Mitochondrial responses to intracellular pulses of photosynthetic oxygen

(oxygen evolution/green algae/mitochondrion/cytochrome c/cytochrome c oxidase)

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ABSTRACT When submitting anaerobic algal cells to a series of saturating flashes, transient absorption changes of mitochondrial origin were detected, showing the characteristic flash-number dependence of photosynthetic oxygen evolution. The faster kinetic event is the oxidation of heme a_3 of the cytochrome-c oxidase, which reaches a maximum at ≈ 3.5 ms before again being reduced within 20 ms. The oxidation of cytochrome c involves an initial submillisecond lag, and its half-time is ≈ 3.3 ms. Another component, probably indicating oxidation of heme a, is seen around 607 nm, with a kinetic behavior similar to that of cytochrome c. The fast time scale of these reactions excludes long-range diffusion and supports a direct intracellular trapping of O_2. It is estimated that, under appropriate conditions, the yield of this process is $>30\%$. The linearity of these responses with respect to the amplitude of the oxygen pulse implies that a single turnover of the cytochrome oxidase is involved. These results suggest that the intracellular oxygen pathway may be of physiological importance in green algae. On the other hand, this technique seems promising both as an alternative to polarographic detection of photosynthetic oxygen and as a means of studying the cytochrome oxidase response *in vivo* to single-turnover oxygen pulses.

In unicellular green algae, much cell space is occupied by the two types of energy-conserving organelles, chloroplast and mitochondrion. Most often a single large chloroplast is surrounded by a number of smaller mitochondria. When energetic aspects are considered, the biosphere may be viewed as a symbiotic association of both organelles (1), so that an algal cell then appears as a microscopic model of the biosphere. Encapsulation of chloroplast and mitochondrion within a cell optimizes the efficiency of their metabolic trade; this metabolism concerns chiefly carbohydrates among other interconnecting pathways (2, 3). Another component exchanged between the organelles is molecular oxygen. In our biosphere, the main route for the oxygen cycle, obviously, is through the atmosphere. Nevertheless, one may ask whether the direct intracellular pathway could occur with significant efficiency—that is, could an algal cell in an anaerobic environment respire by trapping molecular oxygen evolved from its own chloroplast? This question may have evolutionary as well as physiological bearings: should the answer be positive, the oxygen-using respiratory pathway could have evolved in a still anoxigenic atmosphere.

The evidence reported in this paper shows that, indeed, a significant fraction of photosynthetic oxygen may be used by mitochondria before the oxygen diffuses out of the cell. By studying light-induced absorption changes in anaerobic suspensions of algae, signals arising from the oxidation of mitochondrial cytochrome-c oxidase and cytochrome c were seen. This finding provides a new method both for photo-

synthetic oxygen detection and for studying the cytochrome oxidase response to short oxygen pulses.

MATERIALS AND METHODS

Two mutant strains, S30 and S56, of the green alga *Chlorella sorokiniana*, which have been described (4, 5), were used. These mutants, isolated and provided by P. Bennoun in this laboratory, are both "pale-green" strains that lack the major part of the chlorophyll-protein light-harvesting complexes, greatly facilitating detection of absorption changes associated with electron transfer (5) because of reduced background absorption. S56 is a double mutant stemming from S30 and lacks photosystem I (PSI) centers.

The algae were pretreated with benzoquinone as follows: p-benzoquinone was added to the algal culture at 0.3 mM immediately before 5-min centrifugation for pelleting the cells; the supernatant was discarded, and the algae were centrifuged again after resuspension in a quinone-free medium.

The algae were finally resuspended at 10–40 μg of chlorophyll per ml (depending on the spectral region investigated) in 50 mM phosphate buffer, pH 6.5/10% Ficoll. The ionophore dicyclohexyl-18-crown-6 was added at 0.5 mM to obtain fast relaxation of the photochemically induced electric field through the thylakoid membrane, thus minimizing associated absorbance changes. Anaerobic conditions were obtained by bubbling pure argon during 20 min in the reservoir from which the algae were pumped into the measurement cuvette; the experiments were done at room temperature.

Detection of flash-induced absorption changes depended upon the apparatus developed by Joliot and coworkers (6, 7). This machine achieves high sensitivity by using short monochromatic flashes as detecting light with simultaneous detection in measurement and reference paths. Absorbance is thus sampled in a discrete fashion at programmed times with respect to the actinic flashes. The latter consisted of saturating xenon flashes that had a duration of a few microseconds.

RESULTS

Fig. 1 *Left* shows the absorption changes at 548 nm induced by a series of short saturating xenon flashes in anaerobic algae. From considering the spectra (see below), these changes are mostly from the oxidation and re-reduction of mitochondrial cytochrome c. Smaller changes arising from the chloroplast electron-transfer chain were recorded in an aerobic control experiment and subtracted to obtain the signals shown. Fig. 1 *Right* is a plot of the change induced by each actinic flash at 20 ms. This plot displays the characteristic pattern of a flash-induced oxygen evolution sequence—namely, a damped oscillation with periodicity of four flashes,

Abbreviation: PSI and II, photosystem I and II, respectively.

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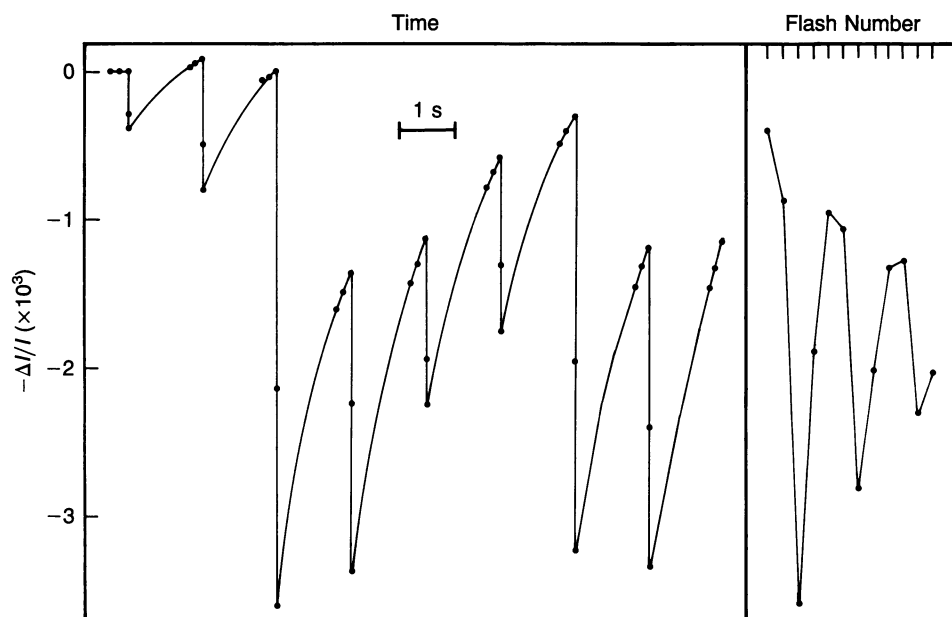
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FIG. 1. At left, absorption changes at 548 nm in response to a series of saturating flashes triggered 1 s apart. A was sampled at 3, 20, 800, 900, and 1000 ms after each flash. Strain S30 was used after benzoquinone treatment and addition of 10 mM sodium ascorbate, 50 μM diaminodurene and 0.5 mM dicyclohexyl-18-crown-6. Data represent the difference between anaerobic minus aerobic conditions. At right is a plot of the changes at 20 ms induced by each flash.

with maxima on the third, seventh, . . . flashes, as originally seen by Joliot (8) and rationalized by the Kok model (9) of the photosynthetic oxygen-evolving system. There appears, however, a small overall downward shift (the first flash yield is zero in a regular oxygen evolution sequence), which is probably due to an uncorrected chloroplast contribution.

For these experiments, the algae were pretreated with p-benzoquinone, as described. This treatment prevents anaerobic dark reduction of the pool of photosystem II (PSII) acceptors (plastoquinones), most probably by inactivating the NAD(P)H–plastoquinone reductase (10, 11), as suggested by Joliot and coworkers (12, 13). The treatment is also responsible for the slow re-reduction kinetics of cytochrome c apparent in Fig. 1 (see also Fig. 3). Flash-induced oxidation of cytochrome c was also seen with untreated algae (data not shown), the amplitude of the transient being severely truncated by the normal fast re-reduction reaction. The latter reaction was completely blocked by 10 μM myxothiazol (inhibiting the mitochondrial $b–c_1$ complex reduction by ubiquinol), causing full restoration of the oxidation kinetics. In benzoquinone-treated algae, it was necessary to add a reductant, such as reduced diamodurene (reduced N,N,N', N'-tetramethyl-p-phenylene diamine or dithionite were also used successfully), to keep the high-potential segment of the mitochondrial chain in a reduced state. Increasing the diaminodurene concentration caused a faster re-reduction kinetics that was inhibited by myxothiazol (data not shown). This result suggests that benzoquinone inhibits the reduction of the ubiquinone pool, similarly to its action on the homologous reaction in the chloroplast. Inactivation of the NAD-(P)H:(quinone-acceptor) oxidoreductase is probably involved in both cases, due to the bifunctional linking of p-benzoquinone to amino acid residues (14) and in line with the strong sequence homology between enzymes reported by Fearnley *et al.* (15).

Fig. 2 shows spectra of the mitochondrial response to a pulse of photosynthetic oxygen, induced by the third flash of a sequence, at 3 and 20 ms. To minimize the contribution of chloroplastic absorption changes—which are particularly disturbing in the blue region—a double mutant strain (S56, pale-green and devoid of PSI centers) was used, and the

changes recorded in a control aerobic experiment were subtracted. Two other sources of distortion or inaccuracy should be noted when examining these spectra. (*i*) the flattening effect (16) is expected to attenuate the changes in regions where the algae present high absorbance (e.g., the chlorophyll bands). This flattening accounts for the relatively small peaks in the blue (Soret bands) compared with the green region. The peak around 607 nm is also expected to be somewhat attenuated. (*ii*) During the several hours necessary for recording the spectra, one sees a continuous decrease of signals, which may be from progressive reduction of the plastoquinone pool causing diminished PSII activity. The data of Fig. 2 were corrected for this drift, as described in the legend.

At least two different kinetic components appear. The bands around 415 nm, 550 nm, and 607 nm (these wavelengths for the peaks were determined, more accurately than for Fig. 3, from individual experiments in each band) have their half-rise time around 3 ms, whereas the 445-nm peak goes through a transient maximum, decreasing between 3 and 20 ms. The changes in the green region fit with the α and β peaks of the oxidized–reduced mitochondrial cytochrome c (17). Peak position at 550 nm allows clear discrimination from the chloroplastic cytochrome f, which peaks at 554 nm. Probably these changes are contributed by both mitochondrial cytochromes c and c_1, which have very close spectra. The band around 415 nm is then expected to be predominantly contributed by the Soret band of these cytochromes and, indeed, has similar kinetic behavior. The transient kinetics of the 445-nm band, on the other hand, can be ascribed to the oxidation and re-reduction of heme a_3 in the cytochrome oxidase (spectra and further references may be found in ref. 18). Contrary to heme a, this cytochrome has a weak differential change in the α band, which agrees with the absence of a transient maximum at 3 ms in the 605-nm region. In addition to the oxidized cytochrome c, the 20-ms spectrum displays a band around 607 nm and persistence of the 445-nm band, which suggest an oxidized heme a in equilibrium with cytochrome c.

More detailed kinetic information (Fig. 3) presents the time courses of the changes at 445 nm, 548 nm, and 607 nm. The
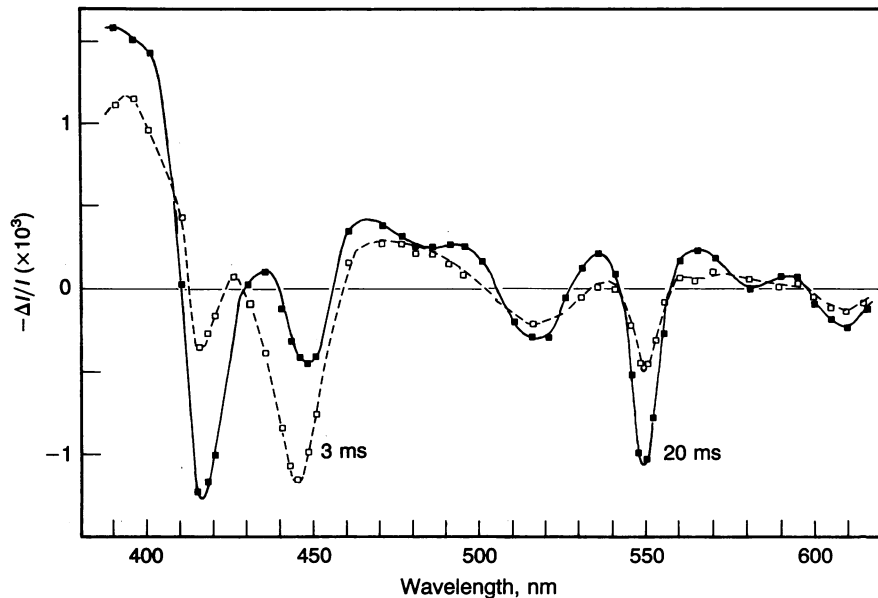
FIG. 2. Spectra of the changes recorded at 3 ms (□) and 20 ms (■) upon the third flash of a series consisting of two flashes 50 ms apart and a third flash 1.5 s later. Sampling of the base line occurred on a 500-ms interval before the third flash. The conditions were the same as for Fig. 1 except that strain S56 was used. Data are presented as the difference between anaerobic and aerobic experiments. Two batches of algae were used, one in the blue region and the other in the longer-wavelength region, with several measurements at 548 nm taken in both cases; these were then interpolated and used to renormalize the data to a constant value for the cytochrome c oxidation at 20 ms.

cytochrome c oxidation recorded at 548 nm displays an initial lag of ≈ 0.5 ms and a half-time of ≈ 3.3 ms. No detectable lag is seen in the a_3 kinetics at 445 nm. The signal peaks around 3.5 ms and then decreases to a nonzero level ($\approx 40\%$ of maximum). The 607-nm kinetics is close to that of cytochrome c, although somewhat faster (half-time around 2.7 ms). This figure also shows a test of the linearity of cytochrome c response with respect to magnitude of the oxygen pulse. The experiment of Fig. 1, showing striking similarity with an O_2 yield sequence, suggested a linear relation. Fig. 3
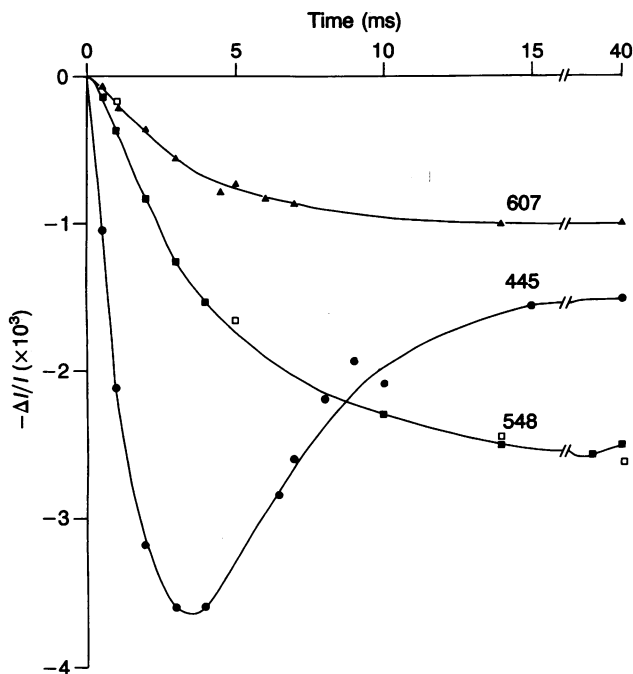

FIG. 3. Time courses of the changes at 445 (●), 548 (■), and 607 (▲) nm upon the third flash. Conditions were the same as for Fig. 2. □, Results obtained at 548 nm with an attenuated flash after renormalization by its photochemical efficiency, as described in text.

shows the kinetics of ΔA_{548} induced by an attenuated third flash (when the two first flashes are saturating). Experimental values were multiplied by a factor of 18, which corresponds to the ratio of the photochemical efficiencies (saturating flash/weak flash). This value was obtained by measuring the field-indicating absorption change induced by the saturating or attenuated (third) flash (strain S56 with no PSI was used in this experiment). Within experimental error, the kinetics are identical in the 0- to 20-ms range, and the amplitude of the cytochrome c oxidation is proportional to the photochemical efficiency of the flash (thus to the magnitude of the O_2 pulse).

To estimate roughly the fraction of photosynthetic oxygen processed by the cytochrome oxidase, we compared the magnitude of cytochrome c oxidation upon the third flash of a sequence in experiments similar to that of Fig. 1, with the amount of oxygen expected to be evolved from PSII. The latter quantity is ≈ 0.58 O_2 molecule per PSII center (9). The concentration of these centers in strain S30 was estimated from that of PSI centers, measured by the ΔA_{703} corresponding to total photooxidation of P700 (with strong continuous light and PSII inhibited). From measuring the charge separation (monitored by the field-indicating ΔA) induced by both photosystems, or by PSI alone (with PSII inhibited), ratio of both types of centers was found to be [PSII]/[PSI] ≈ 0.72. Typical figures for oxidation of cytochrome c at 550 nm on the third flash and for total photooxidation of P700 at 703 nm were, respectively, 1000 and 11,330 (in units of $\Delta I/I = 10^{-6}$). Using 18.5 mM^{-1}·cm^{-1} for the differential extinction coefficient of cytochrome c at 550 nm (17) and 64 mM^{-1}·cm^{-1} for P700 at 703 nm (19), one calculates the following:

$$[\text{PSII centers}] \sim (11330/64) \times 0.72$$

[oxidizing equivalents from O_2]

$$= 4 \times 0.58 \times [\text{PSII centers}]$$

$$[\text{cytochrome } c \text{ oxidized}] \sim 1000/18.5$$

Thus, one finds that $\approx 18\%$ of the O_2 molecules are used for oxidizing the c-type cytochromes. This represents only part of the oxidation process because three other redox centers

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may be expected to equilibrate with these cytochromes: (*i*) heme *a* and (*ii*) Cu_A in the oxidase, and (*iii*) the Fe–S center in the b–c_1 complex. An equilibrium involving heme *a* is, indeed, suggested by the presence of the 607-nm and 445-nm bands in the 20-ms spectrum of Fig. 3. From the extinction coefficients (17, 18), the oxidized heme *a* may be roughly estimated to one-third of the oxidized cytochrome *c*. One expects the amount of oxidized Cu_A to be in the same range. This yields 30% of the photosynthetic O_2 used in mitochondrial oxidations, to which an unknown contribution from the oxidized Fe–S center should be added. The latter is presently difficult to estimate because we do not know the precise values for the equilibrium constant and cytochrome c/b–c_1 complex stoichiometry in these mitochondria. Therefore, 30% is a minimum estimation.

DISCUSSION

It is not surprising that, in an anaerobic algal suspension, the oxygen evolved from the chloroplast is eventually consumed by respiration. More interesting is the finding that a significant part of this process—>30%—occurs through a direct intracellular pathway. That such a pathway is involved in these experiments is easily realized by considering the time scale of the flash-induced mitochondrial changes reported here. Half-time for cytochrome *c* oxidation is $\approx$3 ms. A typical distance for diffusion over time *t* is $\sqrt{2Dt}$, where *D* is the diffusion coefficient (for O_2, $D \approx 2 \times 10^{-5}$ cm²·sec⁻¹). Thus, at 3 ms an oxygen molecule is typically found $\approx$3.5 μm away from its origin. In these experiments, a still smaller distance should be considered because (*i*) the oxygen pulse is not emitted at t_0 but with an exponential kinetics in the ms range (see below) and (*ii*) the kinetics of the 445-nm peak (heme a_3) is faster than that of cytochrome *c*. Because the cells of *C. sorokiniana* have a diameter of $\approx$2 μm and because the average distance between cells in the suspension is several orders of magnitude larger, the observed phenomenon clearly cannot significantly involve intercellular diffusion of O_2 through the suspension medium. Indeed, the fraction of cells located within a few microns of an oxygen-emitting cell and also the solid angle that their mitochondria present to the oxygen wave must be quite small. The intercellular pathway probably occurs as well, though on a slower time scale, and will be masked by the re-reduction reactions. It is noteworthy that the location of mitochondria with respect to the chloroplast, as seen in electron micrographs of these cells [(4); F. A. Wollman and J. Olive, personal communication], is most favorable for efficient interception of a diffusive component emitted from the chloroplast. Indeed, the mitochondria are almost all found against the chloroplast, seemingly in contact, with their long axis parallel to the chloroplast envelope. This pattern, not usually found in higher plant cells (2), suggests an adaptation of green algae for efficient recycling of photosynthetic oxygen, thus allowing mitochondrial respiration in an oxygen-depleted environment.

Clearly, the encapsulation of both a fast light-triggered oxygen emitter and a sensitive fast-responding oxygen detector within cells of a few microns in diameter may offer special opportunities for studying both processes. To date, the only rapidly responding method for detecting flash-induced oxygen evolution has been the Joliot-type electrode (8, 20), in which the photosynthetic material is sedimented on the platinum electrode, thus minimizing diffusion time. Detection of mitochondrial responses provides an interesting alternative, allowing fast oxygen detection under different experimental conditions. The response appears to depend linearly on the magnitude of the oxygen pulse. This dependence implies that a sufficiently small fraction of the oxidases—say <25%, is reacting upon a single flash, whereas saturation is expected (and was seen) when accumulation of

the oxidized state occurs (at high flashing frequency or in the presence of myxothiazol). Fig. 3 shows that the oxidation kinetics remained the same even with a 20-fold-diminished amplitude. This result has an important bearing on the interpretation of the observed responses because it excludes a significant contribution of multiple oxygen turnovers of the oxidase. If, for instance, double turnovers were occurring, i.e., the successive processing of two O_2 molecules by the same oxidase (which should involve the delay of a few ms between the first and second binding of the O_2 molecule), a lower oxygen pulse should decrease probability of the second turnover, affecting both time course and linearity of the response. It thus appears that when the oxidase has completed its first turnover, the local oxygen concentration has had time to drop to a low level.

The rate constant for oxygen release from PSII was first estimated by Joliot *et al.* to 800 s⁻¹ from the phase shift of the polarographic response obtained under excitation with modulated light (20). This result was recently questioned by Plijter *et al.* (21), who reported a much (10- to 100-fold) slower rate. Although a detailed kinetic analysis of the present data is beyond the scope of this paper, they would clearly be very difficult to reconcile with a time constant for oxygen release slower than a few ms and, thus, do not support Plijter's proposal.

Finally, I will discuss the present use of algae as a tool for studying the cytochrome oxidase response to an oxygen pulse. A natural interpretation of the kinetic behaviors seen in Figs. 2 and 3 would be the linear scheme $O_2 \leftarrow a_3 \leftarrow a \rightleftharpoons$ cytochrome *c*. This scheme would fit the data assuming that the 607-nm band is from oxidation of heme *a*. The finding at this wavelength of a delayed reaction with respect to the a_3 oxidation seen at 445 nm and the small advance with respect to the cytochrome *c* kinetics are consistent with the generally admitted idea of a limiting step between hemes a_3 and *a*, with a time constant of $\approx$1.4 ms (18, 22). Persistence of the 445-nm band in equilibrium with the oxidized cytochrome *c* could then be ascribed to the Soret band of heme *a* (the kinetics in this region would thus result from a combination of the transient oxidation/reduction of heme a_3 and oxidation of heme *a* with the same time course as seen at 607 nm). The time resolution in this technique is limited by the O_2 release reaction from the oxygen-evolving system and by diffusion. This limitation makes the method still quite competitive compared with stopped-flow experiments but obviously slow with respect to the procedures involving photodissociation of CO. On the other hand, we have here two features that may prove of particular interest: (*i*) CO is absent, as this molecule is suspected to bind again to a neighboring site after photodissociation and may still affect oxidase reactions (18). (*ii*) The isolation of a single turnover of the oxidase is a specific feature of the present conditions because the oxidase molecules are submitted to a rapidly increasing, then decreasing, oxygen concentration wave.

1. Thomas, L. (1974) *The Lives of a Cell: Notes of a Biology Watcher* (Bantam, New York).
2. Douce, R. (1985) *Mitochondria in Higher Plants: Structure, Function, and Biogenesis* (Academic, New York).
3. Rebeillé, F. & Gans, P. (1988) *Plant Physiol.* **88,** 973–975.
4. Lacambra, M., Larsen, U., Olive, J., Bennoun, P. & Wollman, F.-A. (1984) *Photobiochem. Photobiophys.* **8,** 191–205.
5. Lavergne, J., Delosme, R., Larsen, U. & Bennoun, P. (1984) *Photobiochem. Photobiophys.* **8,** 207–219.
6. Joliot, P., Béal, D. & Frilley, B. (1980) *J. Chem. Phys.* **77,** 209–216.
7. Joliot, P. & Joliot, A. (1984) *Biochim. Biophys. Acta* **765,** 210–218.
8. Joliot, P., Barbieri, G. & Chabaud, R. (1969) *Photochem. Photobiol.* **10,** 309–316.

9. Kok, B., Forbush, B. & McGloin, M. (1970) *Photochem. Photobiol.* **11**, 457–475.
10. Godde, D. & Trebst, A. (1980) *Arch. Microbiol.* **127**, 245–252.
11. Bennoun, P. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4352–4356.
12. Joliot, P. & Joliot, A. (1985) *Biochim. Biophys. Acta* **806**, 398–409.
13. Joliot, P., Vermeglio, A. & Joliot, A. (1989) *Biochim. Biophys. Acta* **975**, 336–345.
14. Sternberg, R., Bindra, D., Wilson, G. & Thévenot, D. (1988) *Anal. Chem.* **60**, 2781–2786.
15. Fearnley, I., Runswick, M. & Walker, J. (1989) *EMBO J.* **8**, 665–672.
16. Duysens, L. N. M. (1956) *Biochim. Biophys. Acta* **19**, 1–12.
17. Margoliash, E. & Frohwirt, N. (1959) *Biochem. J.* **71**, 570–572.
18. Wikström, M., Krab, K. & Saraste, M. (1981) *Cytochrome-Oxidase: A Synthesis* (Academic, New York).
19. Ke, B. (1972) *Arch. Biochem.* **152**, 70–77.
20. Joliot, P., Hofnung, M. & Chabaud, R. (1966) *J. Chem. Phys.* **10**, 1423–1441.
21. Plijter, J., Aalbers, S., Barends, J.-P., Vos, M. & van Gorkom, H. (1988) *Biochim. Biophys. Acta* **935**, 299–311.
22. Hill, B. C., Greenwood, C. & Bonner, W. (1986) *Biochim. Biophys. Acta* **853**, 91–113.