Photosynthesis V. Chloroplast Development
Edited by George Akoyunoglou
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QUENCHING OF THE CHLOROPHYLLIDE FLUORESCENCE BY A SHORT-LIVED INTERMEDIATE IN THE PHOTOREDUCTION OF PROTOCHLOROPHYLLIDE.

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ABSTRACT

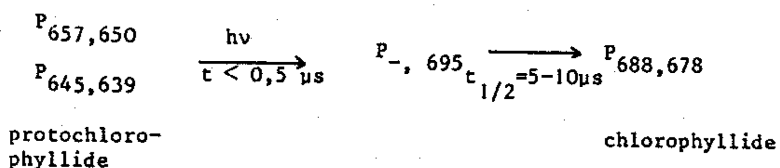
We have recorded the variations of absorption and fluorescence after a flash illumination of an etiolated leaf. This has been performed in fresh leaves frozen at -60°C and in lyophilised leaves at room temperature. In such conditions, a non-fluorescent, long-wavelength-absorbing intermediate was detectable immediately after the flash; it was converted into fluorescent chlorophyllide in darkness after the flash. Using low intensity flashes, we found a quenching of the chlorophyllide fluorescence immediately after the second flash. This fluorescence quenching was concomittent with the presence of the intermediate; it was reversible in darkness during the conversion of the intermediate into a new amount of chlorophyllide. We concluded that the non-fluorescent, long-wavelength-absorbing intermediate is a quencher of the chlorophyllide fluorescence.

INTRODUCTION

Recent studies have shown that at least one short-lived intermediate product is involved in the photoenzymatic reduction of protochlorophyllide into chlorophyllide.

At room temperature, Franck and Mathis[1] have found an intermediate product in an extract 0,5μs (their time resolution) after a short laser flash; it has a main absorption band at 695 nm and it transforms in darkness into the 676 nm absorbing chlorophyllide with a half-time of 5-10μs. Similar results have been obtained by Inoue and al[2] in fresh leaves at room temperature. On the other hand, the room temperature emission spectrum of an etiolated leaf at the end of a 0,5μs saturating flash shows only weak fluorescence

bands in the chlorophyllide region; an intense chlorophyllide fluoresc at 688 nm is seen after a few seconds darkness[3]. Inoue and al[2] have shc that the half-time of the chlorophyllide fluorescence increase was a fe µs in fresh leaves at room temperature. No fluorescence was found to be attributable to the 695 nm absorbing intermediate. From these results, following chart may be written :



(The first subscript refers to the fluorescence maximum of the pigment; the second subscript refers to its red absorption maximum).

By cooling the leaves, it is possible to slow down the dark transformation of the $P_{-,695}$ intermediate into the $P_{688,676}$ chlorophyll At -35°C, the half-time of the 688 nm fluorescence increase is 60 ms in a fresh leaf[4]; at temperatures lower than -150°C, this transformatio is completely inhibited and $P_{-,695}$ is stabilized[5,6]. The life-time of tl intermediate is also strongly increased when the leaf is lyophilized; in the lyophilised leaf, the major part of the protochlorophyllide remai photoreducible and the half-time of the chlorophyllide fluorescence development is 70 ms at room temperature[4].

In this paper, we have recorded the variations of the 688 nm fluorescence induced by a photographic flash (0,1 ms or 5 ms) with a time resolution in the ms range. This has been performed for a series of non-saturating flashes in fresh leaves at -60°C and in lyophilised leave at room temperature. Our main conclusion is that the $P_{-,695}$ intermediate is a quencher of the $P_{688,676}$ chlorophyllide fluorescence.

MATERIAL AND METHODS

Plant material

Primary bean leaves (*Phaseolus vulgaris* var. Commodore) grown in complete darkness were used. Lyophilisation was made using a Virtis lyophilisator according to a method already described[7].

Fluorescence emission kinetics

We recorded the fluorescence emission at 690 nm before and after an actinic blue flash. The fluorescence was excited by an analytical light provided by a He-Ne laser (3×10^{-3} W). For rapid measurements, the

duration of the actinic flash was 3×10^{-4}s and the analytical light was given continuously during 10 ms. For slower measurements, the duration of the actinic flash was 5×10^{-3}s and the analytical light was discontinuous : short analytical pulses (4×10^{-5}s) separated by 8×10^{-3}s dark intervals were given during 1 s. In both cases, the analytical light had no measurable effect on the photoreduction.

The actinic blue light was given as a single flash from a Xenon lamp. The 690 nm fluorescence emission was selected by a Balzer filter (10 nm wide at half-peak) and detected by a PM EMI 9558 B.

Absorption kinetics

We recorded the absorption variations induced by the actinic flash in the same physical conditions than those used for fluorescence measurements. The device used for this purpose has already been described[8]; the time resolution was 40 ms; in these conditions only slow measurements were possible (full scale : 1 s).

Cooling device

The leaf and the light guides rods were introduced in a special metal cuvette through which an adjustable flow of nitrogen vapor was circulating; the temperature at the level of the leaf was controlled by means of a thermocouple.

RESULTS

A. Fresh leaf frozen at -60°C

We have recorded the variations of absorption (ΔA) and fluorescence (ΔF) of etiolated leaves induced by each flash of a series of non-saturating flashes at -60°C. Each flash induced the reduction of 9 to 13% of the protochlorophyllide molecules, depending on the leaf, as measured by the absorption decrease at 650 nm.

Fig. 1 shows the absorption kinetics observed at the first three flashes separated by two 1 min dark periods. The variations of absorption were recorded at 650 nm for the protochlorophyllide $P_{657,650}$, at 680 nm for the chlorophyllide $P_{688,676}$ and at 700 nm for the intermediate $P_{-,695}$. Fig. 2 shows the variations of fluorescence at 690 nm emitted by the chlorophyllide $P_{688,678}$. A first leaf was used for the ΔF at 690 nm, a second one for the ΔA at 650 nm and a pair of opposite leaves for the ΔA at 680 and 700 nm.

The absorption kinetics at 650 nm show that each flash induced the reduction of a certain amount of protochlorophyllide; the 650 nm

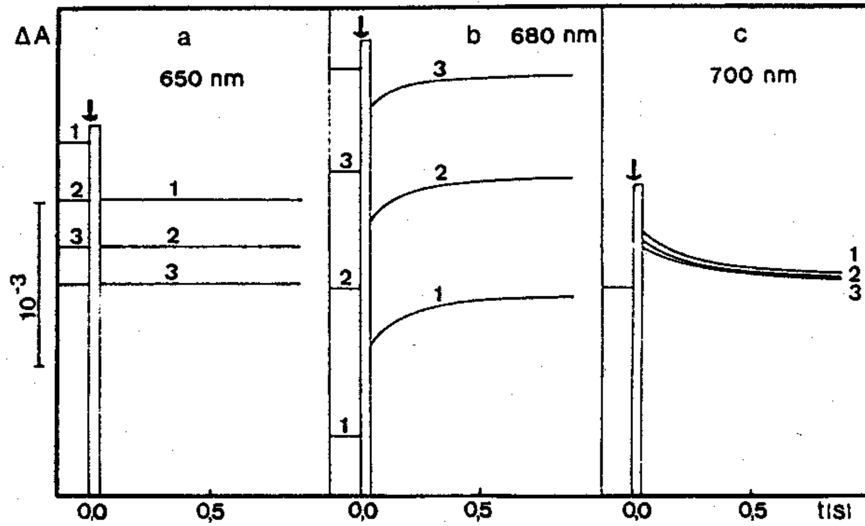


Fig. 1 : Absorption changes at 650, 680 and 700 nm in fresh etiolated leaves at -60°C; first, second and third flash (arrow).

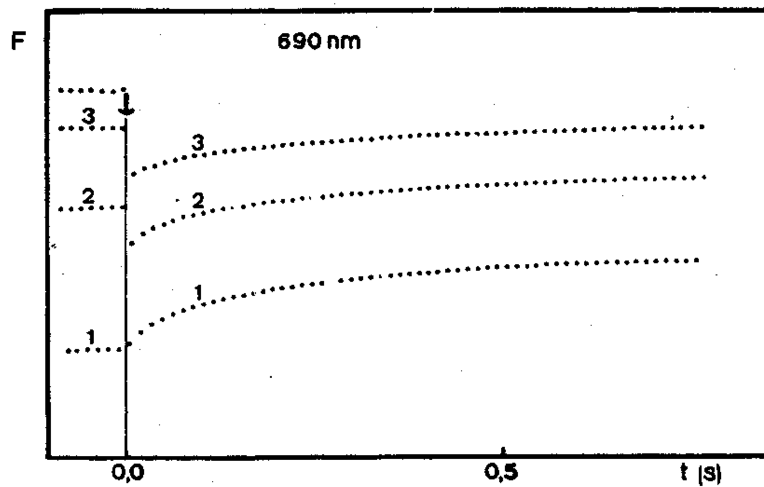


Fig. 2 : Fluorescence changes at 690 nm in a fresh etiolated leaf at -60°C; first, second and third flash (arrow).

absorption decrease at the first flash corresponded to 9 to 13 % of the total protochlorophyllide absorption, depending on the leaf. The absorption decrease was completed in less than 40×10^{-3}s, the time resolution of the apparatus; the absorption remained stationnary afterwards.

The absorption kinetics at 680 nm (chlorophyllide region) showed an initial increase (faster than 40×10^{-3}s) followed by a slow increase with a half-time of 2×10^{-2}s (fig. 1,b); the initial ΔA represents 65 % of the total ΔA. At 700 nm (intermediate region), an initial absorption increase (faster than 40×10^{-3}s) was followed by a slow reversion whose shape was roughly complementary to the slow 680 nm absorption increase; the half-time of this reversion is about 2×10^{-2}s (Fig. 1,c). In both cases (680 nm and 700 nm), the absorption reached a stationnary level after a few s.

From one flash to another, the shape of the absorption kinetics at 650, 680 and 700 nm remained the same but the amplitude of the variations decreased.

Fig. 1 d shows the fluorescence kinetics at 690 nm (chlorophyllide region) recorded in the same conditions than the absorption kinetics; the time resolution was 5×10^{-3}s. At the first flash, the fluorescence intensity was the same immediately after the flash than before the flash; it increased slowly after the flash with a half-time of 3×10^{-2}s and reached a stationnary value after 15 to 20 s, the increase becoming very slow after 2 s. The second flash induced an immediate fluorescence decrease or quenching, completed in less than 5×10^{-3}s; this quenching was reversible within 1 s after the flash. After this reversion, the fluorescence intensity continued to increase smoothly; it reached a stationnary value after 15-20 s which was higher than the stationnary value found before the second flash. The half-time of the whole fluorescence increase was 3×10^{-2}s, a value which was found also at the first flash. At the third flash a similar kinetic was found but the amplitude of the fluorescence quenching was greater than that after the second flash.

The kinetics recorded at the subsequent flashes show that the absolute amplitude of the fluorescence quenching at 690 nm increased with the order of the flash till the 8th flash; than it decreased and became negligeable after 120 flashes. On the other hand, the stationnary intensity of the fluorescence before the flash increased progressively as chlorophyllide $P_{688,678}$ is accumulating; this increase becomes undetectable after 90 flashes.

B. Lyophilised leaf at room temperature

In the lyophilised leaf at room temperature, the variations of absorption due to protochlorophyllide photoreduction were too fast to be recorded using our device. However, we were able to follow the development of the fluorescence at 690 nm due to the transformation of the non-fluorescent intermediate $P_{-,695}$ into chlorophyllide $P_{688,678}$; each actinic flash was able to reduce about 7 % of the initial photoreducible protochlorophyllide.

Fig. 3a shows the fluorescence kinetics at 690 nm in the range with a time resolution of 10^{-2}s. The first flash was followed by an increase of the fluorescence which occured in a biphasic way. The rapid phase was completed in a time shorter than our time resolution (10^{-2}s); the slow phase has a half-time of 7×10^{-2}s. At the second flash, a similar kinetic was found but the relative amplitude of the rapid phase decreased. At the third flash no rapid phase was detectable using this method : the intensity of the fluorescence was the same 10^{-2}s after the flash than before. This situation occured again at all subsequent flashes until all protochlorophyllide was reduced; the intensity of the fluorescence never decreased after the flash.

Using a weak and continuous analytical light, we were able to follow the course of the rapid phase of the fluorescence increase at 690 nm. We recorded the fluorescence during 10 ms after the three first

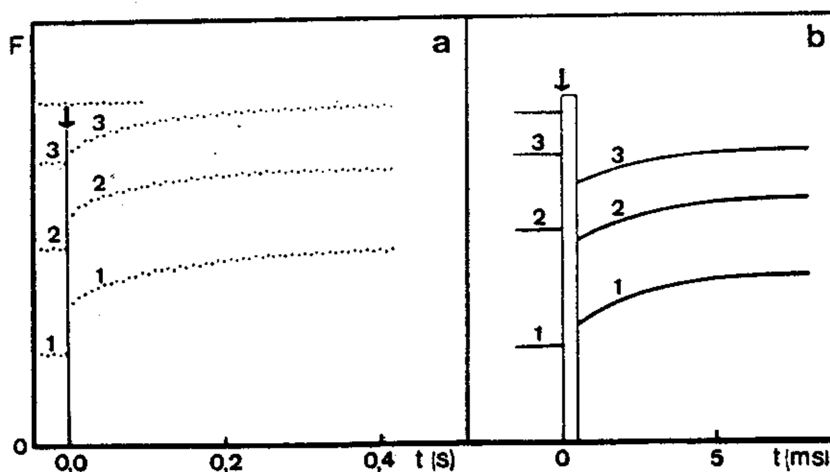


Fig. 3 : Fluorescence changes at 690 nm in lyophilised etiolated leaves at room temperature; first, second and third flash (arrow); a : slow measurements; b : rapid measurements.

actinic flashes separated by a dark period of 30 s; the time resolution was 0,5 ms. After each flash, the slow phase of the fluorescence increase occured in complete darkness and the final stationary intensity was recorded immediately before the following flash. These kinetics are shown in fig. 3b.

The intensity of the fluorescence was somewhat higher 0,5 ms after the flash than before. Then the rapid phase occured with $t_{1/2} \approx$ 1,5 ms; its whole duration was 6-8 ms. The second flash induced a reversible quenching of the fluorescence : the fluorescence intensity first decreased within our time resolution then it increased according to a kinetics similar to the rapid phase observed at the first flash. This reversion was completed in 2-3 ms; the fluorescence continued to rise thereafter. At the third flash, a similar behaviour was found but the amplitude of the fluorescence quenching was higher than at the second flash and its reversion was completed in a time roughly equal to the whole duration of the rapid phase (6-8 ms).

DISCUSSION

Franck and Mathis[1] estimated the life-time of the intermediate $P_{-,695}$ in an extract at different temperatures between +21°C and -50°C by measuring the half-time ($t_{1/2}$) of the absorbance increase at 675 nm due to chlorophyllide formation. They found that, at room temperature, $t_{1/2} \approx 8$ µs and that, at -50°C, $t_{1/2} \approx 110$µs; a value of ≈ 150 µs should be obtained at -60°C (extrapolation of Fig. 7 in ref. 1). We have recorded the absorbance increase at 680 nm due to chlorophyllide $P_{688,678}$ formation in fresh leaves at -60°C and we have found that 70 % of the increase was completed in 40 ms, our time resolution (fig. 1,b); the extrapolation of the kinetic towards shorter times permits to estimate $t_{1/2}$ roughly between 20 and 30 ms. This indicates that the life-time of the intermediate $P_{-,695}$ is probably at least one-hundred times greater in the fresh leaf than in the extract at -60°C. This result shows that the behaviour of $P_{-,695}$ is highly dependent on the membrane characteristics, a conclusion also suggested by the fact that the intermediate is much more stable in lyophilised leaves than in fresh leaves at room temperature[4].

In the etiolated fresh leaf at -60°C, the fluorescence at 690 nm increases after the first flash while the intermediate $P_{-,695}$ is converted into the fluorescent chlorophyllide $P_{688,678}$ as shown by the comparison of the fluorescence kinetics at 690 nm (fig. 2, curve 1) and

the absorption kinetics at 680 and 700 nm (fig. 1, b and c, curves 1). However, the t1/2 of the chlorophyllide fluorescence increase is 300 ms while the t1/2 of the chlorophyllide absorption increase is shorter than 40 ms, our time resolution.

When the leaf receives the second flash, 9 to 13 % of the total potential chlorophyllide has already been accumulated as a consequence of the first flash. Then, the second flash induces an immediate quenching of the chlorophyllide fluorescence (fig. 2, curve 2). During some seconds following the flash, one observes the reversion of this quenching, followed by the appearance of an additionnal amount of fluorescence, while the intermediate is converted into a new amount of chlorophyllide.

We interpret these facts by assuming that the intermediate $P_{-,695}$ is a quencher of the fluorescence emitted by chlorophyllide $P_{688,673}$. This also explains that the fluorescence of chlorophyllide appears much more slowly than its absorption at the first flash. The amplitude of the quenching increases at the third flash because the quantity of chlorophyllide accumulated prior to the flash is nearly two times greater than at the second flash, while nearly the same quantity of intermediate is produced, as the flashes are far from saturation. When all protochlorophyllide has been reduced, no quenching can be observed anymore as no intermediate can be formed.

In the lyophilised leaf, we were able to observe the quenching of the chlorophyllide fluorescence due to the intermediate formation at room temperature. In such conditions, the reversion of this quenching is completed in a few ms only and it corresponds to the rapid phase of the fluorescence increase. The occurence of two well-defined phases (rapid and slow) in the lyophilised leaf at room temperature indicates that the conversion of the intermediate into fluorescent chlorophyllide is a complex reaction.

By illuminating fresh leaves in liquid nitrogen, Dujardin and Correia[9] found that a 690 nm absorbing pigment was produced; simultaneously they observed a decrease of the protochlorophyllide fluorescence. A partial restoration of the protochlorophyllide emission was obtained by heating the sample from 77 K to 220 K, as the 690 nm absorbing pigment was converted into chlorophyllide. They concluded that the 690 nm absorbing pigment was an intermediate step in the reduction of protochlorophyllide and that it was a quencher of the fluorescence emitted by protochlorophyllide. Our results show that the same or a similar intermediate is a quencher of the fluorescence emitted by the newly formed chlorophyl-

lide. This shows that these intermediate pigment-protein complexes are efficient traps for the energy absorbed by all the neighbouring pigments.

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