Respiratory electron transport in plasma and thylakoid membrane preparations from the cyanobacterium *Anacystis nidulans*

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Plasma and thylakoid membranes were separated and purified from cell-free extracts of *Anacystis nidulans* by discontinuous sucrose density gradient centrifugation. Plasma membranes contained less than 0.05% (w/w) chlorophyll per protein. Both plasma and thylakoid membranes oxidized horse heart ferrocytochrome *c* (10 ± 4 and 3 ± 1 nmol/min per mg protein, respectively), with strong inhibition by low concentrations of cyanide, sulfide, azide, carbon monoxide, and salicyl aldoxime. The activity was stimulated 125% by 3.5% Tween 80 and totally suppressed by 100 mM NaCl. Oxidized cytochrome *c* was reduced by both types of membranes in the presence of NAD(P)H. The results indicate the occurrence of a respiratory chain including cytochrome-*c* oxidase in both plasma and thylakoid membranes of *A. nidulans*.

Respiratory chain *Cytochrome-c oxidase* *Cytochrome c reductase* *Plasma membrane* *Thylakoid membrane* (Anacystis nidulans) *Cyanobacteria*

1. INTRODUCTION

Since the first investigations on cyanobacterial respiration [1–3] it has been argued that the cyanobacteria (blue-green algae), whose predominant ecological and physiological feature is oxygenic, plant-type photosynthesis, must be endowed with a respiratory chain comprising some hemoprotein cytochrome-*c* oxidase [3–5]. There is evidence for electron transport components common to both photosynthesis and respiration in the thylakoid membranes (ICM) [6,7]. Spectrophotometric and inhibitor studies on crude membrane preparations of *Anacystis nidulans* comprising both CM and ICM have suggested the presence of aa_3-type terminal oxidase [8]. Oxidation of exogenous *c*-type ferrocytochromes by intact spheroplasts indicated the occurrence of a (H^+-translocating) cyt *c* oxidase in the plasma membrane [9,10]. Similar conclusions were drawn from the H^+-extruding properties of whole cells [11,12], the energetic efficiency of oxidative phosphorylation [13], cytochemical results [14], and still other lines of experimental evidence (review [15]). On the other hand, there are indications (viz. with *Anabaena variabilis*) that the thylakoid membrane may be the only site of electron transport, both photosynthetic and respiratory [16,17]. Clear-cut resolution of this question has not yet been achieved; a major obstacle has been, and still is, the tremendous difficulties in obtaining satisfactory preparations of individual, and yet physiologically active, CM and ICM fractions from cyanobacteria. In the present investigation,

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Abbreviations: cyt, cytochrome; CM, plasma membrane; ICM, thylakoid membrane; Chl, chlorophyll; PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone

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following and improving earlier attempts by Murata and co-workers [18], we succeeded for the first time in isolating and purifying a chlorophyll-free CM fraction from *A. nidulans* that showed comparatively high rates of horse heart ferrocytochrome *c* oxidation. From corresponding inhibition profiles and other characteristics of the reaction we conclude that both CM and ICM of *A. nidulans* contain a hemoprotein terminal oxidase which may be of the aa_3 type according to previous results with crude membranes, intact cells and spheroplasts [15].

2. MATERIALS AND METHODS

Axenic cultures of *A. nidulans*, strain 1402-1 (Göttingen, FRG), were grown photoautotrophically in modified medium D at 35°C to the late logarithmic phase in batch culture as described [12]. Only cultures free of bacterial and other contamination were processed further. Cells were harvested by centrifugation at room temperature, washed twice with sterile growth medium, pH 8.0 (carbonate omitted), and resuspended in 10 mM Hepes/NaOH buffer containing 20% (w/v) sucrose, 5 mM NaCl and 2 mM Na_2EDTA (80 μl packed cells/ml; final pH 7.4). Lysozyme was added (0.25%, w/v) and the suspension incubated at 37°C for 2 h. After centrifugation (3000 × *g*, 20°C, 10 min) spheroplasts and remaining intact cells were resuspended in 10 mM Hepes/NaOH (pH 7.4) containing 5 mM NaCl, 2 mM Na_2EDTA, and 0.01% (w/v) protease inhibitors PMSF, TPCK and/or TLCK, in a total volume corresponding to the original one. The suspension was passed through a pre-cooled French pressure cell at 33 MPa and centrifuged (4000 × *g*, 4°C, 10 min) to remove intact cells and cell debris. The supernatant was centrifuged (175000 × *g*, 4°C, 1 h), the (deep blue) supernatant discarded, and the pellet washed once by recentrifugation under the same conditions. Pelleted and washed crude membranes were suspended in 12 ml of 10% (w/w) sucrose, 10 mM Hepes/NaOH (pH 7.4), 5 mM NaCl, and 2 mM Na_2EDTA, including protease inhibitors as before (approx. 3 mg protein/ml). Aliquots (2 ml each) of the homogeneous membrane suspension were layered on top of discontinuous sucrose gradients (total of 35 ml for each gradient) made up of 42, 35, 30 and 20% (w/w) sucrose uniformly containing 10 mM Hepes/NaOH, 5 mM NaCl, and 2 mM Na_2EDTA (final pH 7.4), together with protease inhibitors as before. The gradients were centrifuged in an SW-27 swinging-bucket rotor (131500 × g_{max}, 16 h; Beckman ultracentrifuge, model L5-50). Individual membrane fractions were withdrawn from the gradient, diluted with 10 mM Hepes/NaOH, 5 mM NaCl and 2 mM Na_2EDTA (final pH 7.4), and centrifuged at 175000 × *g* and 4°C for 1 h. Pelleted membranes were resuspended in small volumes of 10 mM Hepes/NaOH (pH 7.4) and immediately used for the assay of electron transport activities. Usually reactions were started by injecting aliquots of the concentrated membrane suspensions into the spectrophotometer cuvettes containing the assay buffer (10 mM Na-phosphate, pH 7.4) and cyt *c*; in some experiments the membranes were added prior to cyt *c* (see table 3).

Oxidation and reduction of cyt *c* was measured with a Shimadzu UV-300 dual-wavelength spectrophotometer at room temperature using $\Delta\epsilon$ (red − ox) = 19.5 $mM^{-1} \cdot cm^{-1}$ at 550–540 nm [19]. Maximum specific activities were measured at 2–20 μM cyt *c* and membrane protein concentrations < 50 μg/ml. Under these conditions reaction rates were independent of cyt *c* concentration and linear with protein concentration. Horse (or bovine) heart cyt *c*, NADH, NADPH, and SDS were from Sigma, Calbiochem, Serva and Boehringer, respectively; no differences between preparations of different origin could be detected. All other chemicals were of the highest purity grade commercially available. Solutions of cyt *c* and NAD(P)H were always freshly prepared before use. Cyt *c* was reduced with excess Na-ascorbate followed by exhaustive dialysis as described [8]. Protein and chlorophyll were determined according to Bradford [20] and Mackinney [21], respectively.

3. RESULTS

Fig.1 depicts the distribution of individual membrane fractions along the sucrose density gradient after centrifugation. CM fractions were practically devoid of chlorophyll (table 1). Table 2 shows the cyt oxidase activity together with % recoveries of this activity, and of protein, in CM and ICM frac-

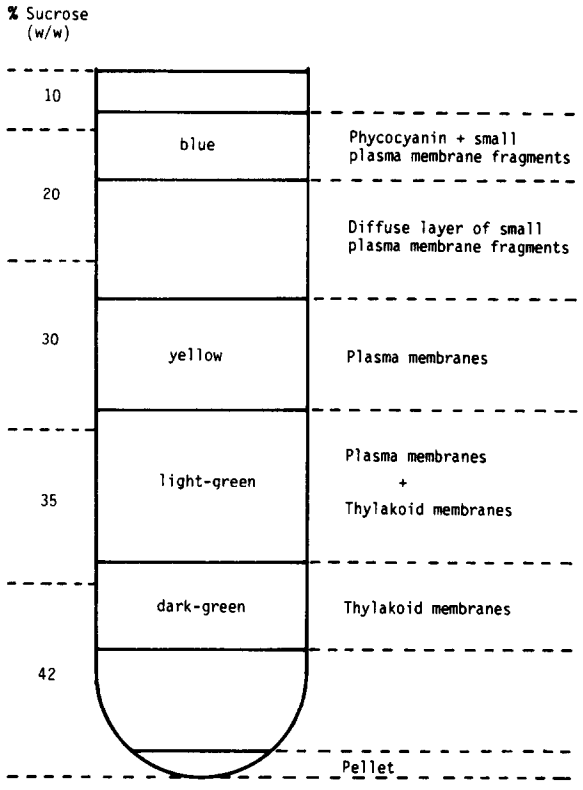

Fig.1. Fractionation of crude membranes from *Anacystis nidulans* by discontinuous sucrose density gradient centrifugation.

Table 1

Fractionation of crude membranes from *A. nidulans* by discontinuous sucrose density gradient centrifugation

Membrane fraction	% (w/w) sucrose	% (w/w) Chl per protein	Assignment
M	10	0.1–0.2[a]	crude membranes
CM	30	<0.05	plasma membrane
CM + ICM	35	0.2	plasma membrane + thylakoid membrane
ICM	35/42	0.2–0.4[a]	thylakoid membrane
P	–	0.1–0.2[a]	pellet

[a] Extremes from 22 independent preparations

For details see section 2; also see fig.1

tions. It is clearly seen that the cyt oxidase activity in the CM preparations was not associated with chlorophyll.

Cyt *c* oxidase activity in both CM and ICM was eliminated by cyanide concentrations as low as 1.2 μM, inhibited >95% by 0.1 mM sulfide, 1.0 mM azide, and 20 mM salicyl aldoxime (a potent copper-chelating agent; cf. [22]), and strongly inhibited also by carbon monoxide (table 3). The reaction was sensitive to elevated ionic strength and stimulated by Tween 80. Qualitatively similar results were obtained from preliminary cyt oxidase assays with isolated CM and ICM from *Plectonema* ATCC 29407, thus confirming [23], while

Table 2

Cytochrome oxidase activity and % recovery of this activity, and of protein, in CM and ICM fractions

Fraction	nmol ferrocytochrome *c* oxidized/min per mg		% activity recovered[ab]	% protein recovered[a]
	Protein	Chlorophyll		
CM (13)	10 ± 4	160 ± 40	0.05–1.5	1– 2
ICM (22)	3 ± 1	12 ± 8	0.25–9.0	15–45

[a] Protein and specific activity in crude membranes = 100% (see table 1)
[b] Specific activity of crude membranes was 20–120 nmol cyt *c*/min per mg protein

Samples contained 15 μM cyt *c* and 15–45 μg protein/ml. For details see section 2; also see table 1. Number of determinations (each from a separate preparation) shown in brackets

Table 3
Inhibition and activation of the cytochrome oxidase activity (nmol cyt *c*/min per mg protein) in CM and ICM preparations

Additions	Final concentration	CM	ICM
None	–	13.6	3.6
CO[a]		2.0	0.5
KCN	1.2 μM	0.0	0.0
Na_2S	0.1 mM	0.3	0.1
NaN_3	1.0 mM	0.5	0.2
Salicylaldoxime	20 mM	0.4	0.1
NaCl	100 mM	0.0	0.0
N_2[b]		0.0	0.0
Tween 80[c]	3.5% (w/v)	30.8	8.0

[a] Membrane suspensions were sparged with 90% (v/v) CO in air for 5 min; other inhibitors were added 1 min prior to cyt *c*. The same inhibition pattern was observed with 12 different batches of membrane preparations

[b] Assays conducted anaerobically in Thunberg cuvettes repeatedly evacuated and flushed with oxygen-free nitrogen

[c] Activity in the presence of Tween 80 was >95% inhibited by 1.2 μM KCN; virtually no cyt *c* oxidation was measured in the absence of membranes thus excluding autoxidation artifacts possibly elicited by Tween 80. By contrast, SDS (0.3%, w/v) elicited high rates of cyt *c* oxidation even in the absence of membranes while completely inhibiting any enzymatic activity (not shown)

Samples contained 15–45 μg protein/ml and 15 μM cyt *c*. For experimental details see section 2

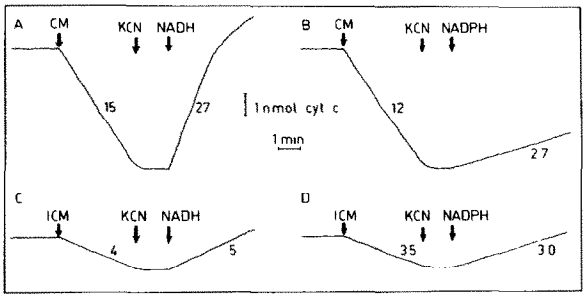

Fig.2. Oxidation (downward deflection) and reduction (upward deflection) of horse heart cyt *c* by CM and ICM preparations. Arrows indicate the addition of membranes (CM or ICM), KCN (5 μM), and NAD(P)H (3.5 mM). Samples contained 45 μg membrane protein/ml and 20 μM cyt *c* 80% of which was present in the reduced form initially.

Table 4
Reduction of ferricytochrome c (2 μM) with 3.5 mM NADH or NADPH in CM and ICM preparations

Fraction	nmol ferricytochrome *c* reduced/min per mg protein	
	NADH	NADPH
CM (13)	16.3 ± 11.1	2.6 ± 0.2
ICM (22)	5.1 ± 4.0	3.1 ± 1.6

Number of determinations (each from a separate preparation) given in brackets. Also see fig.2

no cyt oxidase activity could be measured in CM preparations from *Anabaena* ATCC 29413, in accordance with [16] (V. Molitor and G.A. Peschek, unpublished).

Cyt *c* that was previously oxidized by the cyt oxidase could be reduced, in the presence of KCN, by NAD(P)H in both CM and ICM preparations (fig.2). While this demonstrates that the CM of *A. nidulans* is endowed with a complete respiratory chain including both cyt oxidase and NAD(P)H dehydrogenase(s) any firm conclusions as to the relative share of either nucleotide for electron donation appear to be premature at present in view of quantitative uncertainties between different batches of membrane preparations (cf. fig.2 and table 4).

4. DISCUSSION

Two distinct membrane fractions were separated from crude cell-free extracts of *A. nidulans* by sucrose density gradient centrifugation, both of which were capable of oxidizing (with O_2) and reducing (with NADH and NADPH) horse heart cyt *c*. The light fraction, corresponding to 30% (w/w) sucrose, was essentially free of chlorophyll. Unless these membranes represent a peculiar, chlorophyll-free domain of original ,thylakoid membranes (a possibility that is regarded unlikely; cf. [16,18,24]) it could be the CM. The heavier membrane fraction, corresponding to 42% (w/w) sucrose and containing up to 40% (w/w) chlorophyll per protein, then represents ICM,

which are the site of dual functional photosynthetic/respiratory electron transport systems [6,7,15]. Individual recentrifugation of isolated CM and ICM on identical gradients gave a purified CM band with spectrally undetectable chlorophyll (and pheophytin), yet qualitatively unaltered cyt oxidase and reductase activities (not shown). The lack of both chlorophyll and pheophytin in our CM preparations strongly argues against its possible origin from chlorophyll-containing thylakoid membranes, either native or degraded. Furthermore, possible phase separation in the thylakoid membrane due to preparation at low temperature, thereby leading to artificial enrichment of the cyt oxidase in the chlorophyll-free fraction, must be regarded unlikely, either; note that the same procedure applied to *Anabaena* gave a chlorophyll-free membrane fraction (assumed to represent CM in [16]) devoid of cyt oxidase activity while the ICM fraction did contain the activity (V. Molitor and G.A. Peschek, unpublished). It would be difficult to envisage a phase separation process resulting in such different protein fractionation patterns selectively separating the cytochrome oxidase from chlorophyll in *Anacystis* but not in *Anabaena*. Finally we repeated our membrane preparation and separation procedure with *Anacystis* at 25°C throughout (i.e. well above the lipid phase transition temperature of the membranes [25]), yet arriving at the same fractionation pattern as shown in fig.1 and table 1. Cyt oxidase activity was clearly detectable in the CM fraction obtained at 25°C, albeit at a rate reduced by some 30% when compared with preparations obtained at 4°C (V. Molitor and G.A. Peschek, unpublished). Therefore it is almost certain that our CM band represents isolated and purified plasma membranes of *A. nidulans*, and that this membrane, like the ICM, does contain (aa_3-type) cyt oxidase as was previously suggested (review cf. [15]). Immunological studies on the cyt oxidase in our purified CM and ICM preparations also pointed to an aa_3-type enzyme [26]. It is inadvisable, however, to generalize any such result indiscriminately to other species, or even strains, of cyanobacteria. Recently, a CM preparation was obtained from '*A. nidulans*' devoid of spectrally detectable cyt *a* and cyt oxidase activity [24]. Reasons for this discrepancy may be, apart from possibly different strains of *A. nidulans* used, the tiny yield of CM that was also apparent in our preparations (table 2), the considerable loss of cyt oxidase activity during fractionation and isolation of the membranes (table 2), and/or different preparation and storage procedures involved.

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