Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4352–4356, July 1982
Cell Biology

Evidence for a respiratory chain in the chloroplast

(photosynthesis/respiration/starch degradation/evolution)

PIERRE BENNOUN

Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005, Paris, France

Communicated by Pierre Joliot, April 12, 1982

ABSTRACT **Evidence is given for the existence of an electron transport pathway to oxygen in the thylakoid membranes of chloroplasts (chlororespiration). Plastoquinone is shown to be a redox carrier common to both photosynthetic and chlororespiratory pathways. It is shown that, in dark-adapted chloroplasts, an electrochemical gradient is built up across the thylakoid membrane by transfer of electrons through the chlororespiratory chain as well as by reverse functioning of the chloroplast ATPases. It is proposed that these mechanisms ensure recycling of the ATP and NAD(P)H generated by the glycolytic pathway converting starch into triose phosphates. Chlororespiration is thus an O_2-uptake process distinct from photorespiration and the Mehler reaction. The evolutionary significance of chlororespiration is discussed.**

Respiration is a process common to living cells in which reduced organic substrates are oxidized at the expense of molecular oxygen. The free energy recovered in this process is partly converted into heat and partly recovered by the coupled phosphorylation of ADP into ATP. The hydrolysis of ATP releases free energy available for cell functions. Conversely, photosynthesis generates strong reductants at the expense of the energy of light. This formation of strong reductants corresponds to the main energy-conversion process of photosynthesis. A minor part of the light energy is however used to phosphorylate ADP into ATP. The photosynthetic and respiratory apparatuses show strong similarities: (*i*) both implicate membrane-bound electron transfer chains, (*ii*) several membrane components are present in both systems (cytochromes, iron-sulfur proteins, quinones, ATPases), and (*iii*) both membrane systems form closed vesicles and generate ATP.

Photosynthetic eukaryotic cells possess distinct organelles in which photosynthesis and respiration proceed—namely, chloroplasts and mitochondria. These organelles might be symbiotic prokaryotes that have transferred some of their genetic information to the host cell. In prokaryotic photosynthetic organisms, the photosynthetic and respiratory chains are located in the same membranes and share both electron transport components and coupling factors (1–3). One could thus question whether respiration occurs in the chloroplast as well. I show in this paper that, indeed, chlororespiration exists and is distinct from mitorespiration.

MATERIALS AND METHODS

Chlamydomonas reinhardtii wild-type 137C and mutant strains were grown in TAP medium (4) under an illumination of 200 lux. The mutants used in this work have been described (5–7). *Chlorella pyrenoidosa* was grown phototrophically as described (8).

Open cell preparations were obtained as follows: 200 ml of cell suspension (2×10^6 cells/ml) was collected and suspended in 20 ml of 20 mM *N*-tris(hydroxymethyl)methylglycine(Tricine)/KOH, pH 7.8/10 mM NaCl/10 mM $MgCl_2$/1 mM K_2HPO_4/0.1 M sucrose/5% Ficoll. The cell suspension was passed through a Yeda press operated at 90 kg/cm^2, diluted with 200 ml of Ficoll-lacking buffer, and centrifuged, and the pellet was suspended in the same buffer.

Chlorophyll fluorescence kinetics and luminescence measurements were performed as described (9).

RESULTS

The plastoquinone (PQ) pool of chloroplast is a redox carrier of the photosynthetic electron transport chain. This pool is located between the two photosystems, is reduced by photosystem II, and is oxidized by photosystem I. The number of equivalents present in the PQ pool is an order of magnitude higher than that of the photochemical centers. In dark-adapted cells, the PQ pool is partly oxidized. Diner and Mauzerall (10) proposed that this redox state results from a permanent reduction of the PQ pool by an endogenous reductant and a permanent oxidation by oxygen.

The *in vivo* chlorophyll fluorescence kinetics measured on illumination of dark-adapted cells of the F14 mutant of *C. reinhardtii* devoid of photosystem I reaction centers is shown in Fig. 1. In the absence of photosystem I activity, this kinetics reflects mainly reduction of the PQ pool by photosystem II. A linear relationship exists between the variable part of fluorescence and the rate of photochemical reaction II. Therefore, the area bounded by the fluorescence rise and its asymptote measures the pool size of electron acceptors to photosystem II (11, 12). In addition to allowing easy measurement of the PQ pool, the use of the F14 mutant ensures the absence of chloroplast O_2-uptake processes such as Mehler reaction and photorespiration (13). We see in Fig. 1 that the removal of oxygen in the dark leads to a reduction of the PQ pool. The experiment shown in Fig. 2 suggests that oxidation in the dark of the PQ pool at the expense of oxygen proceeds through a catalytic pathway that depends on the integrity of the chloroplasts. The PQ pool was reduced by prior illumination, and the kinetics of its reoxidation in the dark was studied for whole cells and open-cell preparations. We see that the rate of PQ oxidation is considerably slower in open-cell preparations.

To determine the kind of catalyst involved in the PQ oxidation, we tested the effects of inhibitors of mitochondrial oxidases. Lower eukaryotic organisms possess alternative pathways of mitochondrial respiration. Doussière *et al.* (14) showed, for instance in *Paramecium*, that three pathways of electron transport to oxygen are operating. The main pathway goes to cytochrome aa_3, which is sensitive to CO and to low concentrations of cyanide. An alternative pathway, which is sensitive

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Abbreviations: PQ, plastoquinone; SHAM, salicylhydroxamic acid; DCCD, dicyclohexylcarbodiimide; Bu_3Sn, tri-*n*-butyltin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

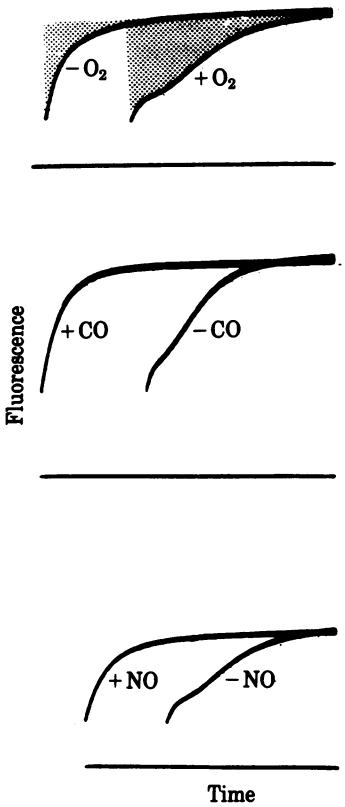

FIG. 1. Chlorophyll fluorescence kinetics of *Chlamydomonas* mutant F14 lacking photosystem I reaction centers. Whole sweep, 2 sec. Shaded areas measure electron acceptors to photosystem II (mainly oxidized PQ) present prior to illumination. Mixtures were incubated in the presence or absence of 20 mM glucose/glucose oxydase at 2 mg/ml in the dark for 4 min (*Upper*), with or without bubbling CO in the dark for 3 min (*Middle*), or with and without bubbling NO in the dark for 30 sec (*Lower*). Under the same conditions, N_2 did not lead to detectable reduction of the PQ pool.

to salicylhydroxamic acid (SHAM), is present as in plant mitochondria. A third pathway goes to a *b* cytochrome, which is likely analogous to bacterial cytochrome *o* (15). This pathway is sensitive to CO and to moderate concentrations of cyanide.

Fig. 1 shows that CO and NO inhibit the dark oxidation of

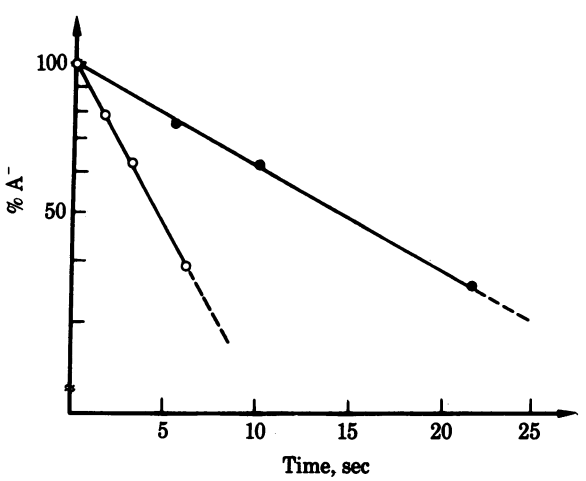

FIG. 2. Kinetics of reoxidation of the pool (A^-) of electron acceptors to photosystem II in *Chlamydomonas* mutant F14. Algae were illuminated to achieve full reduction of the pool and then fluorescence induction was recorded after incubation in the dark for various times. ○, Whole cells; ●, open cells.

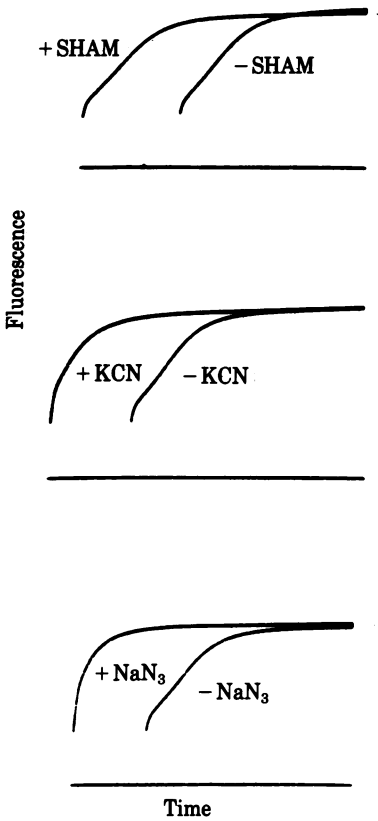

FIG. 3. Chlorophyll fluorescence kinetics of *Chlamydomonas* mutant F14. Whole sweep, 2 sec. Mixtures were incubated in the presence or absence of 4 mM SHAM in the dark for 2 min (*Upper*), in the presence or absence of 10 mM KCN in the dark for 3 min (*Middle*), or in the presence or absence of 10 mM NaN_3 in the dark for 3 min (*Lower*).

the PQ pool in *C. reinhardtii*. In the same organism, high concentrations of cyanide or azide also inhibit this reaction, whereas SHAM has no effect (Fig. 3). Dicyclohexylcarbodiimide (DCCD) is known to act on the membrane sector of the ATPase (16) and also on mitochondrial cytochrome oxidase (17). As shown in Fig. 4, DCCD inhibits oxidation in the dark of the PQ pool in a *Chlamydomonas* mutant lacking chloroplast ATPases. The complex fluorescence rise observed in this mutant is likely related to the high electrochemical gradient built up in the light in the absence of ATPases.

A different situation is observed in *Chlorella pyrenoidosa* in which neither CO (data not shown) nor cyanide lead to reduction of the PQ pool in the dark whereas SHAM does (Fig. 5). Previous data by Maison-Peteri and Etienne (18) indicate that, in *Chlorella*, NaN_3 as well induces reduction of the PQ pool in the dark. We thus observed that specific inhibitors can block

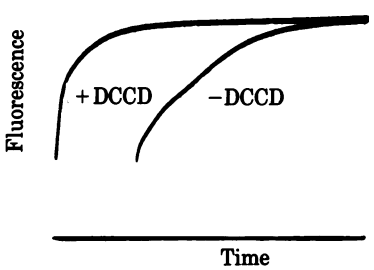

FIG. 4. Chlorophyll fluorescence kinetics of *Chlamydomonas* double mutant F54.F14 lacking system I reaction centers and chloroplast ATPases. Whole sweep, 2 sec. Mixtures were incubated with 20 μM DCCD in the dark for 3 min.

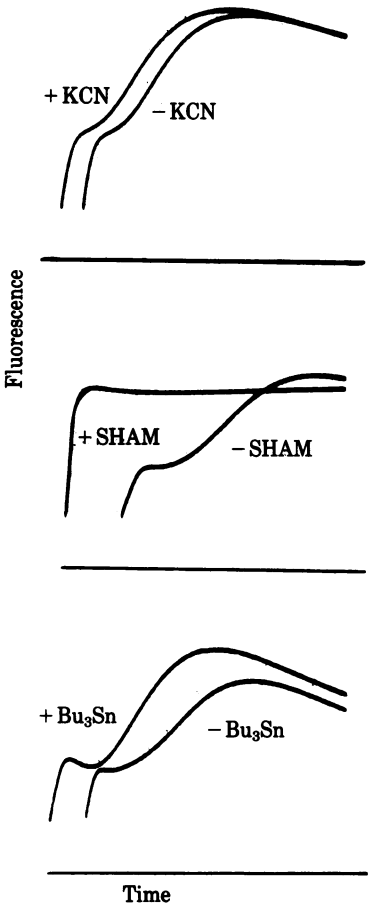

FIG. 5. Chlorophyll fluorescence kinetics of *Chlorella* cells. Whole sweep, 1 sec. Mixtures were incubated in the presence or absence of 10 mM KCN in the dark for 3 min (*Upper*), in the presence or absence of 10 mM SHAM in the dark for 3 min (*Middle*), or in the presence or absence of 2 μM Bu_3Sn in the dark for 5 min (*Lower*).

oxidation in the dark of the PQ pool and that *Chlamydomonas* and *Chlorella* respond to different inhibitors. These inhibitors are known to act on the various oxidases involved in the alternative pathways of mitochondrial respiration (19).

The experiments shown in Fig. 6 show that, in open-cell preparations of *Chlamydomonas*, NADH or NAD(P)H reduces the PQ pool in the dark. This agrees with previous reports by Godde and Trebst (20) and Shahak *et al.* (21). Evidence for a NADH–PQ oxidoreductase bound to the thylakoid membrane of *C. reinhardtii* was given by Godde (22): the spectral properties of this enzyme indicate a flavoprotein containing Fe-S groups. Although analogous to complex I of mitochondria, this enzyme shows a specificity for PQ rather than for ubiquinone. The above experiments favor the conclusion that a respiratory type of electron transfer from NAD(P)H to oxygen proceeds in the chloroplast through the PQ pool involved in the photosynthetic electron transport chain. We will refer to this process as chlororespiration.

We then wondered whether chlororespiration generates an electrochemical gradient across the thylakoid membrane. To answer this question, we used delayed fluorescence (luminescence) as a probe for the presence of such a gradient. Luminescence results from recombination of the charges separated by the system II reaction centers and increases in parallel with the electrochemical gradient. Joliot and Joliot (24) showed that, in-dark adapted algae, a pH gradient is generated across the thylakoid membrane by reverse functioning of the chloroplast

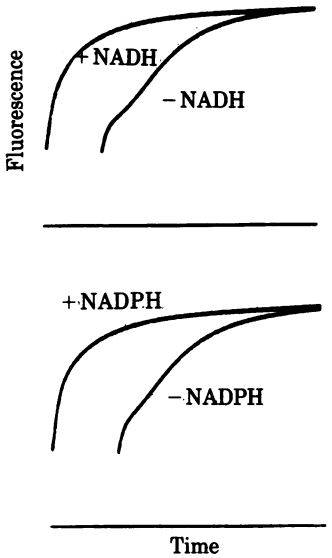

FIG. 6. Chlorophyll fluorescence kinetics of open-cell preparations of *Chlamydomonas* mutant F14. Mixtures were incubated in the presence or absence of 1 mM NADH in the dark for 2 min (*Upper*) or in the presence or absence of 1 mM NADPH in the dark for 2 min (*Lower*).

ATPase: this implies the presence of a pool of ATP in the chloroplast that is converted into ADP by the ATPases with concomitant transfer of protons from the outer to the inner space of the thylakoids. The inhibition of ATPases by tri-*n*-butyltin (Bu_3Sn) was shown by these authors to reduce luminescence emission and hence the electrochemical gradient across the thylakoid membrane. As shown in Fig. 5, Bu_3Sn does not affect chlororespiration: the PQ pool remains oxidized in its presence. We see in Fig. 7 that the luminescence intensity is smaller in the *Chlamydomonas* mutant FUD50 lacking chloroplast ATPases than in the wild type. This agrees with the data by Joliot and Joliot in which the ATPases were blocked by Bu_3Sn. However, Fig. 7 also shows that the luminescence emission in FUD50 is inhibited when chlororespiration is blocked by treatment with CO. In a similar way, Fig. 8 shows that, in *Chlorella*, the luminescence emission observed in the presence of Bu_3Sn is inhibited when chlororespiration is blocked by addition of SHAM. Anaerobiosis that blocks chlororespiration reduces luminescence emission, and addition of Bu_3Sn to the anaerobic sample further reduces the luminescence (Fig. 8). The luminescence data reported above favor the conclusion that an electrochemical gradient is built up across the thylakoid membranes in dark-adapted algae by two different processes: (*i*) reverse functioning of the chloroplast ATPases that hydrolyzes a pool of ATP present in the chloroplast and (*ii*) transfer of electrons in the chlororespiratory chains that oxidizes a pool of NAD(P)H present in the chloroplast.

The effect of partial inhibition of chlororespiration was studied with all the above-mentioned inhibitors. A typical experiment is shown in Fig. 9. The area over the fluorescence rise indicates that the pool of electron acceptors to photosystem II is reduced by 35% after incubation with 2.5 mM cyanide. By comparison, the area over the fluorescence rise in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is 10% of that observed in its absence (not shown). This compound blocks electron transfer between the primary quinone Q of system II and the PQ pool. It is worthwhile to observe that the modifications of the fluorescence rise induced by chlororespiration inhibitors are totally different from those induced by DCMU type inhibitors: (*i*) partial reduction of the PQ pool is observed

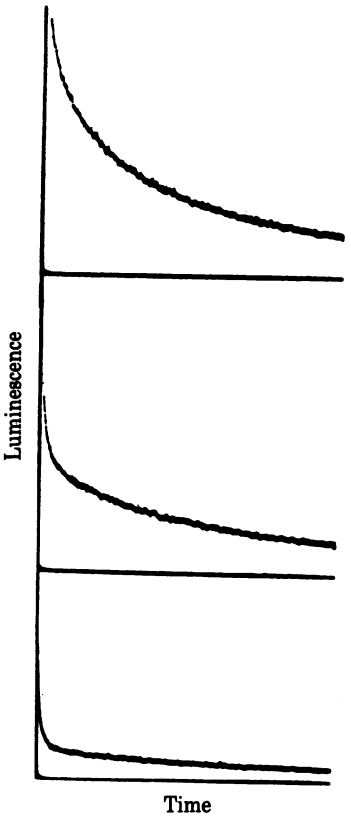

FIG. 7. Luminescence kinetics after illumination by a short flash of very low intensity exciting 2% of the photochemical reaction centers in the presence of 2 μM DCMU in *Chlamydomonas* cells. Whole sweep, 1 sec. (*Upper*) Wild type. (*Middle*) Mutant FUD50 lacking chloroplast ATPases. (*Lower*) Mutant FUD50 mixtures were bubbled with CO in the dark for 3 minutes in the presence of DCMU.

without any increase of the fast fluorescence phase and (*ii*) a decrease of the initial fluorescence yield is observed rather than an increase. This decrease is in agreement with the luminescence experiments showing that the inhibition of chlororespiration decreases the electrochemical gradient across the thylakoid membranes (Figs. 7 and 8): this membrane potential is known to inactivate some of the photosystem II centers into a nonquenching state (25). These centers are reactivated by lowering the membrane potential through inhibition of the ATPases or of the chlororespiratory chains or both.

A kinetical heterogeneity of the PQ pool was previously reported; the two fractions of the pool would show different equi-

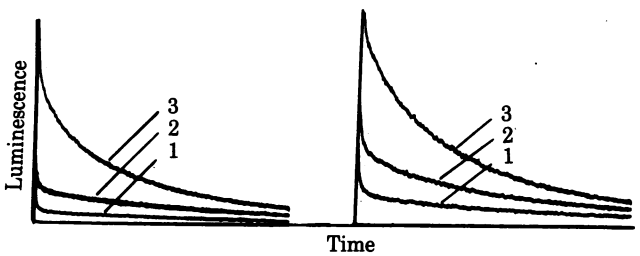

FIG. 8. Luminescence kinetics in *Chlorella* cells. Reaction conditions were as in Fig. 7. (*Left*) Mixtures were incubated with 2 μM Bu_3Sn (trace 2), with 2 μM Bu_3Sn/10 mM SHAM (trace 1), or with neither (control; trace 3) in the dark for 2 min. (*Right*) To determine the effect of oxygen removal, mixtures were incubated with 20 mM glucose/glucose oxydase at 2 mg/ml in the dark for 4 min (trace 2) or after addition of 2 μM Bu_3Sn in the dark to the anaerobic sample (trace 1). Trace 3, control.

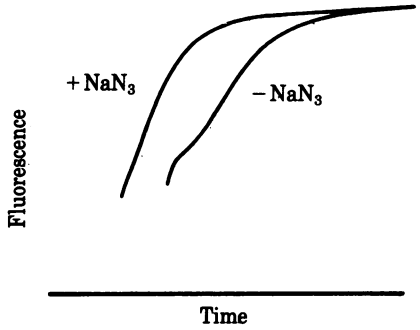

FIG. 9. Chlorophyll fluorescence kinetics of *Chlamydomonas* mutant F14. Mixtures were incubated with or without 2.5 mM NaN_3 in the dark for 2 min prior to illumination. Whole sweep, 2 sec.

librium constants in their reaction with the primary quinone Q (26–28). We see that the lag between the two phases of the fluorescence rise is absent after partial inhibition of chlororespiration (Fig. 9). This indicates that the fraction of the pool that remains oxidized under this condition displays a low equilibrium constant with Q. Thus the chlororespiratory chain is likely connected to the other fraction of the pool displaying a high equilibrium constant with Q. Interestingly, the operation of two pools of quinone was proposed in mitochondria possessing an alternative pathway of respiration (29).

The rate of oxygen uptake due to chlororespiration in *Chlorella* can be deduced from the kinetic studies of Diner and Mauzerall (10) and is ≈10 μmol of O_2 consumed per mg of chlorophyll per hr. This value is to be compared with the total respiration of *Chlorella* estimated as 50 μmol of O_2 per mg of chlorophyll per hr (8) and with the maximal rate of O_2 evolved by photosynthesis, which is ≈250 μmol of O_2 per mg of chlorophyll per hr.

Last, we observed that, after reduction by prior illumination, the rate of reoxidation in the dark of the PQ pool is an order of magnitude faster in intact spinach leaves than in broken spinach chloroplasts. This indicates that chlororespiration probably occurs in higher plants as well as in green algae. That the integrity of the thylakoid membrane is required for chlororespiration is also deduced from the observation that the ionophore dicyclohexyl 18-crown-6 inhibits chlororespiration in *Chlamydomonas* (data not shown).

DISCUSSION

The results reported in this paper demonstrate the existence in the thylakoid membrane of chloroplasts of an electron transfer chain oxidizing NAD(P)H at the expense of oxygen. This chloroplast respiratory chain shares at least one redox carrier, PQ, with the photosynthetic electron transport chain. PQ was also shown recently to be a common link between photosynthesis and respiration in a blue-green alga (30).

It is known that, in eukaryotic cells, electron transport pathways to oxygen are located in the inner membranes of mitochondria and also in the endoplasmic reticulum (31). It is therefore suitable to refer to these different respiratory processes as chlororespiration, mitorespiration, and cytorespiration. Chlororespiration is an O_2-uptake process different from both photorespiration and Mehler reaction. It is currently admitted that the transmembrane electrochemical gradient is the energy source for the phosphorylation of ADP by membrane ATPases as proposed by Mitchell (32). We showed that, in dark-adapted cells, two processes generate an electrochemical gradient across the thylakoid membrane: (*i*) oxidation of NAD(P)H through the chlororespiratory chain and (*ii*) hydrolysis of ATP by chloroplast

ATPases. We are thus led to the question of the origin of the NAD(P)H and ATP pools in dark-adapted chloroplasts. A likely answer to this question lies in metabolism of starch in the dark. Whereas starch is produced through photosynthesis, a glycolytic pathway converts it into triose phosphates that are exported by the chloroplast in exchange for phosphate (33). This glycolytic pathway generates ATP and NAD(P)H from ADP and NAD(P). The former metabolites would then be recycled through the electrogenic pathways described here. It is important to note that the dynamics of such a system is ensured by the export of triose phosphates, which serve as an energy source for the cells.

The oxidases involved in chlororespiration were shown to vary from one organism to another and photosynthetic organisms could thus be classified according to the nature of their chloroplast oxidase. Interestingly, these oxidases appear to be analogous to those involved in the alternative pathways of mitochondrial respiration. This observation stresses once more the analogy between chloroplasts and mitochondria. It is noteworthy that photosynthesis and respiration are linked in prokaryotic photosynthetic organisms as well as in chloroplasts. Whereas pure respiratory apparatuses exist (i.e., pure ATP generators), there is no example of pure photosynthetic apparatuses [i.e., pure NAD(P)H generators]. That the latter are not found may signify that the primitive photosynthetic systems were light-driven ATP generators operating in a way similar to cyclic photophosphorylation, without consuming any oxidant or reductant. These ATP generators would have evolved in two directions: (*i*) they adapted to generate NAD(P)H as well through mechanisms that have been described in photosynthetic bacteria (34), a condition necessary for CO_2 fixation, and (*ii*) they gave rise to ATP generators that function in the dark by substitution of the energy of light by that of appropriate redox compounds. It is interesting to note that a pure light-driven ATP generator exists in *Halobacterium* (35). It consists of a light-driven proton pump with no electron transport chain. Such a system was likely less subject to adaptative evolution.

I thank Drs. P. Joliot, B. Diner, F. A. Wollman, P. Delepelaire, and A. Adoutte for stimulating discussions in the course of this work and M. N. Mannevy for the preparation of the manuscript. This work was supported by Centre National de la Recherche Scientifique Grant ER 016 and Commission of the European Communities Grant ESF-017.

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