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KINETICS OF CHLOROPHYLL FLUORESCENCE AT 77 K IN *CHLORELLA* AND CHLOROPLASTS

EFFECTS OF CCCP, FERRICYANIDE AND DCMU

F. BONNET, C. VERNOTTE, J-M. BRIANTAIS and A-L. ETIENNE

Laboratoire de Photosynthèse du CNRS, 91190 Gif-sur-Yvette (France)

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SUMMARY

The kinetics of chlorophyll fluorescence at 77 K were studied in *Chlorella* cells and spinach chloroplasts.

During a first illumination, the rise is polyphasic with at least three phases. The slowest one is irreversible and corresponds to the cytochrome oxidation.

The dark regeneration of half the variable fluorescence is biphasic, the fast phase being inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) both in *Chlorella* and chloroplasts.

The fluorescence rise during a second illumination is still biphasic.

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) slows down the fluorescence rise in *Chlorella* but has no effect on the dark regeneration. It does not affect the fluorescence of chloroplasts.

Ferricyanide which oxidizes cytochrome *b*-559 at room temperature produces a quenching of the variable fluorescence and an acceleration of the fluorescence rise during the first illumination.

Our results fit the idea of the heterogeneity of the Photosystem II centers at low temperature.

INTRODUCTION

At 77 K, the electron donation to the reaction center chlorophyll (*P* or *P*-680) of Photosystem II is much slower than at room temperature [1]. $P^+ Q^-$ being a quencher of fluorescence (Q: primary electron acceptor) the fluorescence kinetics are slower than the kinetics of Q reduction (as followed by the absorption changes at 550 nm [1]). Whereas at room temperature most electrons come from water, at 77 K several ultimate electron donors are present. Two donors at least are necessary to take into account most experimental results.

Abbreviations: DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP: carbonyl cyanide-*m*-chlorophenylhydrazone.

Different schemes have been proposed by Vermeglio [2]: (1) A model with two photochemical reactions in series; (2) A model with two photochemical reactions in parallel. This latter model presented by Vermeglio [2] was also proposed by Visser with slight modifications [3].

It can be described as follows:

$$\text{cytochrome } b\text{-559 ZPQ} \underset{k_{-1}}{\overset{k^*I}{\rightleftharpoons}} \text{cytochrome } b\text{-559 ZP}^+\text{Q}^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{cytochrome } b\text{-559 Z}^+\text{PQ}^- \overset{k_3}{\rightarrow} \text{cytochrome } b^+\text{-559 ZPQ}^-$$

$$\text{D}_2\text{ZPQ} \underset{k_{-1}}{\overset{k^*I}{\rightleftharpoons}} \text{D}_2\text{ZP}^+\text{Q}^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{D}_2\text{Z}^+\text{PQ}^- \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{D}_2{}^+\text{ZPQ}^-$$

k^*: photochemical rate constant; I: intensity absorbed. Between P and the ultimate donors (cytochrome *b*-559 for half of the centers and D_2 (unidentified donor) for the other half), Visser has introduced an intermediary donor Z in fast equilibrium with P^+ to explain the fluorescence rise (20 % of the variable fluorescence) which occurs within 20 μs after a flash [4] and is much faster than the cytochrome oxidation.

The cytochrome oxidation is irreversible. The oxidized D_2 can be slowly reduced in the dark through a back reaction with Q^-.

In the light of this model, we have studied the fluorescence kinetics during a continuous illumination, the restoration of a variable fluorescence as a function of time after cessation of this illumination and the fluorescence kinetics during a second illumination. Ferricyanide and carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) were used to modify the kinetics at 77 K. Ferricyanide oxidizes cytochrome *b*-559 at room temperature [5]. High concentrations of CCCP inhibit the back-reaction in *Chlorella* [6] and chloroplasts [7], it affects the potential of cytochrome *b*-559 [8] and has several other complex interactions with the donor side [9, 10].

We also studied the effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on the fluorescence decay remaining at 77 K, a temperature at which the electron donation from Q to the plastoquinone pool is not supposed to occur.

MATERIALS AND METHODS

Both chloroplasts and *Chlorella* were used in this study. *Chlorella* were grown and harvested as previously described. Before use, they were centrifuged and resuspended at a concentration of 50 μg chlorophyll total per ml in 0.05 M phosphate buffer containing 10^{-1} M KCl at pH 6.5.

Spinach chloroplasts were prepared as previously described [11] and suspended at 50 μg chlorophyll total/ml in a pH 7.8 tricine buffer. Samples of 0.3 ml are spread over a disk of cheese cloth rigidly positioned on the bottom of a dewar flask. The sample is maintained for a least 1 min in complete darkness before liquid nitrogen is poured into the dewar. CCCP or DCMU are added when needed to the samples at room temperature in the dark. The incubation time is 10 min at least. The fluorescence excited by a blue actinic light (Corning CS 4-96) is collected by a light guide and detected at wavelengths higher than 660 nm (Corning CS 2-64+Wratten 29) by means of an EMI photomultiplier. At 77 K the fluorescence kinetics are controlled only by Photosystem II activity, as shown by Kitajima and Butler [12]. The fluorescence

induction is recorded with a multichannel analyzer (Datalab). The samples can also be illuminated with white saturating 5 μs flashes (General Electric Strobotac) or 250 ms white flash using a mechanical shutter.

Most fluorescence studies at 77 K are done in correlation with absorption changes measurements which require a concentrated sample. The observed kinetics are therefore a mixture of fast and slow kinetics. In our case, care was taken for the homogeneity of the illumination and except for internal reabsorption within each cell or chloroplast, true fluorescence kinetics are detected.

RESULTS

1. Chlorella

(*a*) *Fluorescence rise during the first and the second continuous illuminations.* For dark adapted cells, the fluorescence rise is strongly polyphasic (Fig. 1) with at least three phases as shown by the semi-logarithmic decomposition (Fig. 2, a and b). The ratio of the half time of the slow component to that of the remaining fast phase is about 40 to 50.

Only 50 % of the variable fluorescence is restored in the dark. The fluorescence rise during the second illumination is faster than the first one (Fig. 1), it is also polyphasic (Fig. 2b). On a semi-logarithmic plot, the second induction is superimposed on the curve obtained from the first induction after subtraction of its slowest exponential component (Fig. 2, a and b).

(*b*) *Kinetics of the dark restoration.* The dark restoration of the variable fluorescence is slow. Its kinetics, detected by varying the dark period between the two illuminations, is biphasic (Fig. 3). This restoration is completed within 30 to 40 min.

(*c*) *CCCP effect on the fluorescence kinetics.* The addition of 10^{-5} M CCCP does not change the initial and maximum fluorescence yields observed during the

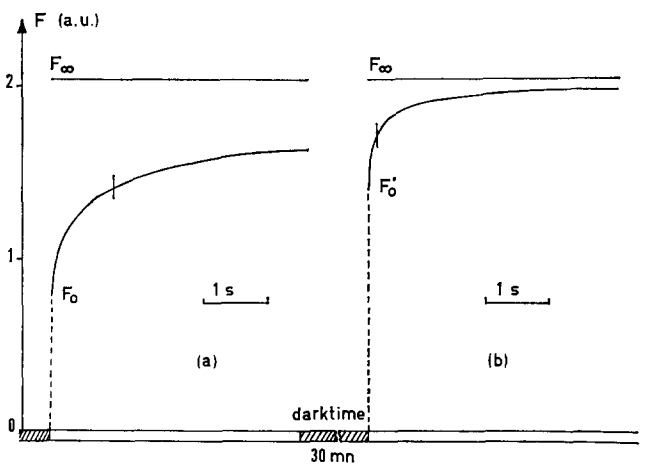

Fig. 1. Curve a: fluorescence induction at 77 K of dark adapted *Chlorella*. Exciting light intensity, 6000 ergs $cm^{-2}s^{-1}$. Curve b: fluorescence induction at 77 K during a second illumination after a dark time of 30 min. The initial fluorescence levels F_0, F'_0 and the maximum fluorescence level F_∞ are indicated for both inductions. Vertical bars show the half-time.

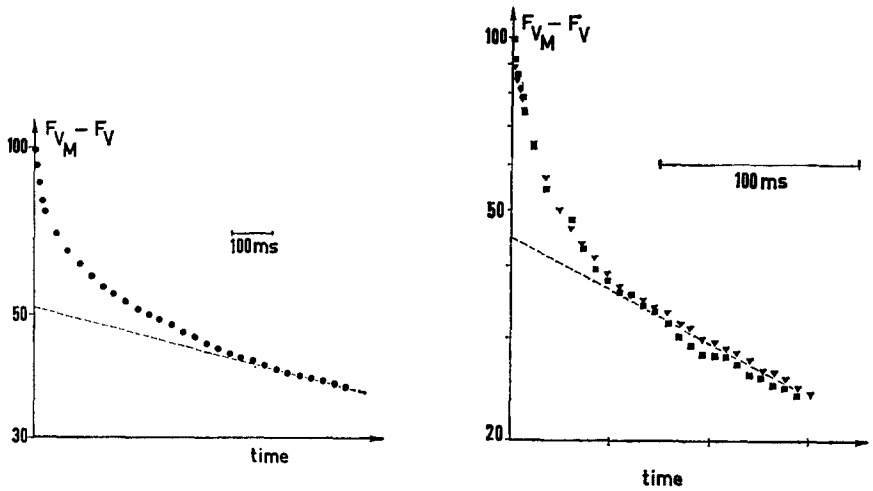

Fig. 2. (a): Semi-log plot of the induction of dark adapted *Chlorella*. Exciting light intensity, 16 000 ergs $cm^{-2}s^{-1}$. The maximum variable fluorescence is normalized to 100. (b): ■, semi-log plot of the second induction. The maximum variable fluorescence is normalized to 100. ▼, semi-log plot of the fast phase of the first induction. The maximum variable fluorescence remaining after subtraction of the slowest phase is normalized to 100.

first or second illumination. The kinetics and amplitude of the dark restoration of the variable fluorescence are not affected by CCCP. However, in the presence of CCCP, the rate of the fluorescence rise is slower by a factor of 1.5 to 1.9 depending on the samples (Fig. 4). The rate constants of all phases seem to be decreased (Fig. 4). This is observed for all the light intensities used and is true also for the induction during the second illumination.

Such an effect can either be due to a change in the competition between the back-reaction (k_{-1}) and forward reaction, which favors the back-reaction or by a change in the photochemical rate k^*I. To discriminate between these two alternatives, we used the experimental procedure first described by Murata et al. [13]: after a

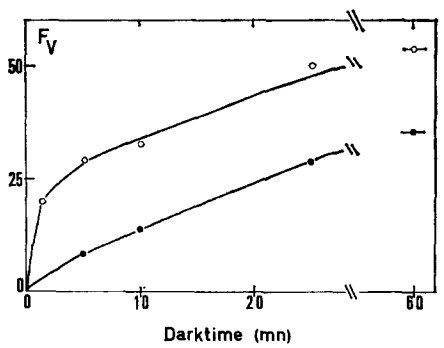

Fig. 3. Dark restoration at 77 K of the variable fluorescence. Exciting light intensity, 6000 ergs cm^{-2} sec^{-1}. The amount of variable fluorescence restored is plotted as a function of the dark time elapsed after the end of the first illumination. ○, untreated algae. ●, algae with 10^{-5}M DCMU.

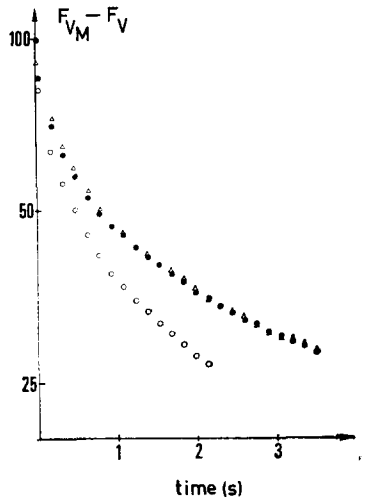

Fig. 4. Semi-log plot of the fluorescence induction of dark adapted *Chlorella* at 77 K. $I = 100$ corresponds to 9500 erg · cm^{-2} · s^{-1}; ○, induction with 100 % light intensity, no addition; ●, induction with 100 % light intensity, CCCP 10^{-5} M present; △, induction with 56 % light intensity, no addition.

short saturating flash, the remaining variable fluorescence: FV_1 will be proportional to the centers reopened by the back reaction, the difference between the maximum variable fluorescence FV_M and FV_1 will be proportional to the centers in a fluorescent state D^+ZPQ^-. Therefore the ratio $FV_1/(FV_M - FV_1)$ gives the ratio of the rate constants of back and forward reactions. It is not modified by CCCP (Table I). Several flashes can also be used. In agreement with Visser and Rijgesberg [14], we found that the first flashes (with or without CCCP) were more efficient in forming the permanent fluorescent states than the following ones. If, instead of short flashes, a longer strong illumination is used, the remaining variable fluorescence will only depend on the forward rate constants, k_{-1} being much smaller than k^*I. CCCP did not change the amount of variable fluorescence remaining after the long flash (Table I).

It seems therefore that CCCP decreases k^*I. Indeed if the light intensity is attenuated so that its ratio to the maximum light intensity is equal to the ratio of the

TABLE I

VARIABLE FLUORESCENCE AT 77 K AFTER *n* FLASHES FOR *CHLORELLA* WITH OR WITHOUT 10^{-5}M CCCP PRESENT

Flash duration	Number of flashes	Variable fluorescence	
		no CCCP	+CCCP
6 μs	0	100	100
	1	89.0±1.0	89.5±1.0
	5	80 ±1.0	78.4±1.0
	20	58.8±1.0	61.9±1.0
250 ms	1	50.4±1.0	52.4±1.0

TABLE II

VARIABLE FLUORESCENCE OF CHLOROPLASTS AND *CHLORELLA* UNDER IDENTICAL EXPERIMENTAL CONDITIONS

		Chlorella	Chloroplasts
Half-time of the fluorescence rise of dark adapted samples (arbitrary units)		1.0	0.38
Flash duration	Number of flashes	Variable fluorescence	
6 μs	0	100	100
	1	89	73.3
	5	80	58.6

half-time with or without CCCP: $I_{max}/I_{low} = (t_{\frac{1}{2}}+\text{CCCP})/(t_{\frac{1}{2}}+\text{no CCCP})$ then the two induction curves (maximum light intensity, +CCCP; attenuated light, no CCCP) are exactly matched on a semi-log scale (Fig. 4).

(*d*) *DCMU effect.* The addition of DCMU to dark-adapted cells slightly increases F_0, does not modify F_{max} and does not change the kinetics of the first fluorescence rise. It strongly affects the kinetics and amplitude of the dark restoration of the variable fluorescence: the fast phase is suppressed and only 35 % of the initial variable fluorescence is restored (Fig. 3).

II. Chloroplasts

(*a*) *Kinetics of untreated chloroplasts.* At 77 K the fluorescence kinetics of chloroplasts are similar to those of *Chlorella*: polyphasicity of the rise, biphasicity

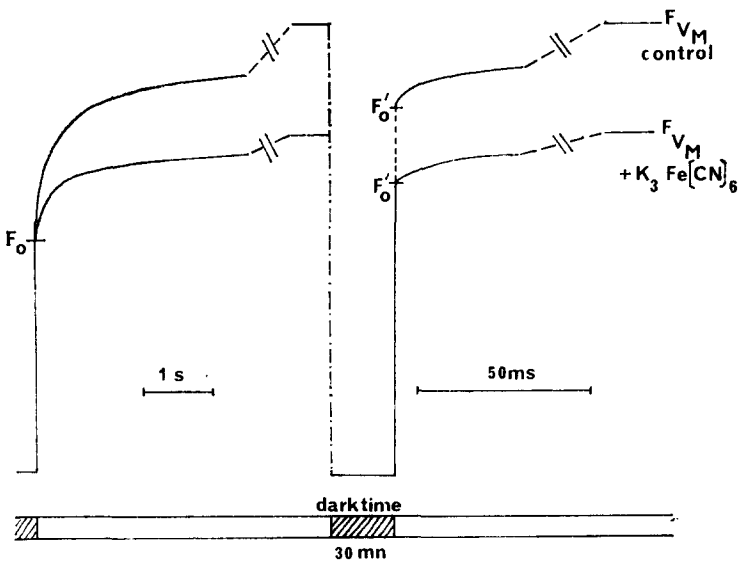

Fig. 5. Fluorescence induction of spinach chloroplasts during the first and second illumination (with a 30 min dark time in between). Exciting light intensity, 6 000 ergs · cm^{-2} · s^{-1}. Upper curves: no addition. Lower curves: with 1 mM $K_3Fe(CN)_6$.

of the dark restoration; however there are two main differences: (i) for identical light intensity and chlorophyll concentration, the fluorescence rise for chloroplasts is 2 or 3 times faster than for *Chlorella*; (ii) only 35 to 40 % of the variable fluorescence is restored in the dark. Table II shows the variable fluorescence remaining after some short saturating flashes. The values are much smaller than for *Chlorella*. In other words, the back reaction is less competitive and more centers are stabilized in a fluorescent state. This is in agreement with the faster kinetics of the rise.

(*b*) *DCMU effect.* It is identical to that observed with *Chlorella*: the fast phase of the dark restoration is inhibited and only 20 to 30 % of the variable fluorescence is restored.

(*c*) *CCCP effect.* The kinetic of the fluorescence rise is not modified. However, F_0 is increased; therefore only 80 % of the variable fluorescence is observed.

(*d*) *Ferricyanide effect.* With 1 mM ferricyanide present, only half the variable fluorescence is left (a red illumination is used so that ferricyanide does not decrease the incident light intensity). The initial level is unchanged. This was also found by Okayama and Butler [5] and by Visser [3].

With FeCN, the half time of the fluorescence rise during the first illumination is half that of untreated chloroplasts.

50 % of the variable fluorescence can be restored in the dark and the half time of the second fluorescence rise is identical to that of untreated chloroplasts (Fig. 5).

DISCUSSION AND CONCLUSION

Our results fit the idea of heterogeneity of the System II reaction centers at 77 K. The slowest phase of the first fluorescence rise must correspond to the irreversible cytochrome oxidation since it is not regenerated in the dark. The fluorescence rise during the second illumination is supposed to be only due to the oxidation of the unidentified donor D_2. A kinetic study of Visser's model shows that the ratio of the slopes of the slow components corresponds approximately to the ratio of the electron donation reactions: k_3, k_4. We found that k_4/k_3 was equal to 10 for *Chlorella*.

This fast equilibrium (k_2, k_{-2}) between fluorescing (Z^+PQ^-) and non-fluorescing (ZP^+Q^-) species provides an explanation for the fast phase observed at the beginning of the fluorescence rise. However, it is difficult to understand why the amplitude of that phase is not reduced during the second illumination. One has to suppose an heterogeneity of the centers also at the Z level.

The first phase of the dark regeneration suppressed by DCMU cannot be easily interpreted as reflecting a transfer of electrons from Q^- to the plastoquinone pool: firstly, A. Joliot has shown that this reaction was blocked at −60 °C [15]; secondly if it was due to a reoxidation of Q^- by the plastoquinone pool, a pathway different from the back reaction is needed for the reduction of D_2. Therefore it is more likely that there is also some kind of heterogeneity for the acceptor side and that DCMU has a stabilizing effect on some of the centers.

Several authors have studied the absorption changes and fluorescence kinetics of ferricyanide treated chloroplasts at 77 K [3, 4, 14]. The C-550 reduction is not modified. During the second illumination only half the C-550 change occurs as in untreated chloroplasts. To explain the effect observed on fluorescence, we have to assume in agreement with Visser that the chemically oxidized cyto-

chrome is replaced by another irreversible donor D_3. The donation is faster in that case, since the half-time of the fluorescence rise during the first illumination is two times smaller than that of untreated chloroplasts. The ferricyanide does not change the half time of the second fluorescence rise and therefore does not seem to affect the electron donation from D_2.

The fluorescence quenching observed in the presence of ferricyanide cannot simply be attributed to the oxidized donor D_3 which has been identified by Visser as an oxidized chlorophyll.

The effect of CCCP on the fluorescence kinetics at 77 K is very different from that occurring at room temperature. In chloroplasts, it does not change the fluorescence kinetics as could be expected if the cytochrome *b*-559 was in its low potential form unable to donate electron to System II; it does not inhibit the back reaction. In *Chlorella*, it does not inhibit the back reaction and we have shown that only a change in the photochemical rate constant k^*I could explain our data. CCCP can favor the competitive formation of a triplet state [16]. Other experiments will be needed to check that hypothesis.

The slower kinetics observed with *Chlorella* as compared with chloroplast kinetics show that in whole algae the back-reaction competes more efficiently with the electron donation than in chloroplasts. In conclusion, we have confirmed the existence of two electron donors which do not compete for the chlorophyll center reduction. We have shown that the centers are also heterogeneous on the acceptor side. Whether this heterogeneity exists at room temperature is still an open question.

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