Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 71-78

LIGHT-INDEPENDENT NADPH-PROTOCHLOROPHYLLIDE OXIDOREDUCTASE ACTIVITY IN PURIFIED PLASMA MEMBRANE FROM THE CYANOBACTERIUM ANACYSTIS NIDULANS

Günter A. Peschek[1], Barbara Hinterstoisser, Bernard Pineau[2] and Albert Missbichler[3]

Biophysical Chemistry Group, Institute of Physical Chemistry, University of Vienna, Währingerstrasse 42, A-1090 Wien, Austria

Received May 31, 1989

SUMMARY: A light plasma membrane fraction corresponding to a buoyant density of 1.087 ± 0.005 g/cm^3 and devoid of chlorophyll was prepared and purified from Anacystis nidulans according to a recently published procedure (G.A.Peschek, V.Molitor, M.Trnka, M.Wastyn and W.Erber (1988) Methods Enzymol. 167, 437-449). Besides major amounts of carotenoids the plasma membranes contained a small but significant pool of chlorophyllide a and protochlorophyllide a as verified by room temperature and 77K spectrofluorimetry and analytical separation and identification by high performance liquid chromatography using authentic standards. Incubation of the plasma membranes in strict darkness in the presence of NADPH was accompanied by the gradual and stoichiometric replacement of protochlorophyllide by chlorophyllide, $NADP^+$ effecting the reverse transition. The reaction was completely insensitive to illumination (5-20 w/m^2 tungsten light) but abolished after heating of the membranes (90°C, 5 min) or in the presence of 10 mM EGTA, and was specifically stimulated by calcium ions. Our results indicate the occurrence of light-independent NADPH:protochlorophyllide oxidoreductase activity in the plasma membrane of Anacystis nidulans.

INTRODUCTION: Cyanobacteria are phototrophic prokaryotes uniquely capable of oxygenic, chlorophyll a-based photosynthesis (1,2) and aerobic, cytochrome oxidase-based respiration (3,4). Basically, the photosynthetic machinery

[1] Corresponding author (Institute of Physical Chemistry, University of Vienna, Währingerstrasse 42, A-1090 Wien, Austria).

[2] Permanent address: Laboratoire de Bioénergétique Cellulaire et Cytophysiologie CNRS, Avenue de la Terrasse, B.P.1, F-9110 Gif-sur-Yvette, France.

[3] Institute of General Biochemistry and Ludwig-Boltzmann-Forschungsstelle für Biochemie, University of Vienna, Währingerstrasse 38, A-1090 Wien, Austria.

ABBREVIATIONS: CM, cytoplasmic (plasma) membrane; ICM, intracytoplasmic (thylakoid) membrane; EGTA, ethyleneglycol-bis-(ß-aminoethyl ether)-N,N,N',N'-tetraacetate; HPLC, high performance liquid chromatography; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DABS, diazobenzosulfonate; λ_{em}, emission wavelength; λ_{ex}, excitation wavelength.

0006-291X/89 $1.50

of cyanobacteria is of the same type as in chloroplasts of higher plants and algae (2). For both chloroplasts (5) and cyanobacteria (6-8) the preparation and separation of (chloroplast) envelope or (cyanobacterial) plasma and thylakoid membranes is a fairly recent analytical achievement. With most of the cyanobacterial species investigated so far a major respiratory role could be established for the chlorophyll-free plasma membrane containing, in particular, an aa_3-like cytochrome oxidase (8-10). With chloroplasts on the other hand the likewise chlorophyll-free envelope membranes were recently shown to be the site of various enzymes involved in lipid and chlorophyll biosynthesis (11-14). This paper presents evidence that chlorophyll-free plasma membrane preparations of the cyanobacterium Anacystis nidulans, in contrast to thylakoid membranes, contain a small but significant pool of protochlorophyllide and chlorophyllide, together with a light-independent NADPH:protochlorophyllide oxidoreductase. Similar to chloroplast envelope membranes, the plasma membrane of cyanobacteria may thus play an important role in the biosynthesis of chlorophyll.

MATERIALS AND METHODS

Growth of the Organism. Axenic cultures of Anacystis nidulans (Synechococcus PCC 6301 = ATCC 27144) were grown photoautotrophically in modified Kratz and Myers medium D (15) gassed with 1.5% (v/v) CO_2 in sterile air at 38°C and 20 w/m^2 warm white fluorescent light (measured with a YSI radiometer, model 65, at the surface of the growth vessel) as described (7). Cells were grown to the linear growth phase (3-4 µl packed cells/ml culture fluid) (9) and harvested by centrifugation at room temperature.

Membrane Isolation and Characterization. Harvested and washed cells were subjected to lysozyme/EDTA treatment and French pressure cell extrusion (7-9). CM and ICM were separated from crude cell-free extracts by discontinuous sucrose density gradient centrifugation (8-10). CM and ICM were identified by use of membrane-impermeant covalent protein marker ^{35}S-DABS (9,16,17).

Spectrofluorimetry. 77K fluorescence spectra were measured on a home-made spectrofluorimeter as previously described (12,18). UV light was excluded by use of a proper filter (J335, Metallisations et Traitements Optiques, Massy, France) which was placed between the 250-w xenon bulb and the exciting monochromator (M25, Jobin et Yvon, Longjumeau, France). A cut-off filter (OG 590, Schott AG, Mainz, FRG) was placed between the sample and the analysing monochromator (M25, Jobin et Yvon). The sample (10 µl) was placed on a filter paper disk at the bottom of a cylindrical microcell (10 mm diameter). The top of the microcell was covered with a two-arm light pipe, the first arm originating from the exciting monochromator and the second one leading to the analysing monochromator equipped with a red-sensitive photo-

multiplier (RTC XP 2203 B, La Radiotechnique, France). Excitation spectra were corrected for energy variations of the xenon light source. Fluorescence emission intensities were linear with membrane protein concentrations up to 3 mg/ml.

Incubation of Plasma Membrane with Pyridine Nucleotides. Purified CM was incubated at 30°C either in strict darkness or at various intensities of tungsten light (5-20 w/m^2 at the surface of the stirred reaction mixture as measured with a YSI Radiometer, model 65) in a medium containing 30 mM Hepes-KOH buffer (pH 7.4) and 0.3 M mannitol, occasionally supplemented with 5 mM $CaCl_2$ or $MgCl_2$, or 10 mM Na_2EDTA or Na_2EGTA (at pH 7.4). Reactions were started by the addition of 3.5 mM NAD(P)H or $NAD(P)^+$ (final concentration). At time intervals of 5, 10 and/or 20 min thereafter 10-µl-aliquots of the membrane suspension were quickly withdrawn from the reaction mixture, placed on the pre-cooled filter paper disk in the spectrophotometer microcell, immediately frozen in liquid nitrogen and assayed for fluorescence emission (cf. above; also cf. Fig.3). Further aliquots from the same suspension were subjected to HPLC (cf. Figs.4 and 5).

Reversed Phase High Performance Liquid Chromatography. 20, 10 and 5 µl acetone or methanol extracts of CM (80-120 µg protein/ml) and ICM (0.9-1.3 mg protein/ml), and pure chlorophyll *a* and chlorophyllide *a* standards (prepared from *A. nidulans* thylakoid membranes according to Refs.19-21 and dissolved in acetone or methanol) were subjected to HPLC using a Beckman HPLC system comprising a Controller, model 421 A, a Solvent Delivery Module, model 114 M, an Absorbance Detector, model 160 (operating at 254 nm), an Organizer, model 340 (100 µl loop), an ERMA-ERC Degasser, model 3020, and a Shimadzu C-R34 Chromatopac Integrator, with a separating column (40 + 250 x 4 mm) filled with Nucleosil 300-5C8 (5 µm) reversed phase material (Machery & Nagel, FRG). Elution was with a gradient of 70-98% (v/v) aqueous methanol at 22°C during 20 min in the order of decreasing polarity. By comparison with authentic standards (cf. Ref.22 and Figs.2-4) the retention time (R_f values) found were assigned to protochlorophyllide *a* (13.06 min), chlorophyllide *a* (13.64 min), pheophorbide *a* (14.07 min), chlorophyll *a* (15.5 min) and pheophytin *a* (18.1 min). In order to confirm the identity of the different fractions obtained these were individually collected from the column and analysed by spectrofluorimetry after proper dilution with acetone (cf. Figs.4 and 1). Protein and chlorophyll were determined according to Bradford (23) and Mackinney (24), respectively. All chemicals used were of the highest purity grade commercially available.

Standards and Controls. Chlorophyll *a* was extracted and purified from *A. nidulans* thylakoid membranes (19). Chlorophyllide *a* was prepared from this chlorophyll *a* by reaction with chlorophyllase isolated and partly purified from senescing leaves of *Ailanthus altissima* (20,21). The only contaminants detected chromatographically in our chlorophyll and chlorophyllide standards were negligible quantities of pheophytin and pheophorbide originating from chlorophyll and chlorophyllide through loss of Mg and/or phytol, presumably by contact with acidic laboratory vapors (cf. Fig.2C,D; also cf. Ref.25). The characteristically complementary changes in fluorescence and chromatography patterns of protochlorophyllide and chlorophyllide in pyridine nucleotide-incubated membranes (cf. Figs.3-5) were not observed when (i) ICM were used instead of CM, (ii) the membranes were heated at 90°C for 5 min prior to pyridine nucleotide incubation, (iii) 10 mM EGTA was included in the reaction mixture, or (iv) the CM were incubated without pyridine nucleotides (cf. Fig.5). Care was taken to keep light strictly off the reaction mixture during the whole incubation and sampling procedure. On the other hand, illumination with 5-20 w/m^2 white light (from a slide projector) was found to be without any influence on the protochlorophyllide-chlorophyllide transformation.

RESULTS AND DISCUSSION

Fig.1 shows 77K fluorescence emission and excitation spectra of CM and ICM. The presence in CM of pigment(s) emitting at 636 and 682 nm (Fig.1A) contrasted with the absence of such pigments from the chlorophyll a-containing ICM (Fig.1B). Strikingly different excitation spectra of the 636 and 682 nm emission peaks (Fig.1C,D) indicated that the latter might originate from two chemically distinct pigments. HPLC resolution of acetonic or methanolic CM and ICM extracts revealed chlorophyll a as the predominant ICM pigment and chlorophyllide a as the major CM pigment (Fig.2; also cf. Ref.25).

Physiologically more relevant, the two CM pigments fluorescing at 636 and 682 nm (Fig.1) could be reversibly converted into each other by incubation of the membranes in the presence of NADPH or $NADP^+$ (Fig.3). Comparison of HPLC with fluorescence data (Fig.4) strongly suggested that the two pigments were indeed chlorophyllide and the still more polar protochlorophyllide, respectively (cf. Fig.1A and Fig.4, slightly different peak

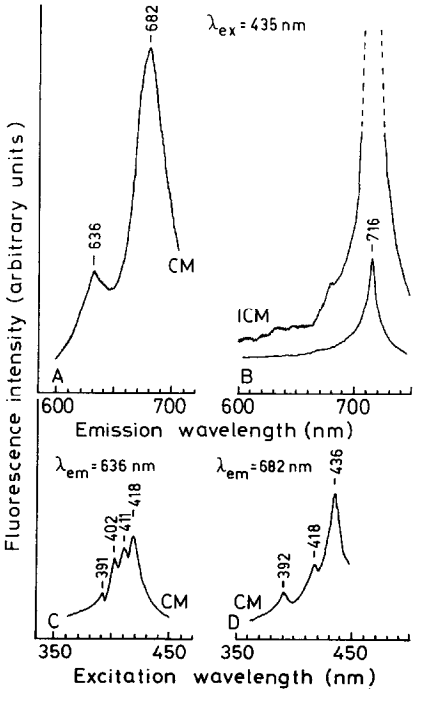

Fig.1 77K fluorescence emission (A,B) and excitation (C,D) spectra of aqueous suspensions of purified plasma (CM) and thylakoid (ICM) membranes. Protein concentration was 2.1 mg/ml throughout (except for the inset of Fig.1B, corresponding to 0.21 mg protein/ml).

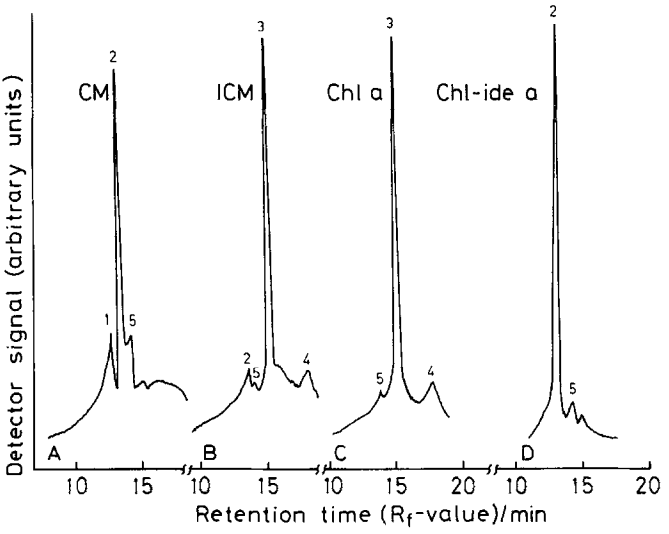

Fig.2 Separation of CM (A) and ICM (B) pigments, extracted with acetone or methanol, and authentic chlorophyll a (C) and chlorophyllide a (D) standards, dissolved in acetone or methanol, by HPLC (cf. Materials and Methods). Peak numbers (R_f values or retention times in min) correspond to the following compounds (also cf. Figs. 3-5 and Refs. 12,22,25): 1 = protochlorophyllide (13.06), 2 (13.64) = chlorophyllide, 5 (14.07) = pheophorbide, 3 (15.5) = chlorophyll and 4 (18.1) = pheophytin, in the order of decreasing polarity.

wavelengths probably owing to the different physical state of the pigments in the membrane (Fig.1) and in methanolic/acetonic solution (Fig.4) (also cf. Fig.2 and Refs.12 and 25).

Fig.5 shows the time course of the protochlorophyllide-chlorophyllide transformation in CM preparations incubated with NAD(P)H or $NAD(P)^+$ as evidenced by the different peak areas no.1 and 2 in corresponding HPL chromatograms (cf. Figs.4A,B and 2; also cf. Figs.4C,D and 3). It is seen that the two compounds were transformed more or less stoichiometrically at the expense of each other (cf.Fig.3), heating of the CM at 90°C for 5 min completely abolished the reaction (Fig.5A, control curve), 5 mM $CaCl_2$ had a markedly stimulating effect (Fig.5B; 5 mM $MgCl_2$ being only about 30% as effective as $CaCl_2$, not shown), and that 10 mM of the strongly calcium-chelating agent EGTA eliminated the reaction (Fig. 5B,C). The changes in HPLC and fluorescence patterns described in Figs.5 and 3 were the same regardless whether the membranes were incubated in strict darkness or under illumination with 5-20 w/m^2 tungsten light. No changes either in fluorescence

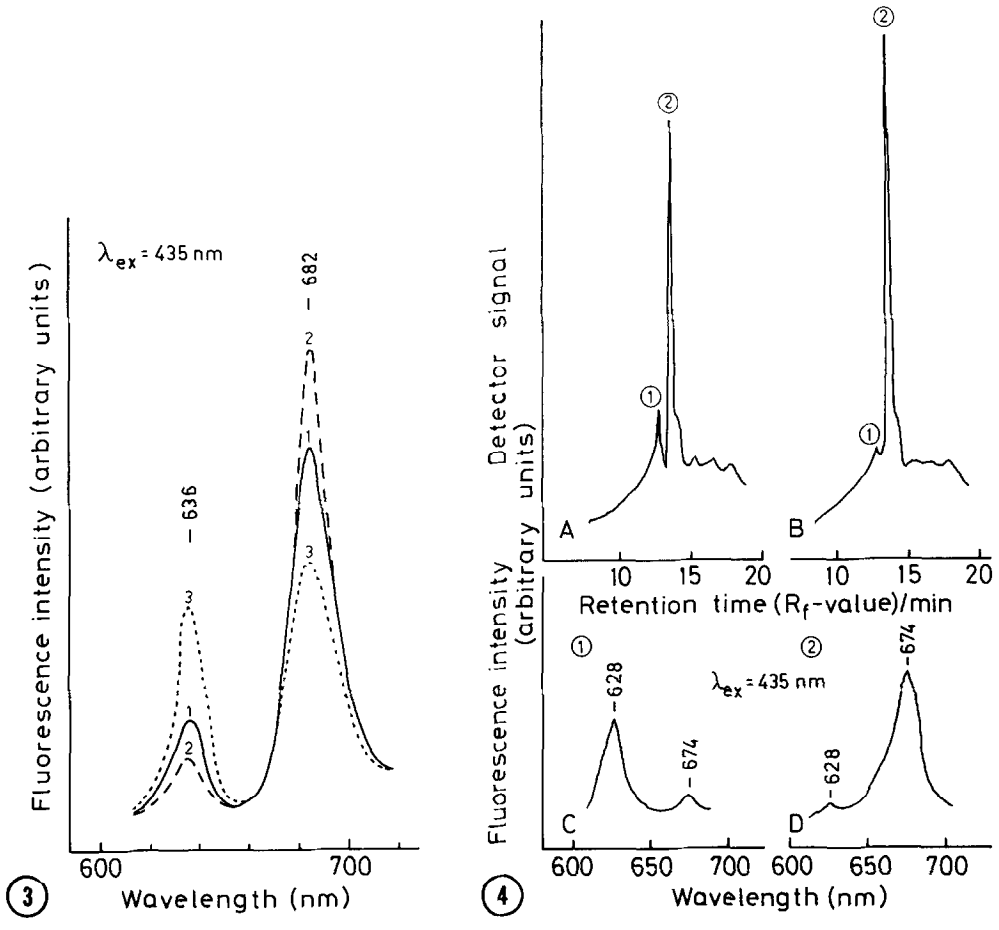

Fig.3 77K fluorescence emission spectra of CM preparations (2.7 mg protein/ml) either freshly prepared (1) or after 10 min incubation with 3.5 mM NADPH (2) or $NADP^+$ (3) at 30°C in complete darkness. No changes in peak heights at 636 and 682 nm were seen when the membranes had been heated at 90°C for 5 min prior to the addition of pyridine nucleotides. For details cf. Materials and Methods; also cf. Figs. 4 and 5.

Fig.4 HPL chromatogram of acetonic or methanolic extracts of plasma membrane preparations before (A) and after (B) 10 min incubation with 3.5 mM NADPH at 30°C in strict darkness; (C) and (D) represent the 77K fluorescence emission spectra of the pigments corresponding to peaks no. 1 and 2, respectively, as collected directly from the HPLC column. For details cf. Materials and Methods. Also cf. the text.

spectra or HPL chromatograms were detected upon incubation of ICM in the presence of NAD(P)H or $NAD(P)^+$ and upon incubation of CM in the absence of pyridine nucleotides. Heat-sensitivity, divalent cation requirement and reversibility of the protochlorophyllide-chlorophyllide transformation in strictly darkened CM (Fig.5) point to the action of (calcium-stimulated) NADPH:protochlorophyllide oxidoreductase, an enzyme which is light-dependent in chloroplast envelope membranes (12) but light-independent in the

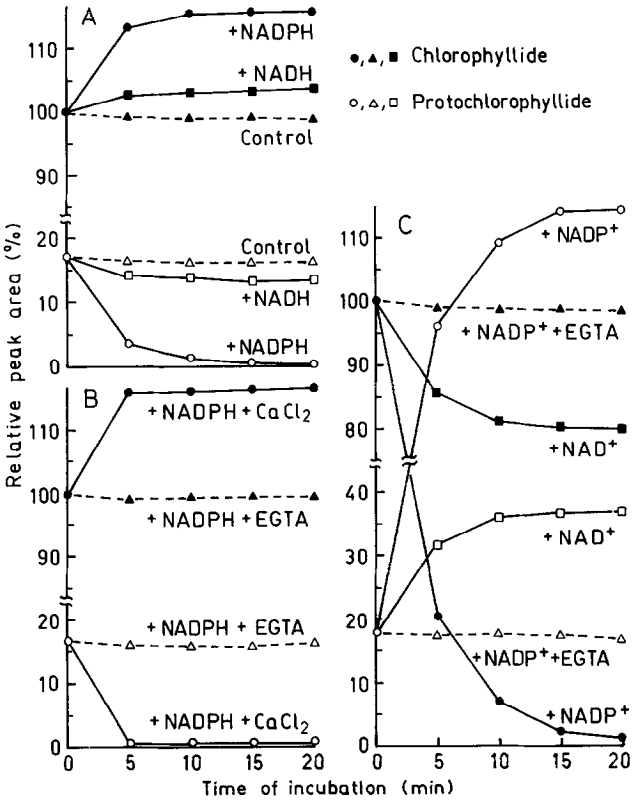

Fig.5 Time course of the protochlorophyllide-chlorophyllide transformation in plasma membranes incubated with 3.5 mM NAD(P)H or $NAD(P)^+$ at 30°C in the absence or presence of 5 mM $CaCl_2$ or 10 mM EGTA. Relative peak areas as displayed by the Chromatopac Integrator were 19351 ± 1533 (= 100%) for chlorophyllide and 3289 ± 397 for protochlorophyllide in freshly prepared membranes from six different cultures (cf. Fig.1). Effects of 5 mM $MgCl_2$ and 10 mM EDTA were about 30 and 50% the effects of $CaCl_2$ and EGTA, respectively (not shown). No changes in HPL chromatograms (cf. Figs.3 and 4) were seen when the pyridine nucleotides were incubated with ICM or heat-denatured CM, or when the CM were incubated in the absence of pyridine nucleotides. For further details cf. Materials and Methods.

cyanobacterial CM preparations. The latter conforms to the well-known observation that chlorophyll synthesis in facultatively chemoheterotrophic cyanobacteria also proceeds in the dark. Yet, similar to the chloroplast system (12) the enzyme distinctly preferred NADP(H) over NAD(H), effects with the latter being less than 20% (Fig.5A,C). Preparative recovery of the distinct pigment fractions resolved by HPLC (Figs.2 and 4A,B), followed by spectrofluorimetric analysis (Fig.4C,D) appeared to leave little doubt as to the identity of the separated pigments with those detected in the original plasma membranes (Fig.1), viz. with protochlorophyllide and chlorophyllide, respectively.

ACKNOWLEDGMENTS

This work was supported by grants from the CNRS and the Austrian Science Foundation. We thank Miss Alexandra Messner and Mr.Otto Kuntner for excellent technical assistance.

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