Occurrence of heme O in photoheterotrophically growing, semi-anaerobic cyanobacterium *Synechocystis* sp. PCC6803

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Abstract Extraction and identification of the non-covalently bound heme groups from crude membrane preparations of photoheterotrophically grown *Synechocystis* sp. PCC 6803 by reversed phase high performance liquid chromatography and optical spectrophotometry led to the detection of heme O in addition to hemes B and A which latter was to be expected from the known presence of aa_3-type cytochrome oxidase in cyanobacteria. In fully aerated cells (245 μM dissolved O_2 in the medium) besides heme B only heme A was found while in low-oxygen cells (< 10 μM dissolved O_2) heme O was present at a concentration even higher than that of heme A. Given the possible role of heme O as a biosynthetic intermediate between heme B and heme A, together with generally much higher K_m values of 5–50 μM O_2 for oxygenase as compared to K_m values of 40–70 nM O_2 for typical cytochrome-*c* oxidase, our findings may permit the conclusion that the conversion of heme O to heme A is an obligately oxygen-requiring process catalyzed by some oxygenase directly introducing oxygen from O_2 into the 8-methyl group of heme O. At the same time thus the occurrence of heme O (cytochrome *o*) in cyanobacteria does of course not imply the existence of an 'alternative oxidase' since according to the well-known 'promiscuity of heme groups' both hemes O and A are likely to combine with one and the same apoprotein.

Key words: Respiratory oxidase; Respiratory electron transport; Heme group; Reversed phase HPLC; Cyanobacteria, *Synechocystis* sp. PCC 6803

1. Introduction

Among the many cytochromes found in living cells [1] cytochrome *a* is unique in being restricted to terminal (respiratory) oxidases [2]. Aerobic respiration could have made sense on earth only after the advent of bulk amounts of dioxygen (O_2) gas which, according to all evidence currently available from comparative biology [3,4], geology [4,5] and paleontology [6] was introduced by the water-splitting, oxygenic photosynthesis of cyanobacteria (blue-green algae) approx. 3.2 billion years ago. As even nowadays oxygen is toxic to strict anaerobes [7] it seems reasonable to assume that the primordial cyanobacteria not only were the first to produce O_2 but also were the first to protect themselves against it [8]. In the long run, after a period of simple detoxification, it must have proven of much greater evolutionary and ecological significance to transform the pre-existing biochemical systems of photosynthesis (most importantly: the membrane-bound electron transport assembly) into aerobic respiration and a respiratory chain ('conversion hypothesis'; see [3,9,10]) and thus to eliminate the O_2 by reducing it back to water.

In this context it is worth noting that all of the cyanobacteria investigated so far (twenty-seven different strains and species tested) synthesize an aa_3-type cytochrome-*c* oxidase ([11]; Peschek et al., unpublished). This enzyme can be found in both plasma and thylakoid membranes of cyanobacteria, relative shares in either membrane strongly depending on growth (viz., 'stress') conditions [12–16]. Biochemical properties of the cyanobacterial cytochrome-*c* oxidase were strikingly similar to the mitochondrial enzyme and to that of *Paracoccus denitrificans* [13,17]. An operon-like genomic region comprising ctaC-D-E genes encoding 'mitochondria-like' subunits II, I and III was recently identified in *Synechococcus vulcanus* [18] and *Synechocystis* sp. PCC6803 [19]. One [18] or two [19] hitherto unidentified open reading frames may belong to the same operon.

The biochemistry of the different heme groups contained in various respiratory oxidases, either cytochrome-*c* or quinol oxidases [20], has been extensively investigated with several heterotrophic eubacteria and archaea [2,21–24]. Although the biosynthetic pathway leading to heme A (2-hydroxyethyl-farnesyl-8-formyl/iron porphyrin) has not yet been unraveled in all details it seems clear that it goes via heme B (2-vinyl-8-methyl/iron prophyrin) and heme O (2-hydroxyethyl farnesy-8-methyl/iron porhyrin) (see [2]). Thus the terminal step of heme A biosynthesis might involve a (hitherto unidentified) oxygenase requiring molecular O_2. As it has become technically feasible by now to extract any type of (acid-labile) heme group from biological tissues, in particular bacterial membranes, and to identify them by reversed phase high performance liquid chromatography [2,21,22] in the present study we have applied these techniques to the cyanobacterium *Synechocystis* sp. PCC6803 after photoheterotrophic growth under low and high oxygen tensions. Our results permit the conclusion that conversion of heme O into heme A requires (rather high levels of) molecular O_2. Similar to other bacteria and conforming to the so-called 'promiscuity of heme groups' [25,26], also in *Synechocystis* heme O and heme A may combine with identical apoproteins. Therefore, detection of heme O (cytochrome *o*) in addition to heme A (cytochrome *a*) does not constitute any evidence for the existence of an 'alternative oxidase' in *Synechocystis*. Last, but not least, the cloning of a 'heme O synthase gene' from *Synechocystis* sp. PCC6803 which was quite recently achieved in the laboratory of Norio Murata (N. Murata, personal communication) deserves mention in the present context.

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Abbreviations: HPLC, high performance liquid chromatography; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HQNO, 2-*n*-heptyl-4-hydroxy-quinoline *N*-oxide; cyt, cytochrome.

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2. Materials and methods

The cyanobacterium *Synechocystis* sp. PCC6803 was obtained through the courtesy of Mme. Rosi Rippka-Herdman, Institut Pasteur, Paris, France, and grown photoheterotrophically at 30°C under 15 $W \cdot m^{-2}$ warm white fluorescent light (measured with a YSI 65 radiometer at the surface of the growth flasks) on 10 mM glucose in axenic batch cultures containing 50 μM DCMU and sparged with either air (21%, v/v, O_2, i.e. 245 μM dissolved O_2) or 1%, v/v, O_2 in bacteriologically pure 'technical' N_2, i.e. < 10 μM dissolved O_2 [27] whose actual concentration was followed throughout growth using a sterilized Clark-type oxygen electrode (YSI 53 oxygen monitor). Cells were harvested by centrifugation at room temperature when their concentration had reached 3.0 μl packed cell mass/ml (light-limited, linear growth phase; see [15]) which in both conditions took the same time, viz. 6.0 days. This observation clearly means that oxygen availability was not growth-limiting in our conditions. Accordingly, the measurement of energy charge values on extracts from both high-oxygen and low-oxygen cells in the light agreeingly gave 0.78 ± 0.05 (data not shown; see [28,29]).

Crude membranes (composed of both yellow plasma and green cytoplasmic membranes) were prepared from harvested and washed cells by low-pressure French pressure cell treatment of lysozyme-pretreated cells as described [12,13]. Non-covalently bound hemes were extracted from a total of 2.0 ml dechlorophyllized [30,31] membrane suspensions (24.5 and 26.2 mg protein/ml, respectively, each from high-oxygen and low-oxygen grown cultures, and devided into 0.05-ml aliquots for experimental handling) with acetone/HCl (19:1, v/v) followed by ethyl acetate/acetonitrile treatment according to the procedure of Lübben et al. [2,21]. The heme composition was analyzed on an ISCO HPLC System 2004i equipped with a Deltapak C18 (3.9 × 150 mm, Zorbax) reversed phase HPLC column. Hemes were eluted with acetonitrile/0.5% trifluoroacetic acid/water gradients according to [25] (also, see [2] and Fig. 1) and detected spectrophotometrically at 406 nm (UVIS 205 detector, ISCO). The hemes were identified by comparison with heme A and B standards prepared by extraction of commercially available bovine cytochrome-*c* oxidase and reductase, respectively (Sigma Chemical Company, St. Louis, MO, USA), by comparison with a heme BO standard prepared by extraction of membranes from a cyt bo_3-overproducing *E. coli* strain (courtesy of Dr. M. Lübben), by transforming hemes which eluted after > 30 min from the reversed phase HPLC column (see Fig. 1) into alkaline pyridine hemochromes [30,32] whose reduced-minus-oxidized difference spectra were recorded (see Fig. 3), and by comparison with data given in the literature describing HPLC separation of heme groups under identical experimental conditions [2,21]. Spectra were recorded at 30°C on a Shimadzu UV-Vis 300 dual wavelength spectrophotometer equipped with a SAPCOM computer for baseline correction. Oxidation of reduced horse heart cytochrome-*c* (type VI from Sigma) was followed at 550 minus 540 nm, the 3.0-ml cuvettes containing approx. 10 μg membrane protein/ml [33].

Quantitation of the heme content was not possible since it was not known how much of the non-covalently bound heme groups was removed together with chlorophyll in the low-temperature pre-extraction of the heavily pigmented membranes [30,31] which reduced the chlorophyll content of the membranes from approx. 0.1 mg/mg protein to 0.01 mg/mg protein thus, by establishing a reasonable signal-to-noise ratio, finally permitting at least the qualitative identification of heme groups in the membranes (see Fig. 1).

3. Results

Fig. 1 shows reversed-phase HPL chromatograms of the heme groups present in extracts of partially dechlorophyllized membranes prepared from high-oxygen (A) and low-oxygen (B) *Synechocystis* photoheterotrophically grown and specified as given in section 2. Extremely high noise levels and background contaminations detected at 406 nm on chromatograms of extracts from freshly prepared membranes obviously stem from chlorophyll (and perhaps also other pigments') degradation products and obscure the identification of heme groups ([21]; Mathias Lübben, personal communication). However, when 'dechlorophyllized' membranes pretreated (pre-extracted) with aqueous acetone [30] or *n*-pentane [31] at −25°C to −20°C were used, which had lost up to 90% of the original 0.1 mg chlorophyll per mg membrane protein, identification of the heme groups on reversed phase HPL chromatograms was possible (Fig. 1) and the comparison with authentic hemes B, O and A (see Fig. 1C and [21]) permitted fairly clear-cut identification of the heme groups although in this case it remained unknown how much of the non-covalently bound hemes originally present in the membranes might have been lost during the pre-extraction step; at any rate enough heme had apparently been retained to permit qualitative identification (Fig. 1).

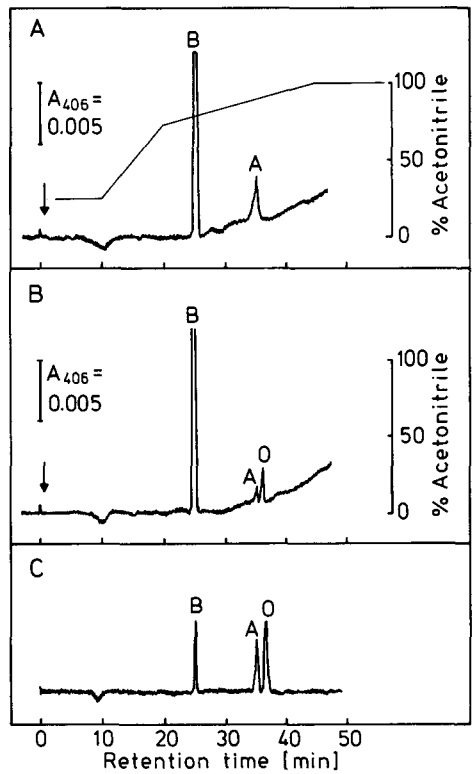

Fig. 1. Reversed-phase HPL chromatograms of non-covalently bound heme groups extracted from dechlorophyllized (pre-extracted) membranes of *Synechocystis* photoheterotrophically grown under high (A) and low (B) oxygen tension. The corresponding chromatogram of authentic heme A, B and O standards is shown in (C). Authentic hemes B and A were extracted from commercially available cytochrome-*c* reductase and oxidase, respectively (obtained from Sigma). Additionally, hemes B and O were extracted from the membranes of a cyt *bo*-overproducing *E. coli* mutant kindly provided by Dr. M. Lübben.

Identification of hemes A and O in dechlorophyllized and solubilized membranes was confirmed by recording CO/reduced-minus-reduced difference spectra on preparations from high-oxygen grown *Synechocystis* (Fig. 2A; peaks at 430 nm and 590 nm pointing to cytochrome a_3 [13,30]) and from low-oxygen grown cells (Fig. 2B; additional spectral features at 415 nm and 555 nm pointing to cytochrome *o* [21]), and by converting the heme groups present in the > 30 min reversed phase HPL chromatographic eluate (Fig. 1) into alkaline pyridine hemochromes and recording oxidized-minus-reduced difference spectra [32] (Fig. 3A; peaks of the heme A derivative at 433 and 588 nm; and Fig. 3B, additional peaks at 415 and 455 nm being attributed to heme O since heme B is no more present in the > 30 min eluate, see Fig. 1; also see [2,21]).

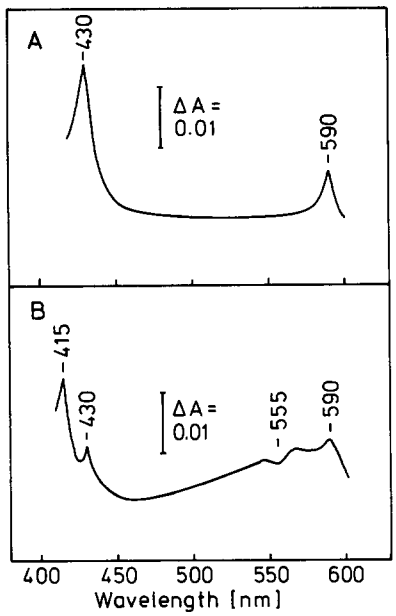

Fig. 2. CO/reduced-minus-reduced difference spectra of 1%-octyl-glucoside solubilized membranes prepared from high-oxygen (A) and low-oxygen (B) grown *Synechocystis*. Reduction of the solubilized cytochromes was achieved by adding a few grains of dithionite. CO bubbling was for a least 20 min prior to measurements.

Fig. 4 shows that native membranes from both high-oxygen (A) and low-oxygen (B) *Synechocystis* catalyze reasonably fast rates of horse heart ferrocytochrome-*c* oxidation which is insensitive to 5 μM HQNO but blocked by 5 μM KCN. Interestingly, the re-reduction of the ferricytochrome *c* previously formed in the cyt-*c* oxidase reaction is inhibited by HQNO (traces 1b and 2b in Fig. 4). This, together with the observation that membranes from wild-type *Synechocystis* (and other cyanobacteria; see [34]) do not oxidize NADH unless a catalytic amount of (horse heart) cytochrome *c* is added (Fig. 4C; polarographic assay) points to a cyt *c* oxidase rather than a quinol oxidase. Since the same cytochrome-*c* dependent NADH oxidation (Fig. 4C) was also observed with membranes from semi-anaerobic *Synechocystis*, where some cytochrome o_3 could act as an 'alternate oxidase' (Fig. 2B), the cyanobacterial respiratory oxidase appears to be a constitutive cytochrome-*c* but not quinol oxidase.

4. Discussion

Crude membrane preparations of cyanobacteria comprising both chlorophyll-free plasma membranes (10–20% in terms of total membrane protein) and chlorophyll-containing thylakoid membranes (80–90%) do not render themselves very suitable for direct spectrophotometry or HPLC owing to severe interference from abundant, mostly lipophilic pigment molecules (chlorophyll) absorbing in the visible region. However, suitable dechlorophyllization (pre-extraction) of the membranes had permitted the spectral detection of cytochrome aa_3 in cyanobacterial membranes fifteen years ago already [30,35]. These results were later confirmed independently in many laboratories and recently corroborated even on the genetic level [18,19]. Thus it seems clear by now that aa_3-type cytochrome-*c* oxidase occurs in both plasma and thylakoid membranes of cyanobacteria, proportions depending strongly on the types and growth conditions of the organisms [11–15]. In the present investigation we used dechlorophyllized membranes [30,31] for qualitative reversed phase HPLC determination of extracted heme groups essentially according to the procedure of Lübben et al. [2,21,22]. These membranes had lost up to 90% of the original chlorophyll content (around 0.1 mg/mg protein) but nevertheless retained substantial amounts of acid-extractable heme groups of types A, B and O, although quantitative measurements on the membranes were no more possible, of course.

Our results clearly show the presence of hemes B and A (and thus of b-type and a-type cytochromes) in membranes from *Synechocystis* sp. PCC6803 grown photoheterotrophically in the presence of ambient oxygen concentration (21%, v/v, or 245 μM O_2 in the growth medium at 30°C; note that the usual supersaturating photosynthetic oxygen production by the cells was avoided by using the photoheterotrophic mode of growth). No other type of heme group was detected in these membranes (Fig. 1A). However, when the oxygen tension during growth was lowered below 10 μM O_2 for at least five generations before harvest (approx. 6 days) our reversed phase HPL chromatograms gave clear-cut evidence for the additional presence of heme O in the extracts (Fig. 1B) which was tentatively proven by comparison with authentic heme standards (see Fig. 1C), by comparison with data published in the literature but obtained with membranes from different bacteria [2,21,22], by CO/reduced-minus-reduced optical difference spectra (Fig. 2), and by reduced-minus-oxidized optical difference spectra of alkaline pyridine hemochromes derived from eluted pigments (Fig. 3). Measurements of cytochrome-*c* and NADH oxidation by the native membranes were fully compatible with the occurrence

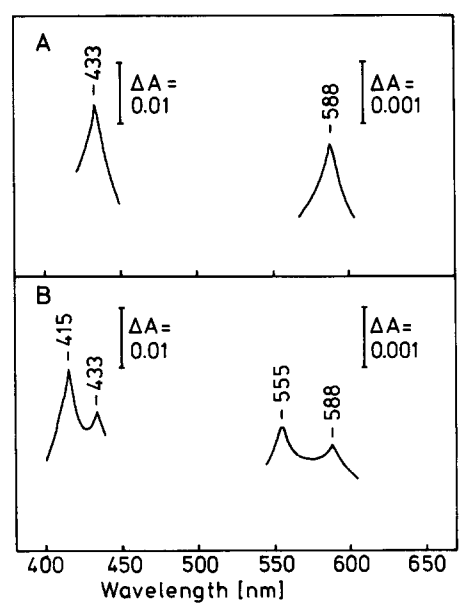

Fig. 3. Alkaline pyridine hemochrome spectra of hemes extracted from the membrane preparations of high-oxygen (A) and low-oxygen (B) grown *Synechocystis* and eluting from the HPLC column well after heme B (see Fig. 1). Heme B-like features of the spectrum (panel B) thus can only be attributed to heme O (see [2,21]). Hemochromes were directly prepared from concentrated fractions of the HPLC eluent with 20% pyridine/0.1 M NaOH and extracted into ether for the spectrophotometric determination after reduction with dithionite and oxidation with ferricyanide.

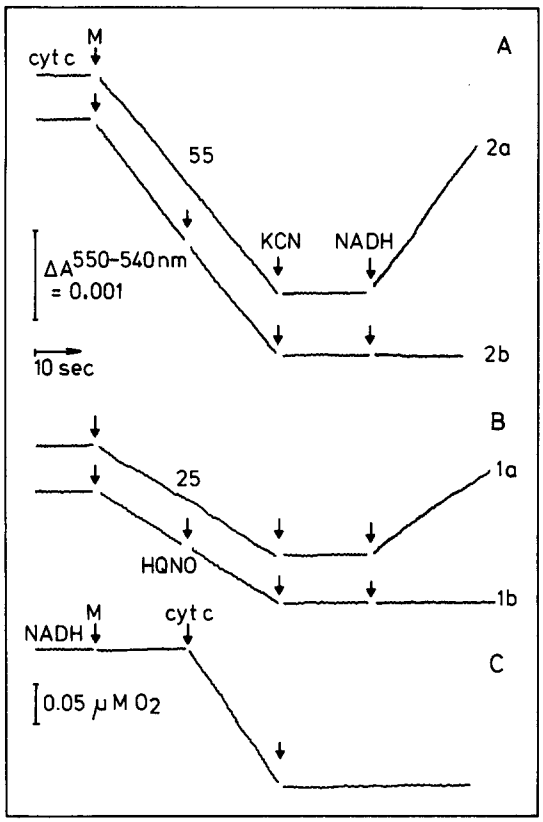

Fig. 4. Recorder traces of horse heart ferrocytochrome-*c* oxidation measured by dual-wavelength spectrophotometry at 550 minus 540 nm (A and B) and of polarographically determined oxygen uptake in the presence of 1.5 mM NADH (C) catalyzed by crude membrane preparations freshly isolated from high-oxygen (A and C) and low-oxygen (B) grown *Synechocystis*. Arrows indicate the addition of concentrated membrane suspension (M) giving a final concentration of 10 μg (A and B) or 65 μg (C) of membrane protein/ml in 2.5 ml K-phosphate buffer (pH 7.0), 5 μM HQNO or KCN, and 1.5 mM NADH, respectively (final concentrations in each case). Figures adjacent to recorder traces give rates of cyt *c* oxidation in nmol/min per mg membrane protein. The arrow marked with 'cyt *c*' on panel C indicates the addition of a catalytic amount (approx. 3 μM final concentration) of horse heart cyt *c*. Initial ferrocytochrome *c* concentration in spectrophotometric assays A and B was 10 μM. 5 μM antimycin-A or piericidin-A blocked the NADH oxidase reaction in isolated and purified cytoplasmic membranes from *Synechocystis* and other cyanobacteria [16,40], strengthening the idea that both 'bacterial' and 'mitochondrial' electron transport complexes III and I occur in the cytoplasmic membrane-bound respiratory chains of cyanobacteria [16]. The NADH oxidase (trace C) in membranes from semi-anaerobic *Synechocystis* showed the same cytochrome-*c* dependence [34] as in membranes from fully aerated cells suggesting that the 'cytochrome o_3' (Fig. 2B) is not functioning as a quinol oxidase under whatever conditions (results not shown).

of a highly KCN-sensitive cytochrome-*c* oxidase in the membranes (Fig. 4) as was previously demonstrated for twenty-seven different strains and species of cyanobacteria ([11,30,35]; Peschek et al., unpublished).

Though our results clearly demonstrate the presence of heme O (cytochrome *o*) in the membranes of *Synechocystis* after prolonged growth in a photoheterotrophic low-oxygen medium they do not favor the occurrence of an o-type (or a-type) quinol oxidase, nor of any other type of 'alternative oxidase' in *Synechocystis*. Rather, we believe that heme O detected in our extracts just reflects an intermediate in the biosynthetic pathway from heme B to heme A [2], which transiently accumulates – most probably attached to the same apoprotein(s) as is heme A [25,26] – when the cells are exposed to oxygen tensions below the O_2-affinity of the (hitherto unidentified) oxygenase enzyme that converts the 8-methyl group of heme O into the 8-formyl group of heme A [2,21]. It may be recalled in this context that K_m (O_2) values of oxygenase enzymes are usually much higher than the $K_m(O_2)$ value of cytochrome-*c* oxidase [36] and that also in the conversion of chlorophyll a (7-methyl-group) to chlorophyll *b* (7-formyl-group) molecular oxygen participates directly in the reaction [37].

Although the enzymes of heme A biosynthesis have not yet been fully characterized in any organism [2,21] a route from heme B via heme O seems likely. At least in the well-studied *Bacillus subtilis*, in this pathway the gene products of ctaA and ctaB genes of the cta operon appear to play key roles [2]. A putative ctaA gene was previously identified in *Synechococcus vulcanus* [18] and a possible ctaB gene (which was called 'coxD' or 'heme O synthase' gene) was quite recently detected in *Synechocystis* sp. PCC6803 though not as part of the cta operon (Norio Murata, personal communication). It is interesting that the ctaB protein of *Bacillus subtilis*, which is thought to be related to a hydroxyethyl farnesyl transferase ('heme O synthase'), bears a striking sequence similarity with the homologous CyoE protein of *E. coli* [38] and the COX10 protein of *Saccharomyces cerevisiae* [39]. Yet, while in wild-type *E. coli* at least one of the alternate respiratory oxidases is of bo-type no cytochrome *o* at all is known to occur in *S. cerevisiae*. On the other hand, using gene vectors containing both ctaA and ctaB genes of *B. subtilis E. coli* can be transformed to synthesize substantial quantities of heme A but no functional a-type respiratory oxidase was found in these transformants [2]. Similar gene manipulation concerning the terminal oxidase of cyanobacteria is eagerly awaited.

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