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OCCURRENCE OF CYTOCHROME aa_3 IN *ANACYSTIS NIDULANS*

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Summary

The cytochrome content of membrane fragments prepared from the blue-green alga (cyanobacterium) *Anacystis nidulans* was examined by difference spectrophotometry. Two *b*-type cytochromes and a hitherto unknown cytochrome *a* could be characterized. In the reduced-minus-oxidised difference spectra the *a*-type cytochrome showed an α-band at 605 nm and a γ-band at 445 nm. These bands shifted to 590 and 430 nm, respectively, in CO difference spectra. NADPH, NADH and ascorbate reduced the cytochrome through added horse heart cytochrome *c* as electron mediator. In presence of KCN the reduced-minus-oxidised spectrum showed a peak at 600 nm and a trough at 604 nm. Photoaction spectra of O_2 uptake and of horse heart cytochrome *c* oxidation by CO-inhibited membranes showed peaks at 590 and 430 nm. These findings are consistent with cytochrome aa_3 being the predominant respiratory cytochrome *c* oxidase in *Anacystis nidulans*.

Introduction

For many years it has been speculated that the slow dark respiratory O_2 uptake by blue-green algae (cyanobacteria) might be intimately associated with the photosynthetic electron transport system [1—3] (cf. Refs. 4—6). However, respiratory electron transport independent of the one operating in photosynthesis might exist as well [3,7—9]. Unfortunately it is still impossible even to sketch molecular and/or mechanistic features of the respiratory chain in blue-green algae as none of their membrane-bound redox compounds could ever be convincingly shown to be involved in respiration [5]. Despite several

Abbrevations: PMS, phenazine methosulfate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

reports on the dark oxidation of horse heart cytochrome *c* by cell-free extracts of blue-green algae [2,10,11] the terminal oxidase responsible for the reaction could never be identified. The present paper discusses spectrophotometric evidence for a cytochrome *a*-type terminal oxidase in membrane preparations of *Anacystis nidulans* strongly resembling mammalian cytochrome aa_3. Some aspects of the respiratory chain of *Anacystis* have already been dealt with in previous publications [8,12,13].

Materials and Methods

Anacystis nidulans was grown axenically at 40°C as described [14]. Purity of the cultures was routinely checked under the phase contrast microscope. Cells were harvested in the early stationary phase, suspended in 50 mM Tris-HCl buffer, pH 8.2, supplemented with 5 mM $MgCl_2$, 1 mM dithiothreitol and 0.3 M mannitol, and passed through a precooled French pressure cell at 20 000 p.s.i. (1400 kg/cm^2). Unbroken cells and debris were removed at 5000 × *g* (4°C; 20 min). A crude membrane fraction was sedimented at 144 000 × *g* (4°C; 120 min). Residual phycocyanin was removed from the membranes by washing them once with fresh breakage medium. Washed particles were suspended in 10 mM sodium-potassium phosphate buffer, pH 7.4, supplemented with 3 mM $MgCl_2$ and 0.8 M sucrose, and immediately examined in thermostatically controlled (4°C) 1-cm cuvettes of a fully computerized Perkin-Elmer dual wavelength spectrophotometer, model 557. Owing to the high chlorophyll content of native ('green') membranes (approx. 0.1 mg chlorophyll/mg protein) these could not be used for spectrophotometry in the regions of high specific chlorophyll absorbance. Hence the membranes were freed from chlorophyll by three brief extractions with −25°C cold 80% aqueous acetone, each one followed by centrifugation at 40 000 × *g* (−20°C; 20 min). The chlorophyll-free ('white') membranes had also lost about 40% of the cytochrome aa_3 originally present as judged from the relative heights of the reduced α-band at 605 nm before and after the treatment (data not shown).

Photoaction spectra of O_2 uptake were determined in a thermostatically controlled (35°C) glass chamber containing 2.5 ml of the diluted membrane suspension equipped with a Clark-type oxygen electrode, and illuminated from one side with an Oriel 1000 W Xenon lamp mounted behind a monochromator on an optical bank. 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, model 65. A mixture of 90% CO and 10% O_2 was slowly bubbled through the membrane suspension. Respiratory O_2 uptake was initiated by injecting a freshly prepared solution of 20 mM sodium ascorbate and 1 mM TMPD (final concentrations) and followed polarographically [8,12]. Photoaction spectra of cytochrome *c* oxidation were determined on CO-gassed membrane suspensions in thermostatically controlled (30°C) 1-cm cuvettes of the spectrophotometer which was adapted for cross-illumination, through a light guide, from the light source specified above. The reaction was started with 10 μM horse heart ferrocytochrome *c* previously prepared by reducing the cytochrome with a 10-fold excess of sodium ascorbate followed by dialysis against 5 l of phosphate buffer, pH 7.4, at 4°C for 16 h. Cytochrome *c* oxidation was

followed by dual wavelength spectrophotometry using a $\Delta\epsilon$ (550 minus 540 nm) of 19.7 $mM^{-1} \cdot cm^{-1}$ [12]. As with O_2 uptake measurements a new batch of membranes was employed at each wavelength of the actinic light. Photodissociation spectra were determined according to Appleby [15]. Protein and chlorophyll were determined as previously described [14].

Heme was extracted directly from freshly prepared membranes using a solvent mixture of methyl ethyl ketone/acetonitrile/3 M HCl (6 : 4 : 1, v/v), and converted to the alkaline pyridine derivatives according to Barrett [16]. Relatively high background absorption caused by the pheophytin formed from chlorophyll through the acid extraction procedure did not interfere with the recording of dithionite-reduced minus H_2O_2-oxidised spectra of the extracts since pheophytin could be neither reduced nor oxidised under the conditions used (cf. Fig. 5).

Results and Discussion

Typical reduced-minus-oxidised difference spectra of green *Anacystis* membranes are shown in Fig. 1. With ascorbate as a reductant the α-band of cytochrome *b*-559 appeared (Fig. 1A). Reduction with dithionite revealed the presence of another *b*-type cytochrome (α-band at 564 nm). In addition a hitherto unknown peak around 605 nm was detected in dithionite- minus ascorbate-reduced spectra (Fig. 1B). Cytochromes *b*-559 and *b*-564 are known

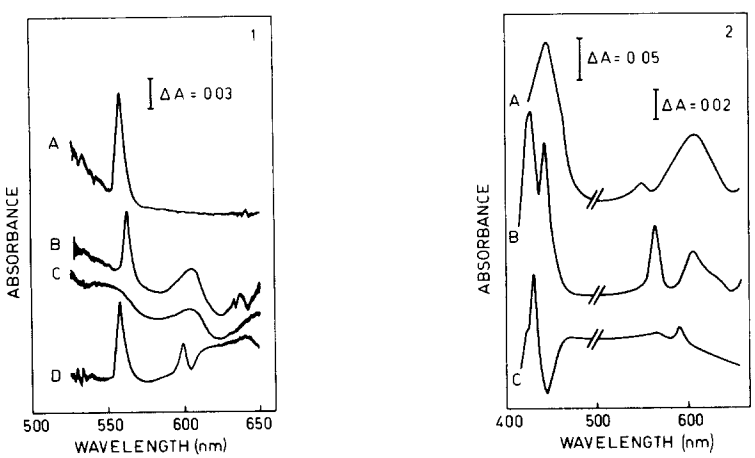

Fig. 1. Difference spectra of chlorophyll-containing *Anacystis* membranes (0.6 mg protein/ml). Baselines were first recorded and stored in the computer of the spectrophotometer; they were then automatically subtracted from the respective difference spectra to be displayed. A, ascorbate minus aerated; B, dithionite minus ascorbate; C, anaerobic plus 3 mM NADPH or NADH minus aerated; D, ascorbate plus TMPD minus aerated. 10 μM oxidised horse heart cytochrome *c* had initially been added to the sample cuvette of (C); both cuvettes of (D) had been preincubated with 0.5 mM KCN for 5 min and briefly aerated before adding 20 mM ascorbate together with 0.1 mM TMPD. Oxidation of the reference cuvette by aeration gave qualitatively similar results as did oxidation by ferricyanide or by 0.1% H_2O_2 (not shown).

Fig. 2. Difference spectra of chlorophyll-free *Anacystis* membranes (A and B, 15 mg protein/ml; C, 22 mg protein/ml). A, ascorbate plus 50 μM PMS minus ascorbate; B, dithionite minus ascorbate; C, dithionite plus CO minus dithionite.

to participate in the photosynthetic electron transport of blue-green algae [17]; analogous *b*-type cytochromes are found in chloroplasts [18].

Besides three soluble *c*-type cytochromes [19] a membrane-bound cytochrome *c*-556.5 supposed to be the counterpart of cytochrome *f* of chloroplasts has been isolated from *Anacystis* [20]. However, as there was no *c*-type cytochrome evident from our spectra (Figs. 1 and 2) the cytochrome *c*-556.5 may have been lost from the membranes during isolation. Characteristically, when horse heart ferricytochrome *c* (obtained by ferricyanide-oxidation and subsequent dialysis of commercially available cytochrome *c*) was added to the membranes the peak at 605 nm appeared even in presence of NADPH, NADH or ascorbate (Fig. 1C). This is consistent with the assumption that the 605 nm pigment might be a terminal oxidase which can be reduced by physiological substrates via added horse heart cytochrome *c* serving as an exogenous electron carrier to cytochrome *c*-depleted *Anacystis* membranes. The spectral characteristics of the reduced *a*-type cytochrome were strikingly modified by KCN (Fig. 1D). With KCN present the peak at 605 nm was replaced by a peak at 600 nm and a trough at 604 nm. Assuming the terminal oxidase in *Anacystis* to be cytochrome aa_3 these findings may be explained on the ground that cyanide-complexed cytochrome a_3 would not be reduced any more through the respiratory chain while cytochrome *a* still would; thus the cytochrome *a* moiety would be reflected by the peak at 600 nm while the trough at 604 nm would stem from cytochrome a_3 effectively excluded from the difference spectrum in presence of KCN (Fig. 1D). This is reminiscent of similar behaviour found with mammalian cytochrome *c* oxidase (cf. Ref. 21).

With green membranes (Fig. 1) spectral observations did not give reliable results in the regions of high chlorophyll absorbance; thus only the α-bands of the cytochromes could be investigated. With extracted (white) membranes, however, the Soret region was successfully examined (Fig. 2). An ascorbate plus PMS-reduced minus ascorbate-reduced spectrum selectively unmasked the *a*-type cytochrome resulting in α-, β- and γ-peaks at 605 nm, 550 and 445 nm, respectively (Fig. 2A). The same peaks were found in dithionite- minus ascorbate-reduced spectra but the large α- and γ-peaks of cytochrome *b*-564 were even more prominent (Fig. 2B; cf. Fig. 1B). Yet by its inability to react with CO cytochrome *b*-564 was excluded from the CO difference spectrum, and only the reduced α- and γ-bands of the CO-complexed *a*-type cytochrome at 590 and 430 nm, respectively, remained (Fig. 2C).

Photoaction spectra of CO-inhibited O_2 uptake supported by ascorbate plus TMPD (Fig. 3A) and of CO-inhibited oxidation of horse heart ferrocytochrome *c* (Fig. 3B) revealed peaks at 590 and 430 nm (and at 550 nm; Fig. 3A) obviously coinciding with the α-, γ- (and β-) bands, respectively, of the CO compound of the reduced oxidase. In photodissociation spectra (Fig. 4) the γ-peak of the CO-complexed oxidase at 430 nm was partially replaced by the γ-peak of the CO-free form at 445 nm during cross-illumination of the sample with 590 and 550 nm light mainly absorbed in the α- and β-regions of the CO complex; virtually no change in the spectrum was observed with 480 or 650 nm light of comparable intensity which is apparently not absorbed by the complex (not shown; cf. Fig. 3A).

Finally, reduced minus oxidised difference spectra of the alkaline pyridine

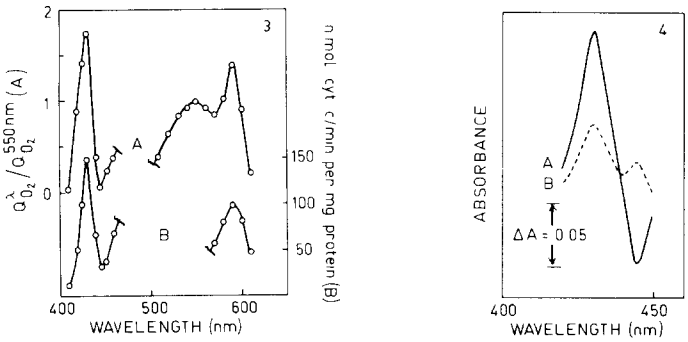

Fig. 3. Photoaction spectra of CO-inhibited O_2 uptake (A) and horse heart cytochrome *c* oxidation (B) by chlorophyll-free *Anacystis* membranes (A, 2.2 mg protein/ml; B, 1.7 mg protein/ml). Dark O_2 uptake was between 33 and 40 nmol/min per mg protein without, and about 3 with CO present. Experimental details are given in the text.

Fig. 4. Photodissociation spectrum for the CO-complexed oxidase in chlorophyll-free *Anacystis* membranes. A, γ-bands of the CO compound of the oxidase; B, same sample as in (A) during cross-illumination with 20 W/m^2 590 nm light; 550 nm light gave similar results but 480 or 650 nm light of equal intensity was virtually ineffective (not shown).

derivatives of heme extracted directly from the membranes are shown in Fig. 5. Heme *a* (cytohemin: α- and γ-bands at 588 and 433 nm, respectively; no β-band) and heme *b* (protoheme: α-, β- and γ-bands at 556, 520 and 422 nm, respectively) can be clearly distinguished. The protoheme obviously resulted from the photosynthetic *b*-type cytochromes shown to be present in the membranes (cf. Figs. 1 and 2).

The *a*-type cytochrome characterized as a functional respiratory cytochrome *c* oxidase in the *Anacystis* membranes thus appears to be cytochrome aa_3 by comparison with spectral properties known of mammalian cytochrome oxidase (cf. Ref. 21). Similar conclusions have recently been drawn from preliminary spectrophotometric investigations on respiratory particles isolated from two other species of blue-green algae, viz. *Nostoc* sp. strain MAC [3,22] and *Anabaena variabilis* [3]. However, the potential presence of minor amounts of

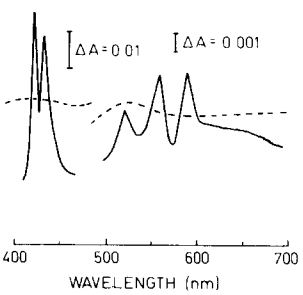

Fig. 5. Reduced minus oxidised difference spectrum of alkaline pyridine hemochromes prepared directly from the *Anacystis* membranes. The sample cuvette was reduced with sodium dithionite while the reference cuvette was oxidised with 0.1 % H_2O_2; background absorption by the pheophytin was not affected by this treatment (for details cf. Materials and Methods). Spectra were recorded at 4°C. Cytohemin (heme *a*, α- and γ-peaks at 588 and 433 nm, respectively; no β-peak) and protoheme (heme *b*, α-, β- and γ-peaks at 556, 520 and 422 nm, respectively) are clearly visible (cf. Ref. 23).

additional types of respiratory cytochrome oxidases in these organisms cannot be rigorously excluded so far.

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