BBA 45628

LASER-INDUCED KINETICS OF CYTOCHROME OXIDATION AND THE 518 mμ ABSORPTION CHANGE IN SPINACH LEAVES AND CHLOROPLASTS

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(Received June 19th, 1967)
(Revised manuscript received September 11th, 1967)

SUMMARY

The Q-switched ruby laser has been used to oxidize cytochrome *f* in several types of intact leaves and the isolated chloroplasts. The halftimes for intact leaf range from 60 to 150 μsec. The cytochrome *f* oxidation rates observed in chloroplasts are from 3 to 100 times slower than the rates in leaves, depending on the type of leaf. A varying intactness of the chlorophyll–cytochrome *f* relationship in chloroplasts, depending on the type of plant, has been postulated. The temperature dependence and quantum yield of cytochrome *f* oxidation has been studied. In addition, the 518 mμ absorption change has been compared in leaves and chloroplasts.

INTRODUCTION

Reactions in spinach leaf and in the isolated chloroplasts have been previously compared by CHANCE AND BONNER[1]. These studies showed that cytochrome *f* oxidation, produced by continuous actinic light, proceeds down to a temperature of 77 °K in both the leaf and chloroplasts.

The present paper describes a comparison of the cytochrome *f* oxidation rate, following a laser flash, in several types of leaves and the isolated chloroplasts. In addition, the kinetics of the 518 mμ absorption change, induced by a laser flash in both the leaf and chloroplasts, are presented. A preliminary report has been presented elsewhere[2].

MATERIALS AND METHODS

The Viroflay variety of spinach (*Spinacia oleracea* L.) and Swiss chard (*Beta vulgaris* L.) were grown in a greenhouse, harvested, and used immediately for experiments. Market spinach was tentatively identified as Hybrid No. 7 (hereafter denoted by H)*. Market spinach used two years previously, in collaboration with Drs. M. AVRON AND B. CHANCE[3] was tentatively identified as Bloomsdale type (hereafter denoted by B). Chloroplasts were isolated from the leaves as previously described[4].

Abbreviation: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

* The identifications of market spinach leaves are by courtesy of Mr. J. SCHERMERHORN, Burpee Seed Co., Philadelphia, Pa.

A piece of leaf (1.5 cm × 1.5 cm) or a turbid suspension of chloroplasts (about 1 mg chlorophyll/ml; 0.16 cm optical path) was illuminated with a 30 nsec flash from the Q-switched ruby laser. The laser wavelength is 694.3 mμ, with an incident intensity at the cuvette from 10^3 to 10^7 W/cm², adjustable by means of a negative lens and neutral density filters. A single beam spectrophotometer (spectral interval = 3 mμ), is used for the measurement of absorption changes. The monochromator lamp can operate at either normal voltage or over-voltage, to give measuring beam intensities of about 0.03 mW/cm² or about 0.1 mW/cm², respectively, at 518 mμ. The signal from the photomultiplier (EMI 9524B) is amplified and displayed on an oscilloscope. A Corning No. 4-96 blue-green filter guards the photomultiplier from the laser flash. The entire apparatus, with timing circuitry for the synchronization of the oscilloscope sweep and laser flash, has been developed by DEVAULT AND CHANCE[5,6].

Reaction halftimes presented in Tables I and II were measured directly from Xerox copies of the photographed oscilloscope traces, after hand-smoothing of the traces. The tabulated uncertainties refer to the standard deviations from the average of ten or more recordings of the halftimes.

RESULTS

Cytochrome f kinetics

Fig. 1 shows recordings of the kinetics of cytochrome *f*, measured in the α-band, 554 mμ. The oxidation of cytochrome *f* following a laser flash in the intact leaf (H) has a halftime of 100 μsec (Fig. 1A). The laser artifact is apparent as an upward spike, that is, the direction of increased light to the photomultiplier. Also, the baseline preceding the laser flash in Fig. 1A is insufficient in length; hence, for an accurate comparison, the trace is reset and an entire baseline is shown. Fig. 1C demonstrates the dark recovery of cytochrome *f* in the leaf, with a halftime of 6 msec.

Figs. 1B and 1D show the kinetics of oxidation and reduction of cytochrome *f*

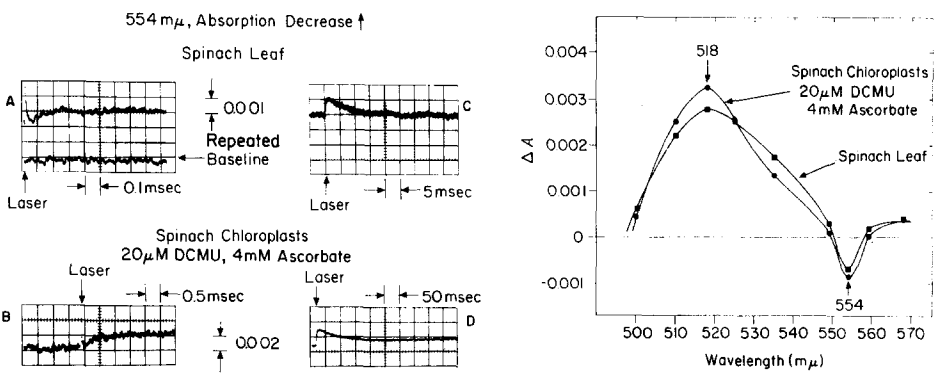

Fig. 1. The oxidation and dark recovery of cytochrome *f*, following a Q-switched ruby laser flash, in spinach leaves (H) and chloroplasts (1.8 mg chlorophyll/ml). $3 \cdot 10^{-9}$ Einstein/cm² at 694.3 mμ; 1.6 mm optical path. Instrument time constants about 10 μsec (Fig. 1A), about 30 μsec (Fig. 1B), about 100 μsec (Fig. 1C), about 1 msec (Fig. 1D).

Fig. 2. Spectra of the laser-induced absorption changes in the spinach leaf (H) and chloroplasts (0.9 mg chlorophyll/ml). The extent of the intermediate (I) recovery phase is plotted for the 518 mμ band.

for the isolated chloroplasts (H). In this case, 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is added to prevent activation of System II of the photosynthetic electron transport chain, by the laser flash at 694 mμ. Ascorbate serves as a dark reductant for cytochrome *f*. The oxidation halftime (Fig. 1B) is 300 μsec, a factor of 3 slower than in the leaf. The recovery halftime is 50 msec (Fig. 1D), a factor of 8 slower than in the leaf. Since photoreduction of cytochrome *f* by System II is blocked by DCMU, the latter recovery represents chemical reduction by the added ascorbate.

A short lag can be detected between the instant of the laser flash and the start of first-order oxidation of cytochrome *f*, in Figs. 1A and 1B. Averaged over 40 photographs, the lag amounted to 25 μsec in the leaf, and 80 μsec in the chloroplasts. This lag may be merely an artifact, from a slight undershoot in the recovery of the photomultiplier from the laser flash. A contributing factor may be a limitation in the instrument response time, which is, for example, 3 times longer in Fig. 1B than in Fig. 1A (see figure legends). Alternatively, the lag may indicate an additional electron carrier (possibly plastocyanin) between cytochrome *f* and the photooxidized trap of System I[7,8]. Neither of these two hypotheses can be eliminated by the present data. The essential problem is that decreasing the instrument response time also decreases the signal-to-noise ratio to the point where accurate measurements of lag and halftime cannot be made.

Fig. 2 presents spectra of the laser-induced absorption changes in the spinach leaf and chloroplasts (H). The α-band of cytochrome *f* is apparent, as well as the broad 518 mμ band, to be discussed later.

The cytochrome *f* oxidation kinetics are summarized in Table I. It will be observed that the market spinach used in identical experiments two years previously (second row, Table I) gave a similar cytochrome *f* oxidation rate for the leaf, but a slower rate by a factor of four for the isolated chloroplasts[3]. The question therefore arose whether the discrepancy was due to a difference in the variety of market spinach used at the two different times, or to some difference in preparation of the chloroplasts.

Hence, the same experiments were carried out on freshly grown Swiss chard and Viroflay spinach leaves and chloroplasts, the results of which are summarized in the last two rows of Table I. The fastest cytochrome *f* oxidation rates are observed in the Viroflay and Swiss chard leaves, $t_{1/2} = 60$ to 90 μsec; however, the rates in the isolated chloroplasts are slower by a factor of 100. Since all chloroplasts were prepared by the same technique[4], the results of Table I indicate a significant sensitivity of the chlorophyll–cytochrome *f* interaction to the isolation procedure for different types of leaves (see DISCUSSION).

TABLE I

HALFTIME FOR CYTOCHROME *f* OXIDATION, FOLLOWING A Q-SWITCHED LASER FLASH

	Intact leaf (μsec)	*Chloroplasts, with 20 μM DCMU, 4 mM ascorbate* (μsec)
Market spinach (probably Hybrid No. 7)	110 ± 40	300 ± 100
Market spinach (probably Bloomsdale)	150 ± 100	1300 ± 300
Swiss chard	90 ± 40	8500 ± 2500
Viroflay spinach	60 ± 30	9000 ± 3000

Preliminary experiments on the temperature dependence of cytochrome *f* oxidation show that in all types of leaf tested, the rate at 0° is slower by a factor of about 3 than the rate at 22°. As previously reported[3], the rate in market spinach chloroplasts (B) is slower by a factor of 2 at 0°. The latter value holds for all types of chloroplasts tested.

Fig. 3 depicts the results of an experiment under light-limited conditions, the laser beam being attenuated, *via* negative lens and neutral density filters, to the incident energies indicated on the graph. From the resulting cytochrome *f* absorption changes, the following quantum requirements can be calculated: 3 ± 1 for market spinach chloroplasts (H) and for Viroflay spinach chloroplasts; 6 ± 2 for the corresponding intact leaves. In the calculation we used the extinction coefficient of 20 mM^{-1} cm^{-1} for cytochrome *f* at 554 mμ[1], and the value of 95 to 98 % of the laser light at 694 mμ absorbed by either the chloroplasts (1.3 mg chlorophyll per ml) or leaf sample. The latter absorptivity values were measured directly in the above-described apparatus, and were corrected for scattering of the sample by a Beer's Law determination.

The 518 mμ absorption change

The absorption increase at 518 mμ following a laser flash occurs with the half-time, $t_{1/2} \leqslant 0.3$ μsec, that is, less than the response time of the apparatus[3]. The subsequent dark recovery proceeds with three distinct phases, as shown in Fig. 4 and summarized in Table II, for the spinach leaf and chloroplasts (H). The rapid (R) recovery phase is similar in both leaf and chloroplasts (Figs. 4A and 4D). The intermediate (I) phase occurs as a plateau in the kinetics of the leaf (Fig. 4B) and as a partial recovery in the chloroplasts (Fig. 4E). The spectra of the 518 mμ band in leaf and chloroplasts, presented in Fig. 2, refer to the I phase. For spectra of the R and S phases, see ref. 3.

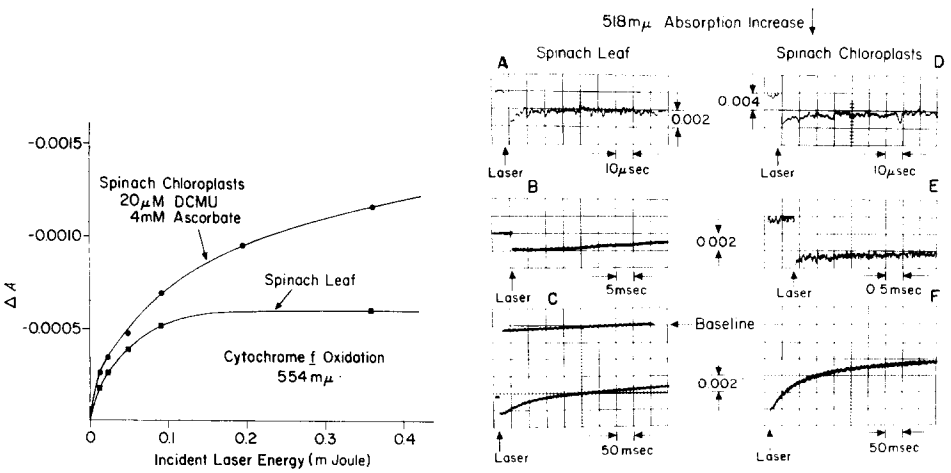

Fig. 3. The extent of cytochrome *f* oxidation under light-limited conditions, for spinach leaf (H) and chloroplasts (1.3 mg chlorophyll/ml).

Fig. 4. The 3 recovery phases of the 518 mμ absorption change in spinach leaf (H) and chloroplasts (1 mg chlorophyll/ml), following a laser flash. Instrument time constants about 1 μsec (Figs. 4A and 4D), about 3 μsec (Fig. 4E), about 30 μsec (Figs. 4B and 4C), about 100 μsec (Fig. 4F).

The final recovery phase (S) is similar in both leaf and chloroplasts (Figs. 4C and 4F) with the following qualification.

After the first laser flash on the leaf, the final recovery halftime at 518 mμ is $t_{1/2} = 10$ msec. Following about four flashes, the final halftime increases to the tabulated value of 75 msec. This observation is interpreted as caused by exhaustion of endogenous substrate in the leaf following flash activation. This hypothesis was verified by exposing the leaf to dim white light and CO_2-enriched atmosphere, which restored the original 518 mμ recovery rate. The same phenomenon was observed in all types of intact leaf tested. The chloroplasts, on the other hand, showed the slower final recovery rate after all laser flashes (Table II). No experiments with dim illumination *plus* CO_2 have yet been attempted with the isolated chloroplasts.

TABLE II

THREE PHASES IN THE RECOVERY OF THE 518 mμ ABSORPTION CHANGE, FOLLOWING A FLASH FROM THE Q-SWITCHED LASER

Phase	*Intact leaf (market spinach)*	*Chloroplasts (market spinach)*
R phase halftime	3.5 ± 1.0 μsec	4.0 ± 1.5 μsec
I phase halftime	—	300 ± 200 μsec
S phase halftime	75 ± 20 msec (following 4 flashes)	100 ± 50 msec

DISCUSSION

Cytochrome f

The chief conclusion from the present work is that the primary electron transfer from cytochrome *f* to the System I trap occurs with a similar rate in the four types of intact leaves examined. However, the corresponding rate in the isolated chloroplasts is slower by factors of 3 to 100 depending on the particular type of plant. A varying integrity of the chlorophyll–cytochrome *f* relationship, following isolation of chloroplasts has already been postulated to explain the data. Alternately, a reducing pool between System II and cytochrome *f* becomes uncoupled upon isolation of chloroplasts to a different extent in different types of plants. This reducing pool can then compete with the laser-induced oxidation of cytochrome *f*, to give slower apparent rates of oxidation in the chloroplasts, as shown in Table I (*cf.* the discussion following ref. 9).

Analogous results have been obtained with photosynthetic bacteria, using the laser flash technique[10]. Thus, *Chromatium* D cells show a halftime for cytochrome *c* oxidation of 2.3 μsec[6], while the chromatophores also have this extremely fast rate, $t_{1/2} = 2$ μsec. On the other hand, *Rhodospirillum rubrum* cells have a halftime for cytochrome *c* oxidation of 25 μsec, while the chromatophores have a halftime of 10–20 msec, that is, a factor of 500 slower[11].

The relatively high quantum requirements, that is, 3 for chloroplasts and 6 for leaves, can be partially explained by the fact that a fraction of the laser quanta at 694 mμ is absorbed by System II. The latter quanta are, therefore, ineffective for cytochrome *f* oxidation. However, absorption by System II would account for roughly

only one half of the incident quanta, leaving a discrepancy from the theoretical quantum requirement of unity.

518 mμ absorption change

The distinctive feature in the 518 mμ kinetics of the intact leaf is the intermediate (I) phase, which occurs as a plateau with a duration of about 10 msec (Fig. 4B). This phenomenon is to be compared with the 518 mμ kinetics in whole cells of Chlorella (Fig. 9, ref. 3). In the latter case, the I phase occurs as an additional increase in absorption with a halftime of about 2 msec following the R phase recovery. The final S phase recovery then occurs in Chlorella with a halftime of about 100 msec.

As postulated in ref. 3, the plateau in the 518 mμ kinetics of the leaf may be maintained by the simultaneous decay of a secondary reactant. This secondary reactant does not contribute to the 518 mμ absorption change, so that the kinetics observed at 518 mμ show only a 10 msec delay before the final recovery (Fig. 4C).

Evidence has been presented elsewhere[3,13] that the rapid (R) recovery phase of the 518 mμ absorption change is associated with a photooxidation, possibly involving carotenoids.

The S recovery phase most probably represents a process distinct from that causing the R phase at 518 mμ. The evidence is as follows: (1) differences in spectral peaks[14,3]; (2) temperature independence of the R phase and dependence of the S phase on temperature[15,12,3]; (3) difference in sensitivity to chemical inhibitors[16,3]. Furthermore, the S phase is most probably related to a photoreduction[3,15], in contrast to the above postulation for the R phase.

ACKNOWLEDGEMENTS

The author is greatly indebted to Dr. B. CHANCE for providing the original motivation for this work and for helpful criticisms of the manuscript. The author also acknowledges valuable discussions with Drs. D. DEVAULT, W. PARSON, J. BIGGINS, H. SCHLEYER, and M. NISHIMURA.

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