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EFFECT OF LOW TEMPERATURE (−30 TO −60 °C) ON THE REOXIDATION OF THE PHOTOSYSTEM II PRIMARY ELECTRON ACCEPTOR IN THE PRESENCE AND ABSENCE OF 3(3,4-DICHLOROPHENYL)-1, 1-DIMETHYLUREA

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SUMMARY

The fluorescence yield has been measured on spinach chloroplasts at low temperature (−30 to −60 °C) for various dark times following a short saturating flash. A decrease in the fluorescence yield linked to the reoxidation of the Photosystem II electron acceptor Q is still observed at −60 °C. Two reactions participate in this reoxidation: a back reaction or charge recombination and the transfer of an electron from Q^- to Pool A. The relative competition between these two reactions at low temperature depends upon the oxidation state of the donor side of the Photosystem II center:

(1) In dark-adapted chloroplasts (i.e. in States S_0+S_1 according to Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475), Q, reduced by a flash at low temperature, is reoxidized by a secondary acceptor and the positive charge is stabilized on the Photosystem II donor Z. Although this reaction is strongly temperature dependent, it still occurs very slowly at −60 °C.

(2) When chloroplasts are placed in the S_2+S_3 states by a two-flash preillumination at room temperature, the reoxidation of Q^- after a flash at low temperature is mainly due to a temperature-independent back reaction which occurs with non-exponential kinetics.

(3) Long continuous illumination of a frozen sample at −30 °C causes 6–7 reducing equivalents to be transferred to the pool. Thus, a sufficient number of oxidizing equivalents should have been generated to produce at least one O_2 molecule.

(4) A study of the back reaction in the presence of 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) shows the superposition of two distinct non-exponential reactions one temperature dependent, the other temperature independent.

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

INTRODUCTION

The System II photoactive center may be represented as Z chlorophyll Q, where Z is a secondary electron donor and Q the primary electron acceptor described by Duysens and Sweers [1]. The dark reoxidation of Q^- following the photochemical step can occur by two possible reactions: by an electron transfer from Q to a secondary acceptor with an advance in the S state according to Kok et al. [2], or by a recombination of the positive and negative charges created by the photochemical act [3].

It is now well known that low temperature slows the Q to A electron transfer [4, 5]. In previous papers [6, 7], it was established that the kinetic characteristics of the fluorescence induction between −40 °C and −70 °C depend on the number of oxidizing equivalents stored on the donor side of System II. Furthermore, a short saturating flash given to the frozen sample was never able to totally destroy the quenching: only a part (Q_F) was suppressed with a high quantum efficiency corresponding to the normal charge separation which leads to O_2 formation. Another fraction of the quenching (Q_S) was destroyed with low quantum efficiency by continuous illumination or several flashes and was probably linked to the oxidation of cytochrome b_{559} [8].

In this paper, the dark recovery of the Q_F quenching at different temperatures (−30 to −60 °C) is shown to be dependent upon the S states of the chloroplasts prior to cooling.

METHODS

Chloroplasts were prepared from market spinach leaves according to Avron [9] and stored at −70 °C, in the presence of 5 % dimethylsulfoxide. Prior to use the chloroplasts were suspended in 0.05 M Tris buffer (pH 7.5) with 0.4 M saccharose and 0.01 M NaCl at a concentration of 200–250 μg of chlorophyll/ml. In some experiments, 54 % glycerol (v/v) was added.

Fluorescence induction at low temperature was measured with the method previously described [7]. Although the principle is the same, the cuvette used here is thinner: it is formed by a circular aperture (13-mm diameter) in a piece of 0.25-mm thick teflon sheet sandwiched between the thermoelectric module (bottom of the cuvette) and the lucite light pipe (top of the cuvette). The thinness of the cuvette has two advantages: a rapid freezing and thawing of the sample (10 s from + 2 °C to −30 °C), and a greater homogeneity of illumination of the sample.

The sample can be illuminated by continuous light (3000 ergs · cm^{-2} · s^{-1}) and/or by flashes (Xenon, Verre and Quartz Co, Model XOD 22. 1.3 J., 5-μs duration). Actinic light is filtered by one (for flashes) or two (for continuous light) 10-mm thick Schott BG 38 blue glass filters. Fluorescence is detected by a radiotechnique XP 1002 photomultiplier through two red filters (Rubylith Ulano and Kodak Wratten No. 70). Florescence induction curves were stored and digitized by a multichannel analyzer (Intertechnique DIDAC 800).

RESULTS

Chloroplasts dark adapted before cooling

When dark-adapted chloroplasts are cooled at −30 °C and receive a short

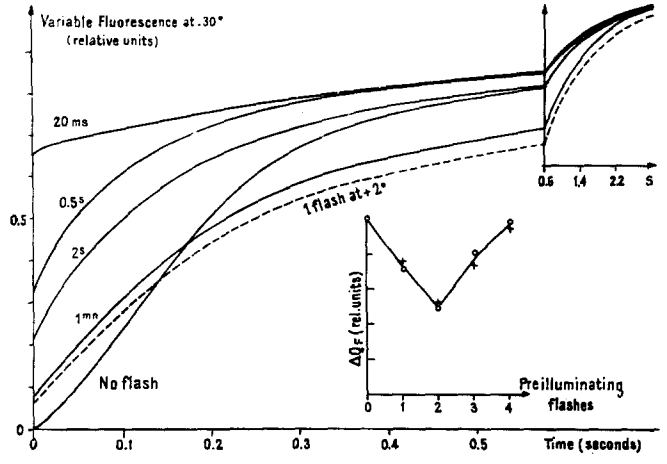

Fig. 1. Fluorescence induction curves performed at indicated times following a saturating flash at −30 °C. Chloroplasts in 54 % glycerol (v/v). - - -, fluorescence curve at −30 °C for chloroplasts preilluminated by one flash at +2 °C before cooling. Insert Fig. 1, ΔQ_F values at low temperature after a varied number of preilluminating flashes (not including the flash used to measure ΔQ_F). Chloroplasts without glycerol. ΔQ_F is the difference in the fluorescence yield before and 20 ms after a saturating flash. ●, ΔQ_F at −50 °C after zero to four preilluminating flashes at +2 °C before cooling; +, one flash at −30 °C, 10 s of dark, warming to +2 °C followed by zero to three flashes, cooling to −50 °C and measure of ΔQ_F.

saturating flash, the fluorescence yield f_i measured 20 ms after the flash is about 65 % of the maximum value attained upon continuous illumination. In Fig. 1 the fluorescence induction curves for increasing dark periods following a flash at low temperature are shown. The fluorescence induction curves reach a limiting value after 1 min at −30 °C. This final curve differs from the control, observed without flash preillumination, in that the regenerated curve does not show an inflection point. However, it closely resembles the curve obtained for chloroplasts given a preilluminating flash at +2 °C and analysed at −30 °C (Fig. 1, dashed curve).

Similar behavior is observed at lower temperatures (e.g. −40 °C) but the total time to reach the final state becomes much longer. Though this dark relaxation is strongly temperature dependent, it does not depend upon the suspending medium: at −30 °C, its rate is similar in the presence of 54 % glycerol (v/v) where the sample remains liquid, and in the absence of glycerol where the sample is frozen.

The effect of a flash given at +2 °C or at −30 °C is compared in the inset of Fig. 1. In the first experiment, the sample is illuminated by zero to four flashes at +2 °C and the amplitude of Q_F quenching (i.e. ΔQ_F at time $t = 0$ of analysis (see legend)) measured at −50 °C. This experiment gives a ΔQ_F sequence which displays an oscillation of period 4 [6]. In a second experiment, the sample is first illuminated by one flash at −30 °C, then is warmed to +2 °C where zero to three flashes are given and then cooled to low temperature where the ΔQ_F amplitude is measured. The ΔQ_F value is a function of the total number of preilluminating flashes given to the chloroplasts. The effect of the first flash is the same independent of whether it is given at +2 °C or at −30 °C.

These results suggest that following a flash excitation of dark-adapted chloro-

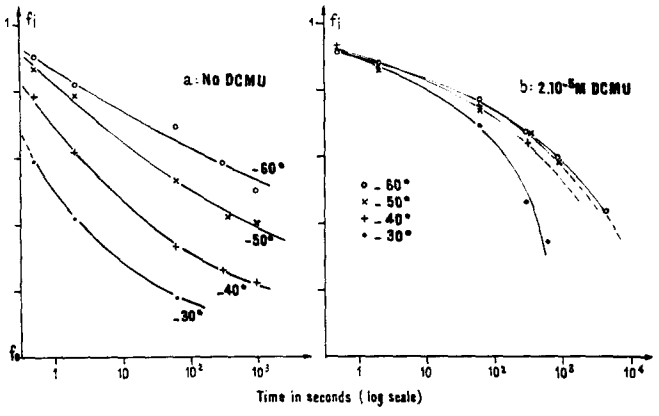

Fig. 2. Initial fluorescence level f_i measured at the onset of the analyzing beam as a function of the dark time following a saturating flash at different temperatures. Chloroplasts without glycerol. (a) No DCMU; (b) $2 \cdot 10^{-5}$ M DCMU. The curves have been normalized to the value of f_i at 20 ms.

plasts at −30 °C, Q^- transfers its electron to a secondary acceptor and an oxidizing equivalent is stabilized on the donor side of System II.

To estimate the rate of reoxidation of Q^- at different temperatures, the recovery of the quenching was measured in the absence and presence of 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Fig. 2). As this inhibitor prevents the transfer of an electron from Q^- to a secondary acceptor, the reoxidation can only occur by a charge recombination. In Fig. 2 the decrease with time of the fluorescence yield f_i following a saturating flash given at the indicated temperatures is plotted. From a comparison of the amplitude of f_i at a given temperature in Figs 2a and 2b, it appears that the recovery is faster in the absence than in the presence of DCMU. This means that as the temperature is raised, a greater fraction of Q^- is reoxidized by Pool A.

Because of the changes in the kinetics between the control and the regenerated curves in the absence of DCMU, and the fact that fluorescence is not linearly related to the concentration of Q [10], these results must be considered only qualitative. Nevertheless, there are two main conclusions which can be drawn from these experiments: (a) the transfer of the electron from Q to A is temperature dependent; (b) at a given temperature, the rate of this reaction is neither exponential nor second order.

Back reactions in presence of DCMU

The temperature dependence of the relaxation observed in the presence of DCMU is shown in Fig. 2b. Charge recombination occurs through both temperature-dependent and temperature-independent processes for this temperature range. The higher the temperature, the more the former process predominates. Fig. 3 shows the fluorescence yield f_i 2 s and 2 min after a saturating flash as a function of temperature. The relative importance of the two processes is clearly shown. 2 s after the flash, the temperature-dependent reaction is largely predominant for temperatures higher than about −5 °C. The longer the dark time, the lower the temperature where the break in the curve appears, that is to say when the temperature-dependent reaction becomes predominant. The limiting factor for the total recovery of f_i in the presence of DCMU

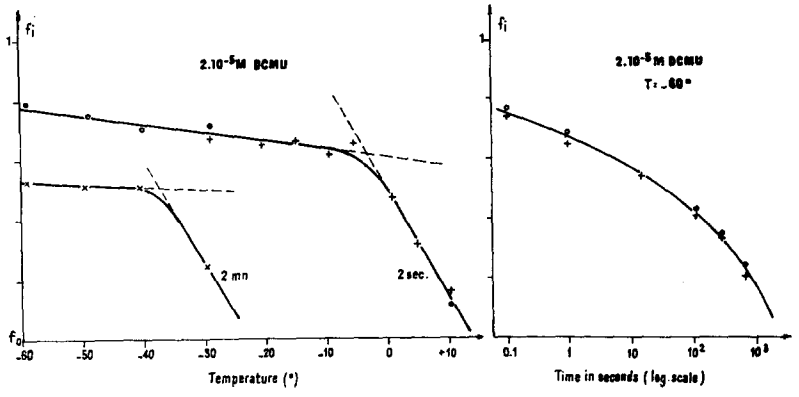

Fig. 3. Initial fluorescence level f_i recovered in 2 s or 2 min of dark after a flash as a function of the temperature. $2 \cdot 10^{-5}$ M DCMU. Chloroplasts without glycerol. The curves have been normalized to the value of f_i at 20 ms.

Fig. 4. Initial fluorescence level f_i recovered as a function of the dark time following a flash at −60 °C. Chloroplasts without glycerol. $2 \cdot 10^{-5}$ M DCMU. The curves have been normalized to the value of f_i at 20 ms. ●, one flash given at −60 °C; +, the second of two flashes given 3 min apart at −60 °C.

seems to be only a question of the duration of the dark period.

Fig. 4 shows the recovery of the initial level of fluorescence for various dark times following either a single flash or the second flash of a group of two flashes given 3 min apart. In both cases, the time course of recovery is the same; this proves that following the second flash, the state of the system is identical to that following the first flash.

Chloroplasts illuminated by flashes before cooling

The variation of the ΔQ_F amplitude at −40 °C has been studied using chloroplasts which either received one or two flashes at +2 °C and one flash at low temperature (Fig. 5, open circles) or which were preilluminated by zero to three flashes at +2 °C (Fig. 5, black circles). Two conclusions can be drawn from this comparison: (1) the ΔQ_F amplitude is identical when the sample is illuminated either by two flashes at +2 °C or by one flash at +2 °C and one flash at −40 °C followed by a sufficient dark time (3 min); this means that at least the centers in the S_1 state before cooling can store a second positive charge leading to the S_2 state after a sufficient dark time where reoxidation of Q^- by Pool A takes place. Since States S_2 and S_3 are indistinguishable as seen from the fluorescence induction curves [6], it is not possible to determine whether centers in the S_2 states are transformed to State S_3. (2) Chloroplasts which have received two flashes at +2 °C cannot stabilize additional oxidizing equivalents when given an additional flash at −40 °C (Fig. 5, dashed line).

Another aspect of the same phenomenon is depicted in Fig. 6. The chloroplasts are preilluminated by two flashes before cooling and one flash at low temperature. The fluorescence induction curve is studied for various dark times following the flash. For dark times longer than 1 min, the fluorescence curve is similar to the control. An advance in the S states towards States S_3+S_0 would give a fluorescence curve with greater amplitude and faster rise. Thus, for these conditions, the recovery of the

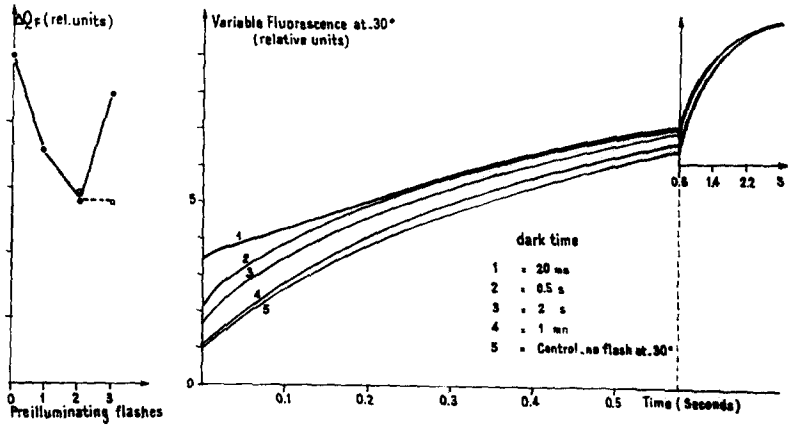

Fig. 5. ΔQ_F values at -40 °C as a function of the total number of preilluminating flashes (i.e. not including the flash used to measure ΔQ_F). Chloroplasts without glycerol. ΔQ_F is the difference in the fluorescence yield before and 20 ms after a saturating flash. ●, after 0 to 3 preilluminating flashes given at $+2$ °C; ○, after 1 or 2 flashes at $+2$ °C, cooling to -40 °C, one flash $+3$ min of dark period.

Fig. 6. Fluorescence induction curves performed at indicated times following a saturating flash at -30 °C. Chloroplasts in 54 % glycerol (v/v) have been preilluminated by two saturating flashes at $+2$ °C just before cooling.

Q_F quenching at low temperature is chiefly due to a charge recombination between Q^- and the positive charge created during the flash. It should be added that the kinetics of the recovery of Q_F quenching is not altered by the presence of 10^{-3} M ferricyanide (data not shown), which is known to oxidize cytochrome b_{559} [11].

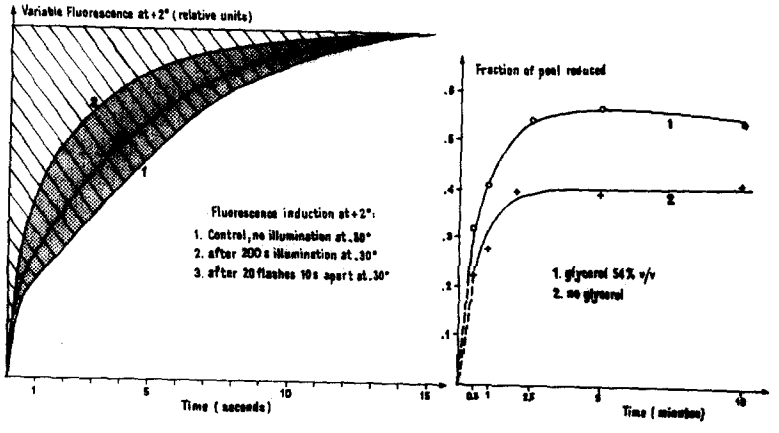

Fig. 7. Fluorescence inductions at $+2$ °C. Chloroplasts in 54 % glycerol (v/v). (1) Control. (2) 200-s continuous illumination at -30 °C. (3) 20 flashes 10 s apart at -30 °C. The slashed area corresponds to the concentration of oxidized Pool A at time zero of analysis. The dotted area corresponds to the concentration of Pool A, which has been reduced upon 200 s of continuous illumination at -30 °C.

Fig. 8. The fraction of Pool A reduced by continuous illumination at -30 °C as a function of the duration of this illumination. Chloroplasts 1, 54 % glycerol (v/v); Chloroplasts 2, no glycerol.

Experiments presented in Fig. 7 show that upon continuous illumination, it is still possible to reduce Pool A at low temperature: the slashed area over the fluorescence curve observed at +2 °C measures the concentration of oxidized Pool A present at time zero. This area is smaller when the chloroplasts are cooled to −30 °C, given continuous illumination and warmed to +2 °C (Curve 2). The difference between these two areas gives the concentration of Pool A reduced during the illumination at −30 °C (Fig. 7, dotted area). In Fig. 8 the percentage of Pool A reduced versus the duration of the continuous illumination is plotted: it thus appears that at −30 °C, it is possible to reduce about 60 % of Pool A in the presence of 54 % glycerol (v/v) and 40 % in the absence of glycerol. If one accepts that Pool A represents 15–20 oxidizing equivalents [12], this would correspond to the transfer of 9–12 electrons in the presence of glycerol and 6–8 electrons in its absence.

The comparison between the effect of a series of twenty flashes 10 s apart and a continuous illumination given for the same period of time shows that the sequence of flashes is much less effective in reducing Pool A (Fig. 7, Curve 3). In this experiment, the number of flashes and their spacing were chosen to permit the slow transfer of an electron from Q to A; under these conditions, the flashes reduced only 22 % of the total A pool while 52 % was reduced upon 200 s of continuous illumination at −30 °C. It can be concluded that during a series of flashes an appreciable amount of the electrons is involved in a back reaction which occurs mainly when the centers are in the $S_2 + S_3$ states, as shown in Fig. 6.

The temperature dependence of the back reaction observed for chloroplasts in the S_2+S_3 states is shown in Fig. 9. This reaction is very weakly temperature dependent for temperatures below −30 °C. Nevertheless, the relaxation kinetics are non-exponential, extending over a very large time scale, as observed in dark-adapted chloroplasts in the presence of DCMU.

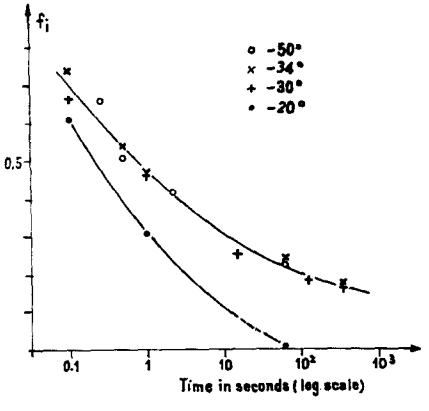

Fig. 9. Initial level of fluorescence f_i measured as a function of dark time following a saturating flash at the indicated temperatures. The chloroplasts are preilluminated by two flashes at +2 °C before cooling. No glycerol. The curves have been normalized to the value of f_i at 20 ms.

DISCUSSION

From fluorescence measurements performed at room temperature, Forbush and Kok [13] measured a 0.6-ms half-time for the reoxidation of Q^-. More recently, Zankel [14] obtained an even shorter value (0.3 ms). Malkin and Michaeli [5] further suggested that as the temperature is decreased from +20 °C to −40 °C, an increasing fraction of the primary acceptor Q is progressively disconnected from the secondary acceptor A. Contrary to this hypothesis, our results prove that the transfer of electrons from Q to Pool A still occurs below −30 °C but very slowly. In dark-adapted chloroplasts (S_3+S_1 states), we can roughly estimate the increase of the half-time of the Q to A reaction from +20 °C (0.3 ms) to −30 °C (2 s, see Fig. 2a) to about a factor of 600, which leads to an average Q_{10} of 3.7. The slow electron transfer from Q to A observed here must occur in a complex very tightly bound to the photocenter without any intervention of diffusion.

Several hypotheses may be proposed to account for the limit to a complete reduction of the Pool A observed here: the reaction of O_2 formation could have been the limiting step, but the possibility to produce more than the 3 or 4 oxidizing equivalents required to evolve an O_2 molecule requires this assumption to be rejected. Besides, the fluorescence yield stays high, i.e. Q stays reduced, upon continuous illumination at −30 °C even though Pool A is not completely reduced (data not shown). If the limiting step was on the donor side, we would expect a low fluorescence level as is generally observed when the System II donor side is inactivated (ultraviolet-treated [15, 16] or Tris-washed chloroplasts [17]).

A limiting step on the acceptor side must thus be assumed but it can occur in different ways: (1) a part of Pool A is not accessible to the electron flow; (2) A^- is reoxidized by System I and one must admit that O_2 can still accept the electrons coming from System I, as no external acceptor is added to the chloroplasts; (3) a third possibility is that Pool A can be totally reduced but that a part of it is reoxidized during the heating of the sample from −30 to +2 °C.

Back reactions

Either in the presence of DCMU or in chloroplasts preilluminated by two flashes (S_2+S_3 states), the Q^- reoxidation after an additional flash at low temperature occurs primarily through back reactions. First, it must be noted that these back reactions are clearly different from the one described by several authors [18–20] at liquid nitrogen temperature as a fast charge recombination which occurs with a 4.5-ms half-time. In the experiments presented here, the shortest time measured after the flash is 20 ms. This means that in our case, the positive charge is already stabilized on a secondary donor in a reaction competitive with the immediate chlorophyll$^+$ Q^- recombination.

In the presence of DCMU, the temperature-dependent reaction is very likely the one previously described by Bennoun [3], where the negative charge reacts with the positive charge stored on the donor Z. This reaction has an activation energy equal to 14 kcal/mole per °K. Although this reaction showed apparent second-order kinetics, Bennoun's experiments further suggested that there is no cooperation between the centers [21]. The temperature-independent reaction could be due to a tunnelling effect. The rates of charge recombination are distributed continuously over the en-

semble of the centers with the recombination time varying over a factor of 100.

The experiment of Fig. 4 shows that the relaxation of the fluorescence yield in the presence of DCMU was identical following a first flash and a second given 3 min after the first. The 20 % of the centers that recovered during the 3-min period between flashes obviously had a faster recovery than those centers that did not recover during this time. If during the second flash these 20 % of the centers were redistributed over the wide range of relaxation times observed when the relaxation curve is allowed to go to completion, then the second flash should have increased, relative to the first flash, the concentration of slowly recovering centers. The observation that the relaxation after the second flash follows an identical time course as that following the first, indicates that the concentration of slow centers did not increase and that no redistribution occurred. Thus, a center that recovered at a particular rate following the first flash recovers at the same rate following the second.

These results indicate the existence of a heterogeneity of the centers which can be due to different sources: (a) the positive charge can occupy different positions on the donor side of the center; since no distinct phases appear in the kinetics, this hypothesis is unlikely unless each of these reactions is in turn heterogeneous; (b) a structural alteration could take place, varying the distance or the properties of the environment between the photoactive chlorophyll and the secondary donor. The back reaction studied here might be controlled by the transfer of the positive charge between a secondary donor and the chlorophyll.

When the chloroplasts are in the S_2+S_3 states, the back reaction which reoxidizes Q^- after a flash at low temperature is also temperature independent below -30 °C. The fact that neither the Q_F amplitude [7] nor the rate of reoxidation of Q_F are ferricyanide sensitive proves that the positive charge created during the flash has been transferred to a donor Z distinct from cytochrome b_{559}, which is oxidized under these conditions. The back reaction presents common features with the one observed in the presence of DCMU: it occurs by a tunnelling and extends over a very large time scale; however, it is faster than in DCMU.

The fact that a back reaction is more probable when the centers are in the S_2+S_3 states than in the S_0+S_1 states may suggest that either the rate of transfer of the positive charge between Z and chlorophyll is slower or the equilibrium is displaced towards chlorophyll$^+$ in the higher S states. Though no intermediary donor (Y) is required to account for the results presented here, ideas similar to this last possibility have been already suggested [22, 23].

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