Immunologically cross-reactive and redox-competent cytochrome b_6/f-complexes in the chlorophyll-free plasma membrane of cyanobacteria

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Plasma and thylakoid membranes were separated and purified from cell-free extracts of the cyanobacteria *Anacystis nidulans*, *Synechocystis* 6714, *Anabaena variabilis* and *Nostoc* sp. strain Mac. Immunoblots of the membrane proteins using antisera raised against subunits I–IV of the chloroplast b_6/f-complex gave evidence for the presence of a homologous complex in both plasma and thylakoid membranes from the four species of cyanobacteria investigated. Both plasma and thylakoid membranes catalyzed the electron transfer from (exogenous) plastoquinol-9 and NADH to horse heart ferricytochrome *c*. However, while with plasma membranes these reactions were severely inhibited by low concentrations of antimycin A and rotenone, respectively, the inhibitors were without major effect on thylakoid membranes. The results will be discussed in terms of a possible similarity (analogy and/or homology?) of cyanobacterial plasma membranes to the inner mitochondrial membrane.

Respiratory chain; Cytochrome b_6/f-complex; Cytochrome-*c* reductase; Inhibitor; Plasma membrane; Thylakoid membrane; Cyanobacteria

1. INTRODUCTION

Cyanobacteria typically contain two distinct bioenergetically competent membrane systems, the cytoplasmic or plasma membrane (CM) and the intracytoplasmic or thylakoid membrane (ICM). Though very likely to exist from the viewpoint of comparative cytology and membrane function [1,2] even sophisticated electron microscopic techniques [3] have not yet revealed a clear-cut continuity of CM and ICM in a cyanobacterial cell. Since the preparative separation and purification of individual CM and ICM from cyanobacteria is a fairly recent achievement [4,5], and also since the overall amount of ICM in a cyanobacterial cell exceeds that of CM by factors of 10 or more, there is still some controversy as to the electron transport properties of cyanobacterial plasma membranes [6–9]. On the other hand, it has become clear that CM do not contain chlorophyll (but may contain biosynthetic chlorophyll precursors such as chlorophyllide [10] and protochlorophyllide [11]); also some respiratory chain comprising NAD(P)H dehydrogenase, plastoquinone, *b*- and *c*-type cytochromes (7) and aa_3-type cyt oxidase [9,12] as well as cyt-*c* reductase [8,13] activities could be detected in purified CM preparations from a wide variety of cyanobacteria [14]. Periplasmic cyt c_6 identical to the intrathylakoidal cyt [15,16] appears to function as a respiratory electron donor to the CM-bound cyt-*c* oxidase. The pivotal role of the CM and its respiratory chain for the adaptation of cyanobacterial growth to stress conditions has been pointed out in numerous physiological [17–19] and ultrastructural [20,21] studies.

While apart from the cyt-*c* oxidase reaction [5], the NAD(P)H-cyt-*c* reductase reaction has been repeatedly documented with isolated and purified CM of several cyanobacteria [8,13,14], the electron transport complexes involved in the latter have not yet been clearly identified. With ICM there is no doubt that a chloroplast-like cyt *b_6/f-complex is responsible for the* plastoquinol-cyt-*c* (or plastocyanin) oxidoreductase activity; this complex has been characterized and purified from *Anabaena* [22], and the genes coding for its subunits were sequenced for *Nostoc* [23,24]. In the present communication, by using monospecific antibodies against the chloroplast b_6/f complex, we want to show for the first time that isolated and purified CM from cyanobacteria, alike to ICM, do contain an immunologically cross-reactive cyt b_6/f-complex homologous to the chloroplast complex.

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Abbreviations: CM, cytoplasmic (plasma) membrane; ICM, intracytoplasmic (thylakoid) membrane; PCC, Pasteur Culture Collection, Paris, France; ATCC, American Type Culture Collection; cyt, cytochrome; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

Axenic batch cultures of *Anacystis nidulans (Synechococcus* PCC 6301, ATCC 27144), *Synechocystis* PCC 6714 (ATCC 27178), *Anabaena variabilis* PCC 7937 (ATCC 29413) and *Nostoc* sp. strain Mac (PCC 8009) were grown photoautotrophically and harvested from light-limited (linearly growing) cultures as described [5]. Only cultures free of bacterial and other contamination were processed further. CM and ICM were prepared and purified as in [4,5,25]; the CM preparations were free of spectroscopically detectable chlorophyll *a* [11], i.e. they were not contaminated with ICM; this was also seen from distinctly different polypeptide patterns of CM and ICM, respectively, subjected to SDS-PAGE (not shown here but cf. [13,22]). SDS-PAGE, Western blotting and immunoblotting was performed as described for the cyt-*c* oxidase [5,26] but finally using antisera raised against cyt *f*, cyt b_6, the Rieske FeS protein and subunit IV of the chloroplast cyt b_6/f complex [27–29]; the antibodies were kindly donated by Dr R. Malkin (Berkeley, USA), and used at dilutions of 1:200 to 1:500. Apparent molecular weights were determined by comparison with a Sigma MW-SDS-70L marker kit. Protein and chlorophyll were determined according to Bradford [30] and Mackinney [31], respectively.

The reduction of horse heart ferricytochrome *c* (type VI of Sigma) was measured with a Shimadzu UV-300 dual-wavelength spectrophotometer at 25°C using $\Delta\epsilon$ (redox) = 19.5 $mM^{-1} \cdot cm^{-1}$ at 550–540 nm [32]. Reactions were started by the addition of membranes (2–75 μg protein/ml, final concentration) to the complete reaction mixture containing 7–10 μM cyt *c* and 1.5 mM NADH (Boehringer, Mannheim, FRG) or 60–90 μM plastoquinol-9 (freshly prepared from spinach or *Anacystis nidulans* and reduced according to [33]) in 10 mM K-phosphate buffer and 2 mM Na_2EDTA (pH 7.0). Temperature of measurements was 25°C throughout; although this is considerably below the optimum growth temperature of the cyanobacteria used [5] it was a necessary precaution in order to slow down the rate of nonenzymatic cyt *c* reduction which was always measured for several minutes prior to the addition of membranes (cf. Fig. 1). Antimycin A and rotenone (from Sigma) were added as freshly prepared stock solutions in analytical grade dimethyl sulfoxide; concentrations of the latter never exceeded 0.2% (v/v) in the reaction mixture. Stock solutions of plastoquinol-9 were prepared and applied according to [27].

3. RESULTS

Spectrophotometric recorder traces of the CM and ICM catalyzed reduction of horse heart ferricytochrome *c* by NADH and plastoquinol-9 are shown in Fig. 1. As our CM preparations are perfectly pure and not contaminated with any ICM [5,9,11] it must be concluded that both CM and ICM of cyanobacteria contain intrinsic cyt *c* reductase activity with either NADH (via a membrane-bound dehydrogenase) or plastoquinol as (physiological) electron donors. Qualitatively identical graphs were obtained with CM and ICM from *Anacystis, Synechocystis, Anabaena* and *Nostoc* (not shown; cf. Table I). However, a striking difference between CM and ICM was noticed in the behavior towards inhibitors antimycin A (Fig. 1B) and rotenone (Fig. 1A). These inhibitors which are well known to block mitochondrial electron transport at sites III (by virtue of the antimycin A-binding cyt b_{562} [34], a component of the b/c_1-complex [29,35,36] and I (NADH dehydrogenase), respectively, at the very low concentrations employed, clearly did inhibit the CM-catalyzed electron transfer through b_2/f-complex and NADH

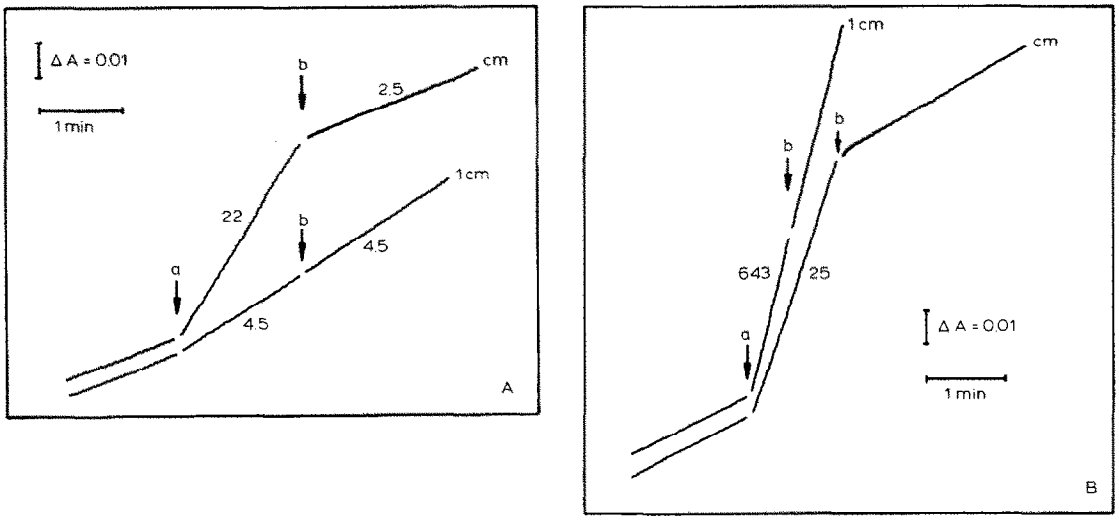

Fig. 1. Spectrophotometric recorder traces for the reduction of 10 μM horse heart ferricyt-*c* (550–540 nm) with 1.5 mM NADH (A) and 80 μM plastoquinol-9 (B) in 10 mM K-phosphate buffer (pH 7.0) at 25°C as catalyzed by the isolated and purified plasma (CM) and thylakoid (ICM) membrane from *Anacystis nidulans*. Final concentration of membrane protein was 75 μg/ml throughout, except for B/ICM which contained 3.5 μg/ml. Numbers adjacent to the traces are nmol cyt *c* reduced/min per mg membrane protein corrected for noncatalyzed rates measured prior to the addition of membranes. Arrows 'a' and 'b' indicate the addition of membranes and of 10 μM rotenone (A) or 5 μM antimycin A (B), respectively. Effects of the latter on NADH-cyt *c* reduction were the same as on the plastoquinol supported reaction, i.e. almost no inhibition with ICM but 80–100% inhibition with CM, suggesting that the cyt b_6/f-complex is an electron transport intermediate between NADH and cyt *c*. Effects of the inhibitors were not increased by 5 min preincubation with membranes. Higher concentrations of the inhibitors (50–150 μM) also inhibited ICM catalyzed reactions to some extent (never beyond 30–50%) but in a barely reproducible way with great variation between different batches of membrane preparations (not shown). Qualitatively identical graphs were obtained with CM and ICM from *Synechocystis, Anabaena* and *Nostoc* (cf. Table I).

Table I

Reduction of horse heart ferricytochrome *c* with NADH or plastoquinol-9 as catalyzed by purified preparations of cyanobacterial plasma (CM) and thylakoid (ICM) membranes

Species	Membrane	Electron donor	
		NADH	Plastoquinol-9
Anacystis	CM	23	28
	ICM	5	665
Synechocystis	CM	34	87
	ICM	11	948
Anabaena	CM	380	410
	ICM	8	1066
Nostoc	CM	17	36
	ICM	6	720

Assays were conducted by dual-wavelength spectrophotometry as detailed in section 2. Control runs assured that reaction rates were linear with protein concentration and independent of the concentration of cyt *c* and plastoquinol-9 under the conditions described. Rates are expressed as nmol cyt *c* reduced/min per mg membrane protein and averaged from 3–5 separate membrane preparations, standard deviations ranging from 5% to 15% of the corresponding mean for each type of membrane. The rates given in the table are corrected for noncatalyzed cyt *c* reduction in the absence of membranes (cf. Fig. 1). While antimycin A (5 μM) and rotenone (10 μM) inhibited CM-catalyzed reductions of cyt *c* with plastoquinol-9 and NADH, respectively, by 80–100% the inhibitors were without major effect (0–25% inhibition at most) on ICM irrespective of the source organism (results not shown; cf. Fig. 1).

dehydrogenase while its ICM-catalyzed counterpart was left almost unaffected (Fig. 1 and Table I).

When purified CM and ICM from *Anacystis* (Fig. 2A) and *Synechocystis* (Fig. 2B), as well as *Anabaena* and *Nostoc* (not shown), were immunoblotted with (polyclonal) monospecific antibodies raised against cyt *f*, cyt b_6, the Rieske protein and/or subunit IV of the chloroplast b_6/f-complex, a fairly specific and consistent pattern of immunological cross-reactions was obtained. With each of the 4 cyanobacteria the apparent molecular masses of 3 of the cross-reacting polypeptides from both CM and ICM neatly corresponded to what is known from work with the isolated complex [22,27] or the genes coding for it [23,24], viz. 31–38 kDa for cyt *f*, 22–25 kDa for cyt b_6, and 16–18 kDa for subunit IV. Antiserum against the Rieske protein, however, consistently gave cross-reacting bands corresponding to about 30–36 kDa with all membranes tested (Fig. 2A, lane 2), much higher than the reported value of 19–20 kDa. Whether this is due to incomplete solubilization and dissociation or to irreversible aggregation of the Rieske protein with some other (low-molecular weight) membrane protein under our SDS-PAGE conditions [5,26], or simply to some malfunction of the antibody, cannot be said at present. What can be said, however, is that the fairly specific and reproducible pattern of immunological cross-reactivity of the cyt b_6/f components was the same with highly purified CM and ICM from the 4 different species of cyanobacteria studied here.

4. DISCUSSION

Physiological activity and immunology of isolated and highly purified plasma and thylakoid membranes from two unicellular and two filamentous cyanobacteria as documented in the previous section with respect to the NADH-cyt *c* oxidoreductase (including the cyt b_6/f-complex) appear to corroborate the view that a complete respiratory chain is present not only in thykaloid membranes (in the form of a dual-functional photosynthetic/respiratory electron transport assembly) but also in cyanobacterial plasma membranes which, due to the absence of chlorophyll [4,5,11], are photosynthetically incompetent. It has long been argued that this CM-bound respiratory chain primarily serves immediate energetic needs of the plasma membrane by building up a trans-CM protonmotive force [37,38]. This view has gained much support from observations on stress-exposed (e.g. salt-adapting) cyanobacteria [5,17–19,39], particular emphasis resting on the cyt-*c* oxidase. The latter could be shown to be an 'apparently indistinguishable' aa_3-type enzyme in both CM and ICM isolated from >20 different strains grown under a variety of different conditions which greatly influenced the quantitative expression of cyt-*c* oxidase and reductase activities in CM and ICM, respectively [5,17,19,39] (G.A. Peschek et al., unpublished). However, since we have also found quite recently that in *Synechocystis* PCC 6803 a single gene codes for subunit I of the cyt-*c* oxidase (G. Schmetterer, D. Alge and G.A. Peschek, unpublished, cf. [40]) the question will have to be answered how a seemingly identical enzyme/protein finds its way, in a regulated and coordinate fashion, into different cellular membranes/compartments. Yet, for cyanobacteria the same question must hold for the cyt c_6 [41] which appears to occur in both periplasmic and intrathylakoidal spaces [15,16]. And finally, as the present investigation shows, it might also apply to the cyanobacterial cyt b_6/f-complex thus being of more widespread implication than previously assumed.

Some further conclusions to be drawn from the activity data (Fig. 1 and Table I) deserve closer attention. (i) The rate-limiting step for the reduction of cyt *c* with NADH appears to be the NADH dehydrogenase, proper, and not the cyt b_6/f-complex. This is particularly evident with ICM. (ii) Since the specific activity of the plastoquinol-cyt *c* oxidoreductase (per mg membrane protein) was always much greater in ICM than in CM it is reasonable to assume that the concentration of the cyt b_6/f-complex also is much greater in ICM than in CM, consistent with a primarily photosynthetic function of the ICM. (iii) Since antimycin A and rotenone, both 'classical inhibitors' of the mitochondrial respiratory chain, viz. at complexes III and I, respectively, affected cyanobacterial CM only, without major effect on ICM, it is tempting to speculate whether in general cyano-

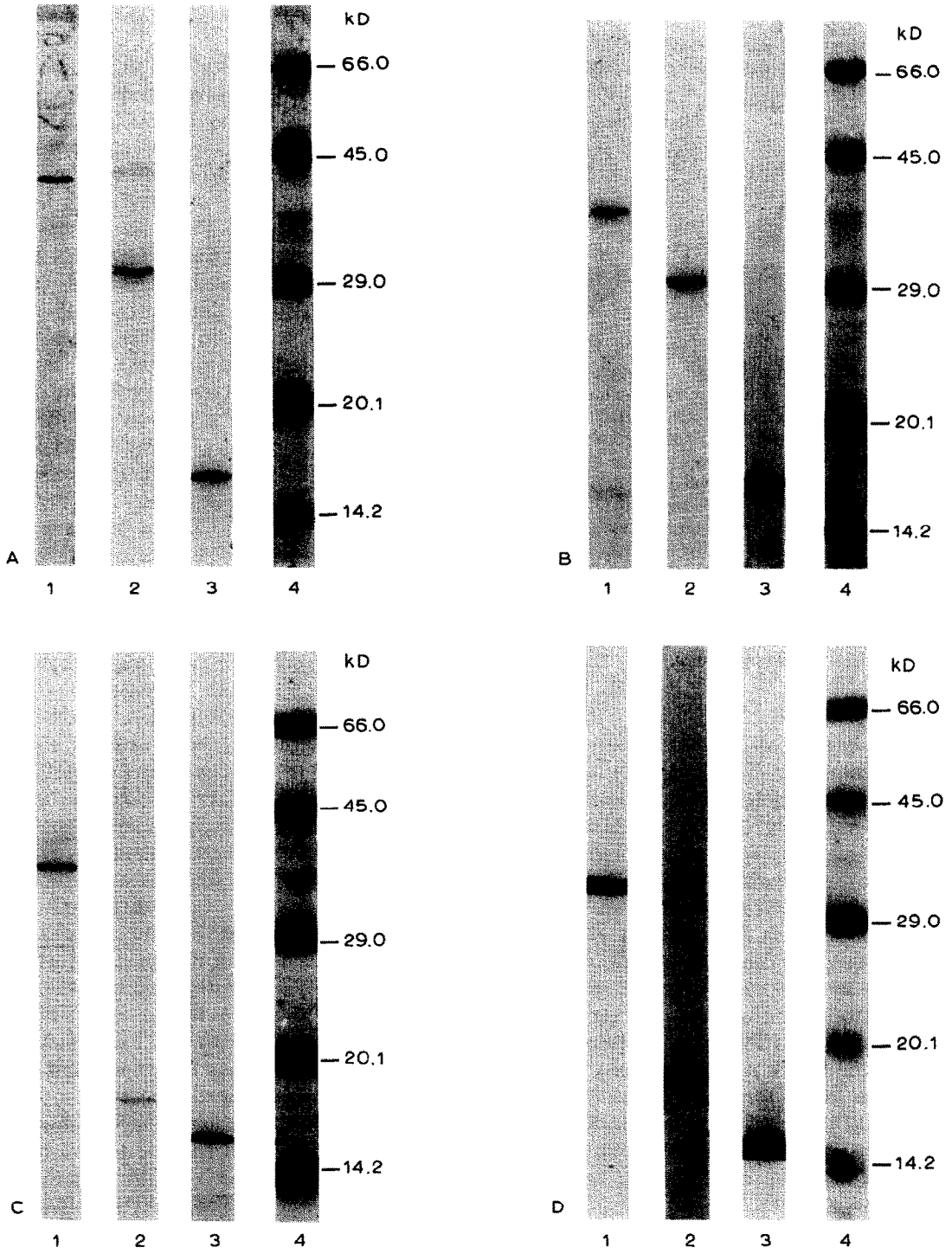

Fig. 2. Immunoblots of SDS-PAGE separated and nitrocellulose transferred polypeptides of isolated and purified plasma (A,C) and thylakoid (B,D) membranes from *Anacystis* (A,B) and *Synechocystis* (C,D) with antisera raised against the following components of the chloroplast cyt b_6/f-complex; cyt *f* (lane 1), cyt b_6 (lane 2 of C and D), Rieske protein (lane 2 of A and B), and subunit IV (lane 3).Cross-reacted bands were made visible with goat anti-rabbit horseradish peroxidase-conjugate as a second antibody and 4-chloro-1-naphthol/hydrogen peroxide staining as described [5,26]. Molecular weight markers of the Sigma MW-SDS 70-L-kit were used. The following amount of membrane protein was applied to the lanes: 60 and 80 μg of ICM and CM from *Anacystis*, and 50 and 110 μg of ICM and CM from *Synechocystis*, respectively. Antisera were used at dilutions of 1:200 to 1:500. Similar patterns of immunological cross-reaction were obtained with CM and ICM from *Anabaena* and *Nostoc* (not shown). However, the limited amounts of antisera available permitted a limited number of experimental runs only. For experimental details and interpretation cf. section 2 and the text.

bacterial plasma membranes have preserved more 'mitochondrial features' than thylakoid membranes; e.g. (part of) the CM-b_6/f-complexes might be intrinsically associated with a 'mitochondria-like' antimycin A-binding site while the ICM-b_6/f-complex, otherwise both functionally and immunologically very similar, might not; similarly, (part of) the CM-NADH dehydrogenase may be of the 'mitochondrial' (rotenone-sensitive) type while the analogous ICM enzyme may be of the bacterial [42] or chloroplast [43] type which was recently isolated also from cyanobacteria [44,45]; for the latter investigations (also [22]) whole cells or crude membranes (CM and ICM) were used and thus minor quantities of 'exotic' (viz. mitochondria-like) enzymes situated exclusively or additionally in the CM might have escaped the investigators.

Following the discussion above it is perhaps not unfeasible to assume an evolutionary origin of *both* chloroplasts and mitochondria from an engulfed cyanobacterium, invoking some kind of 'unified endosymbiont hypothesis': basically, the transformation of the endosymbiont's photosynthetic into a respiratory electron flow chain (according to the 'conversion hypothesis'; cf. [46]) within a host cell should not have been much more difficult than in a free-living cell before it became an endosymbiont.

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