Biochimica et Biophysica Acta 810 (1985) 106–109
Elsevier

BBA 40058

BBA Report

Characterization of a photosynthetic mutant of *Lemna* lacking the cytochrome b_6-f complex

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(Received April 12th, 1985)
(Revised manuscript received July 8th, 1985)

Key words: Photosystem II; Chlorophyll-protein complex; Cytochrome b_6-f complex; Plastoquinone; (*Lemna*)

A photosynthetic mutant of *Lemna perpusilla* (no. 1073) has been examined by spectrophotometric and immunoblotting techniques in order to localize the site of defect. In contrast to previous conclusions (Shahak, Y., Posner, H.B. and Avron, M. (1976) Plant Physiol. 57, 577–679), neither cytochrome *f* nor cytochrome b_6 could be detected spectrophotometrically in the mutant. Furthermore, immunoblotting using antibodies specific for each of the four constituent subunits of the cytochrome b_6-f complex demonstrate that the entire complex is absent in the mutant. The light-harvesting chlorophyll-protein complex of Photosystem II is present in similar amounts in wild-type and mutant *Lemna*. However, the total amount of plastoquinone-9 is reduced by approx. 65% in the mutant strain, while the photoreducible plastoquinone-9 pool is comparable in wild-type and mutant *Lemna*.

Mutants deficient in specific components of photosynthesis have been valuable in attempting to understand structure-function relationships in this complex process [1,2]. For higher plants, a collection of nuclear mutants of maize which are selected on the basis of high fluorescence have been described [2]. These mutants lack a group of specific polypeptides associated with an individual membrane protein complex, although the mutations are caused by a single lesion in the nuclear genome [3,4]. The pleiotropic characteristics of such mutants suggest the importance of certain constituent polypeptides in each protein complex for their assembly and stabilization. Although quite useful, the maize mutants are lethal variants that cannot grow to maturity, and it is therefore difficult to characterize them on a biophysical and biochemical level.

In the present report, we have examined a photosynthetic mutant of *Lemna* which has previously been shown to lack a functional Rieske Fe-S center [5]. There are two reasons for a more detailed characterization of this mutant. (1) The original description of this mutant reported the presence of cytochrome *f*, as determined by light-induced absorbance changes in the α-band region of the cytochrome [6]. However, no spectrum of this absorbance change was presented. If cytochrome *f* is present in this mutant, then one might have a mutant which is specifically missing one component of the cytochrome b_6-f complex, the Rieske Fe-S center, while other components, i.e., cytochrome *f* and presumably cytochrome b_6, would still be present. (2) Most photosynthetic mutants from higher plants are not viable. The

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Abbreviations: LHCP II, light-harvesting chlorophyll-protein complex of Photosystem II; Cyt, cytochrome; Chl, chlorophyll.

Lemna mutant, as well as the wild-type strain, can be grown heterotrophically, and large amounts of material can be obtained for analysis under non-photosynthetic growth conditions. Thus, this system might afford unique opportunities for the study of the light reaction components independent of the functional cytochrome complex.

Lemna wild-type (*Lemna gibba*) and mutant 1073 (*Lemna perpusilla*) were obtained from Drs. Elaine Tobin (University of California at Los Angeles) and Herbert Posner (SUNY-Binghamton), respectively. Both strains were grown in sterilized E medium [7] containing 10 μg/ml Benlate R (Dupont) as fungicide. Cultures were grown at 25° in dim light. Thylakoid membranes were prepared using a blending solution containing 0.3 M sucrose/50 mM Tris-HCl (pH 7.8)/15 mM NaCl/5 mM $MgCl_2$ [8]. The procedures used for SDS-polyacrylamide gel electrophoresis of membrane proteins and immunoblotting with CNBr-impregnated filter paper have been described [9]. Antibodies specific for individual polypeptides of the cytochrome b_6-f complex were prepared from electrophoretically purified proteins from the isolated maize complex and were a gift from Dr. Alice Barkan (University of California, Berkeley). Antisera to the light-harvesting chlorophyll protein complex of Photosystem II (LHCP II) were from Dr. Stephen Mayfield (University of California, Berkeley). HPLC analysis of quinones was done on an LKB apparatus using an LKB Lichrosorb RP-18 (5 μm) reversed phase column and a mobile phase of methanol/isopropanol (1 : 1). Standard plastoquinone-9 from spinach was purified from spinach thylakoids and was a gift from Richard Chain of our laboratory.

Cytochromes were determined in a solution containing 1% Triton X-100 to convert cytochrome *b*-559 to a low-potential form (non-hydroquinone-reducible). Cytochrome *f* was then estimated from the hydroquinone-minus-ferricyanide difference spectrum using a Cary 219 spectophotometer. The remaining thylakoid cytochromes were estimated from the dithionite-minus-hydroquinone difference spectrum. The extinction coefficients used for cytochrome *b*-559 was 20 $mM^{-1} \cdot cm^{-1}$ (559–540 nm) and for cytochrome *f*, a value of 18 $mM^{-1} \cdot cm^{-1}$ was used (554–540 nm).

In agreement with previous results on the *Lemna* mutant [6], membranes prepared from this strain show Photosystem I and Photosystem II activities, but whole-chain electron transport from H_2O to either methyl viologen, ferricyanide or NADP was totally absent. The chlorophyll *a*/*b* ratio was similar in the wild-type and mutant thylakoids indicating there was no substantial alteration of chlorophyll pigments in the mutant.

Spectrophotometric analysis of cytochromes using chemical difference spectra is shown in Fig. 1. The wild-type membranes show an absorbance maximum at 554 nm, indicative of cytochrome *f* in the hydroquinone-ferricyanide spectrum. A Chl/Cyt *f* ratio of 500 : 1 was calculated. In contrast, no cytochrome *f* was detected in the thylakoids from the mutant. Examination of dithionite-minus-hydroquinone difference spectra indicated the mutant was also devoid of cytochrome b_6 and that the only cytochrome in the membranes was cytochrome *b*-559. The concentration of this cytochrome was one per 450 Chl. Thus,

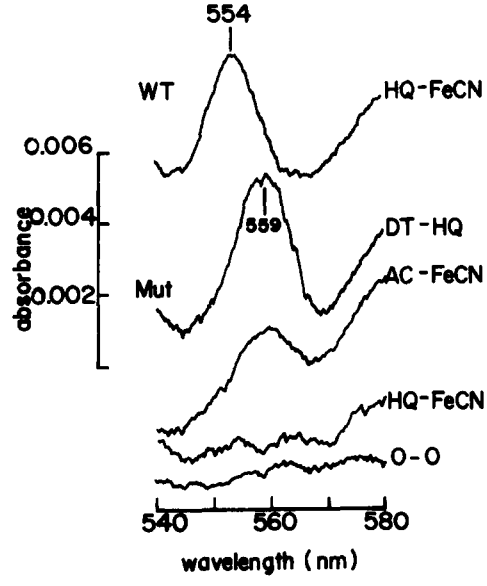

Fig. 1. Redox difference spectra of wild-type and mutant *Lemna* thylakoids. The reaction mixtures contained 1% Triton X-100, 50 mM Hepes (pH 7.5) and 0.1 mg Chl/ml. The spectra were recorded with a Cary 219 spectrophotometer with automatic baseline correction. A few grains of the following were added as indicated: HQ, hydroquinone; FeCN, ferricyanide: AC, ascorbate; DT, dithionite. O, no addition.

with the previous observation that the mutant thylakoids lack the Rieske Fe-S center [3], it is clear that the prosthetic groups known to be associated with components of the cytochrome b_6-f complex are absent in the mutant *Lemna*.

Fig. 2A compares the polypeptides composition between thylakoids of wild-type *Lemna* (lane 1), mutant *Lemna* (lane 2), and purified spinach cytochrome b_6-f complex (lane 3). The latter contains four subunits: 35 kDa (Cyt f); 20 kDa (Cyt b_6), 18 kDa (Rieske Fe-S) and 16 kDa (Subunit IV) [10]. Also shown in Fig. 2B and 2C are immunoblots with antibodies against cytochrome f and Subunit IV (Fig. 2B) and cytochrome b_6 and the Rieske Fe-S center (Fig. 2C) of wild-type and mutant *Lemna*. An antibody against LHCP II has been used in Fig. 2D. It is evident that although similar amounts of LHCP II are present in both membranes, the four subunits of the cytochrome complex are absent from the mutant. The fact that no cross-reacting material is detected in the mutant thylakoids indicates there is no assembly of the cytochrome complex in these membranes.

In addition to the disappearance of a protein band in the 33–35 kDa region and bands at 19 and 14 kDa, which probably correspond to cytochrome f, cytochrome b_6 and subunit IV, respectively, there are several other differences in the polypeptide pattern between mutant and wild-type thylakoids. A decrease in a band at approx. 55 kDa suggest the amount of coupling factor is reduced in the mutant (compare arrows in Fig. 2A, lanes 1 and 2). Furthermore, polypeptides of apparent molecular weights of 36, 35 and 21 kDa were not detected in the mutant. Interestingly, two polypeptides (17 and 17.5 kDa) were present in the mutant but were absent in wild-type membranes. At present, we are not able to identify these polypeptide changes, but it is unlikely they play any role in photosynthetic electron transport, since both Photosystems I and II are separately active in the mutant.

Fig. 3 shows an HPLC analysis of the PQ-9 content of *Lemna* thylakoids. In the petroleum ether extract [11], at least six prominent peaks can be resolved and the fifth peak was identified as PQ-9 by comparison with a standard PQ-9 sample. It was found that the mutant thylakoids contained less PQ-9 than the wild-type thylakoids on a Chl basis. Quantitation gives Chl/PQ ratios of 100 and 29 in the mutant and wild-type, respectively. However, the acceptor pool of Photosystem II,

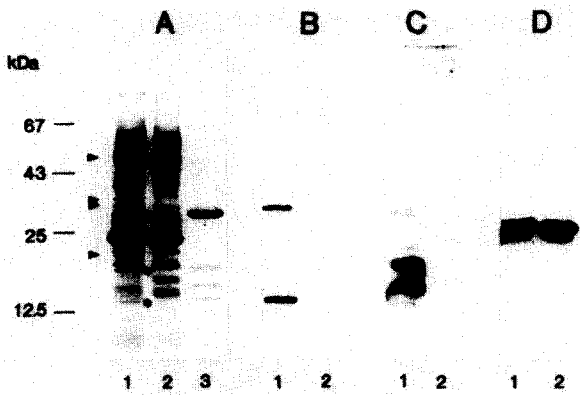

Fig. 2. Analysis of protein components in wild-type and mutant *Lemna* thylakoids. The isolated thylakoids were analyzed for their polypeptide composition by SDS-PAGE. (A) Coomassie blue stained gel; (B) immunoblot with cytochrome f and subunit IV antisera; (C) immunoblot with antisera against cytochrome b_6 and Rieske Fe-S protein; (D) immunoblot with LHCP II antisera. Lane 1, wild-type *Lemna*; lane 2, mutant *Lemna* and lane 3, spinach cytochrome b_6-f complex. * denotes positions of polypeptides of the cytochrome b_6-f complex that are missing in the mutant. ▶ denotes portions of other polypeptides affected in the mutant.

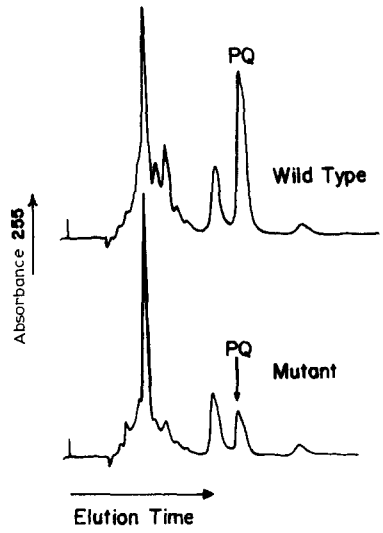

Fig. 3. HPLC analysis of wild-type and mutant *Lemna* thylakoid extracts. PQ, plastoquinone-9.

estimated from fluorescence induction kinetics curve, was not significantly different in the two membranes (see also Ref. 4). These findings suggest the photoreducible plastoquinone pool, which has been associated with the secondary acceptor pool, is not altered in the mutant in spite of a decrease in total PQ-9 of approx. 70%.

In the present report, we have demonstrated that in mutant 1073 of *Lemna*, all four polypeptides of the cytochrome b_6-f complex are missing in the isolated thylakoids. The only cytochrome present in such thylakoids is cytochrome *b*-559. At present we are investigating whether the defect in this mutant is at the transcriptional or translational level, and such further studies may yield insights as to the mechanism of assembly of this integral membrane protein complex.

This work was supported in part by a grant from the National Institutes of Health. We would like to thank Drs. A. Barkan and S. Mayfield for antibodies and R.K. Chain for a sample of purified PQ-9.

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