures; see Peschek et al. (1988) and Möltor et al. (1986)) aliquots of the culture were harvested by centrifugation at room temperature, washed three times with distilled water, and finally suspended in the desired preparation or assay medium. A second aliquot of the culture was supplemented with 20 μM DCMU and sparged with bacteriologically pure, “technical” nitrogen gas (containing approximately 1% O₂) under continued illumination for another 24 h until harvest. A third aliquot of the culture was derived from DCMU-poisoned O₂ cells by switching to a gassing regime with normal air (21%, v/v, O₂) for a final 12-h period. During each condition of incubation the O₂ concentration in the cell suspensions was continuously monitored with a Clark-type oxygen electrode (YSI oxygen monitor, model 53). Cells from O₂-oversaturated (310–360 μM O₂), semi-anaerobic (<10 μM O₂), and normally re-aerated (210–215 μM O₂) cultures were designated “phase A,” “phase B,” and “phase C” cells, respectively (see Fig. 1).

Membrane Isolation and Respiratory Measurements—Cytosolic membranes were isolated and purified from harvested and washed cells after lysozyme treatment and French pressure cell extrusion as described (Peschek et al., 1989a, 1989c) and resuspended in 10 mM potassium phosphate buffer, pH 7.0 or 7.6, for spectrophotometric and polarographic assays, respectively. Oxidation of horse heart ferrocytochrome c (7 μM initial concentration) was measured in a Shimadzu UV-300 dual wavelength spectrophotometer (Mölitor and Peschek, 1986), and O₂ uptake by the membranes in the presence of 60 mM sodium ascorbate, 0.1 mM TMPD, 3.5 mM NADH, 3 or 25 μM horse heart cytochrome c, 60 μM plastocyanin-I, and/or 60 μM 2-methylpyridinequinone was measured polarographically as described (Nicholls et al., 1992; Kraushaar et al., 1990). Temperature of measurements was 30°C.

RESULTS AND DISCUSSION

Fig. 1D shows the reversed-phase HPLC chromatogram of heme B, A, and O standards prepared as described under “Experimental Procedures.” Retention times of the standards were close to 26, 35, and 37 min, respectively, for hemes B, A, and O and in the order of increasing lipophilicity, reasonably reproducible (within ±3%) from one extraction to the other, and independent of the source of the heme. This clear-cut identification of the heme standards permitted a reliable analysis of non-covalently bound heme groups in cytoplasmic membranes isolated from phase A (oxygendegenerated, photosynthetically grown), phase B (semi-anaerobic), and phase C (re-aerated) Anacystis (Fig. 1, A–C, respectively). The most abundant heme is heme B corresponding to the ubiquitous occurrence of cytochrome b at high levels in almost any type of biological membrane (Lübben and Morand, 1994; Lemberg and Barrett, 1973).
The only other acid-labile heme detectable in these membranes is heme A (Fig. 1A) from the aa₃-type cytochrome c oxidase, which has been characterized in cytoplasmic and/or thylakoid membranes from 27 different cyanobacterial species (Peschek et al., 1989a, 1989b; Wastyn et al., 1988).

After a 24-h incubation period in the light under low oxygen tension (<10 μM final concentration of O₂ in the medium, which is still above the K_m of typical cytochrome c oxidases but below the K_m of most oxygenases (Jones, 1981)) heme A concentration in the membranes was diminished, and a new peak of heme O was seen (Fig. 1B). The non-growing cells, equilibrated with 1% (v/v) O₂ in the light, exhibited stable energy charge values of up to 0.8 (Nitschmann, 1985; Nitschmann and Peschek, 1986), which means that they went on being sufficiently energized to meet all of their maintenance requirements. Re-aeration of the semi-anaerobic and illuminated yet non-growing cell suspensions finally produced an increase of the membranes’ heme A content at the expense of heme O. This shows that the transitions between hemes A and O were fully reversible and most probably dependent on the availability of sufficient O₂ only (Fig. 1C). When de-aeration and re-aeration of the cells were performed in strict darkness or in the presence of 25 μM horse heart cytochrome c, the reversibility of the transition was not observed (Nitschmann, 1985). Efficient synthesis of ATP (by photophosphorylation) and of proteins is a prerequisite also for the maintenance metabolism (steady-state protein turnover). (Control experiments not shown.)

Both hemes A and O represented functional cytochromes (viz. cytochromes a₃ and o₃, respectively) in the semi-anaerobic and re-aerated phase B and C cells as can be seen from CO-reduced-minus-reduced optical difference spectra of n-octyl glucoside-solubilized membranes (Fig. 2A), photoaction spectra of the CO-inhibited O₂ uptake by native membranes in the presence of ascorbate and cytochrome c (Fig. 3), and by polargraphic measurement of different oxygen affinities of the two different cell types (Fig. 4). Semi-anaerobic cells contain two different CO-reactive cytochromes, one of a₃-type with peaks at 430 and 590 nm and another one with peaks at 415 and 555 nm, which probably stem from the "o-type oxidase" (Fig. 2A; note that spectral features of b-type and o-type cytochromes cannot be easily distinguished due to an almost identical electronic situation in the heme core of either cytochrome; see Lübben and Morand (1994)). The same problem of spectrally indistinguishable hemes B and O would also apply to the alkaline pyridine hemochrome spectra shown in Fig. 2B. However, in this case the hemochromes were prepared from a fraction eluting from the reversed-phase HPLC column well after heme B (i.e. >30 min; see Fig. 1). Thus the "b-type" spectral features in Fig. 2B (hemochrome difference spectrum with peaks at 415 and 555 nm) must be attributed to heme O, while 433- and 588-nm peaks clearly belong to heme A (Lübben and Morand, 1994; Peschek, 1981). The final proof that in semi-anaerobic Anacystis both hemes A and O are part of a functional respiratory oxidase rests on the following data. (i) Photoaction spectra of CO-inhibited oxygen uptake (Fig. 3B) showed peaks at 415 and 555 nm (cytochrome o₃) while the same experiments
Immunoblots of membrane proteins from semi-anaerobic and re-aerated cells with monospecific antibodies raised against subunits I of $a_3$-type cytochrome $c$ oxidase of $P$. denitrificans and $bo_3$-type quinol oxidase of E. coli are shown in Fig. 5. Independent of the antibody used and of the oxygen status of the cells, always only one single band was obtained, which, according to its apparent molecular mass of about 55 kDa, corresponds to subunit I of the well known $a_3$-type cytochrome $c$ oxidase of Anacystis (Peschek et al., 1988, 1989a; Molitor et al., 1990). Therefore it seems most likely that both hemes A and O, which are synthesized in Anacystis from heme B as in other living cells (Lübben and Morand, 1994; Hansson and von Wachenfeldt, 1993) and whose relative proportions just vary with oxygen concentrations available (Fig. 1), can combine with one and the same apoprotein. This would give rise to the spectral and functional attitude of both an $a_3$-type and an $o_3$-type enzyme (Figs. 2–4).

Similar promiscuity of the heme groups appears to obtain for both high spin, $O_2$-reducing hemes and low spin hemes as was found for the "conjugated" $ca_o/ca_{a_3}$-type, the $bo_{bo}$/bo$_3$-type, and the $o_{o_3}$/bo$_3$-type cytochrome oxidase pairs in Bacillus PS3 (Sone and Fujiwara, 1991), Acetobacter aceti (Matsushita et al., 1992), and E. coli (Puustinen and Wikström, 1993; Puustinen et al., 1992), respectively. Yet, this does not imply the various promiscuous types of oxidases in cyanobacteria representing true alternative oxidases in the classical sense; Table I gives the rates of oxygen uptake catalyzed by the cytoplasmic membranes from Anacystis phase B cells (see Fig. 1) incubated in the presence of different artificial and "physiological" electron donors and inhibitors. The results can best be reconciled with the occurrence of a functionally (with respect to the acceptor side) uniform cytochrome c (but not quinol) oxidase in the non-branched respiratory electron transport system of Anacystis and most probably of cyanobacteria in general.

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Transient Accumulation of Heme O (Cytochrome o) in the Cytoplasmic Membrane of Semi-anaerobic *Anacystis nidulans*: EVIDENCE FOR OXYGENASE-CATALYZED HEME O/A TRANSFORMATION

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