Biochimica et Biophysica Acta, 725 (1983) 376–383
Elsevier

BBA 41399

MULTIPLE EFFECTS OF LINOLENIC ACID ADDITION TO PEA THYLAKOIDS

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(Received March 31st, 1983)
(Revised manuscript received August 11th, 1983)

Key words: Fatty acid; Photosystem II; Fluorescence; Linolenic acid; Electron transfer; (Pea chloroplast)

The addition of linolenic acid to thylakoids produces various pH-dependent effects. We have demonstrated a binding site near the Photosystem (PS) II center with a pK_a of 6.5: when linolenic acid is unprotonated it induces in the dark a rise of the initial fluorescence level, the latter being similar to the maximum fluorescence obtained during illumination of untreated thylakoids. The comparison of the fluorescence lifetimes in the presence and absence of linolenic acid leads us to conclude that the charge stabilisation on the primary acceptor, Q, is prevented by linolenic acid. A second binding site on the protein carrying B, the secondary acceptor of PS II, has also been demonstrated for linolenic acid. It has a 3-(3,4-dichlorophenyl)-1,1-dimethylurea-type effect both in the protonated and unprotonated forms. Finally, measurements of electrophoretic mobility of the thylakoids indicate several other sites of linolenic acid inclusion with an average pK_a of 5.7. At alkaline pH the presence of unprotonated linolenic acid increases the charge density on the membrane. As a result a higher concentration of divalent cations is needed to obtain fluorescence and stacking changes than for untreated thylakoids. The presence, at acidic pH values, of the unprotonated form of linolenic acid leads to the inhibition of cation-induced fluorescence changes, probably by preventing the movement of chlorophyll-protein complexes in the membrane.

Introduction

Damage to photosynthetic membranes and inhibition of photosynthetic activity during stress and ageing have been correlated to the appearance of free fatty acids, specially unsaturated fatty acids, released from lipids by endogenous lipases (for a review, see Ref. 1). These authors have extensively studied the effect of linolenic acid on the activity of isolated chloroplasts. They provided evidence for a new type of PS II inhibition, probably due to a blocking of the charge separation within the PS II center.

Furthermore, Scoufflaire et al. [2] observed a linolenic acid-induced loss of cation-induced stacking and fluorescence changes. They detected also an increase of negative charges at the membrane surface due to incorporation into the membrane of linolenic acid.

In this paper, we show that these fatty acid-induced effects are pH dependent. This leads us to discuss the various effects of linolenic acid and their pH dependence in terms of the position within the photosynthetic membrane of the protonated and unprotonated forms of the fatty acid.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1 dimethylurea; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 4-morpholineethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Chl, chlorophyll.

Material and Methods

Broken chloroplasts were isolated from pea leaves according to a procedure previously described [3]. The final resuspending medium contained 0.3 M sorbitol, 10 mM NaCl, 10 mM Tricine or Hepes, ph 6.8–7.5, or 10 mM Mes, pH 5.0–6.7, and 10^{-6} M gramicidin D.

Chlorophyll fluorescence was measured using 2.5-ml samples of chloroplast suspension at a concentration of 5 μg Chl/ml. The excitation beam was passed through a Corning 4-96 filter; the fluorescence emitted at 90° to the exciting beam was selected using red filters (Corning 2-64 plus Wratten 92). The excitation intensity was about 30–50 photons/center per s. The shutter opening time was less than 1 ms. The signal was recorded utilizing a multichannel analyser (Sein).

In the presence of DCMU, the induction curve of dark-adapted chloroplasts presents a single phase from the F_0 to the F_m level corresponding to oxidized and reduced Q, respectively.

In the absence of DCMU, the induction curve shows two distinct phases: a fast rise from initial fluorescence yield F_0 (Q oxidized) to a plateau F_i followed by a slow rise to F_m (Q reduced). The slow rise $F_i \rightarrow F_m$ reflects the reduction of the plastoquinone pool. This phase is suppressed by DCMU-type inhibitors which block electron transfer after Q, the F_i level being then raised to the F_m level.

Triggered luminescence in dark-adapted chloroplasts was measured as described by Etienne and Lavorel [4].

Surface charge densities of the thylakoid samples were determined from their electrophoretic mobility, detected using a laser Doppler velocimetry technique [5]. Chloroplast samples, at a concentration of 5 μg Chl/ml, contained 10^{-5} M DCMU to avoid light effects on the charge density of the thylakoids. A 6328 Å helium-neon laser was used for sample illumination.

Fluorescence lifetime determinations were done by phase fluorimetry at 82 MHz [6]. Both phase shift and demodulation were measured. The excitation wavelength was 514.5 and the fluorescence analysed at 685 nm ($\Delta\lambda = 6$ nm).

Fatty acids and their methyl esters were purchased from Sigma Chemical Co. Linolenic acid (*cis*, *cis*, cis-9,12,15-octadecatrienoic), linoleic acid (*cis*,*cis*-9,12-octadecadienoic), oleic acid (*cis*-9-octadenenoic) and stearic acid (octadecanoic) and their methyl esters acid were dissolved in ethanol such that the final concentration of ethanol in the thylakoid suspension was less than 1%.

Results and Discussion

Effect of linolenic acid addition on the F_0 level

Golbeck et al. [1] have observed that linolenic acid added to thylakoids modifies the PS II fluorescence characteristics: it raises the F_0 level up to the F_m level of fluorescence, the latter level not being affected. F_0 is the initial level of fluorescence after dark adaptation of the thylakoids, corresponding to all PS II centers being open, i.e., with the primary stable acceptor Q oxidized; the F_m level is the maximum level of fluorescence obtained after illumination has closed all the centers, i.e., reduction of Q. These authors suggested that the fatty acid prevents the primary charge separation, the centers consequently having the same fluorescence yield as closed centers which are no longer capable of this charge separation.

Fluorescence lifetimes of thylakoids incubated with or without 100 μM linolenic acid were measured. The lifetime of the F_m fluorescence of both

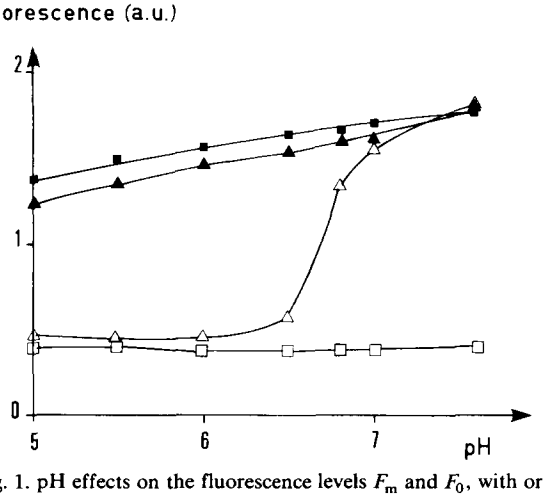

Fig. 1. pH effects on the fluorescence levels F_m and F_0, with or without 100 μM linolenic acid added to the thylakoids. All samples were incubated, in the presence of 10 mM $MgCl_2$, for 4 min at each pH, then 1 min with or without linolenic acid and the fluorescence induction was recorded. (□, ■) F_0 and F_m, respectively of the control, (△, ▲) F_0 and F_m, respectively with 100 μM linolenic acid.

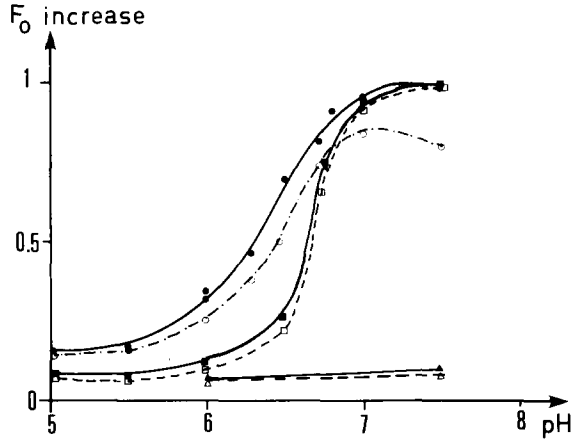

Fig. 2. Linolenic acid-induced F_0 increase versus pH. The F_0 increase is given by F_0/F_m in the presence of linolenic acid minus F_0/F_m of the control, divided by $1-(F_o/F_m)$ control. (●, ○) 100 and 50 μM linolenic acid, respectively, in the absence of $MgCl_2$. (□, ■) 100 and 50 μM linolenic acid, respectively, in the presence of 10 mM $MgCl_2$. (▲, △) 100 and 50 μM methyl ester of linolenic acid, in the presence of 10 mM $MgCl_2$.

samples present the same characteristics: $\tau_p = \tau_m = 1.50 \pm 0.05$ ns.

As shown in Fig. 1, the F_0 increase induced by linolenic acid addition is pH dependent: at pH 7.5 in the presence of 10 mM $MgCl_2$ in the medium, 100 μM linolenic acid raises F_0 to F_m, whereas at pH 6.0, practically no rise of F_0 occurs. Fig. 2 shows that the pH dependence is modified by the presence or absence of divalent cations in the medium, the curves having been normalized (see figure legend) for a better comparison. A great part of the difference between samples in the presence or absence of $MgCl_2$ may be due to the fact that, whereas in a low-salt medium, the fluorescence is proportional to the concentration of closed centers, this is not the case in the presence of a high cation concentration, because of exciton transfer between PS II units [7].

The pH dependence of the linoleic acid-induced F_0 rise could be due either to the effect of pH on membrane components (i.e., a pH-induced conformational change of the membrane may make it sensitive to linolenic acid addition) or to the effect of pH on the linolenic acid incorporated into the membrane; in the latter case it is possible that the ionisation of the carboxy group of linolenic acid is involved. Indeed, although the pK_a of linolenic acid in solution is near 4.8. Egret-Charlier et al. [8] and Ptak et al. [9] pointed out that when stearic acid is incorporated into vesicles, the apparent pK_a is shifted towards alkaline pH, the more so when the membrane bears negative charges. Then linolenic acid included in the thylakoid membrane bearing negative charges at pH above 4.3 may have an apparent pK around 6.5.

To test the latter hypothesis we performed the following experiments.

Firstly, we added linolenic acid methyl ester, which is uncharged at every pH, and we found (Fig. 2) that its addition does not affect the F_0 level, even at pH 7.5.

Secondly, we measured the electrophoretic mobility of thylakoids. Fig. 3 shows the values of $\Delta\nu$ (Hz) for thylakoid suspensions with and without 100 μM linolenic acid at various pH values; the electric field strength was 20 V/cm. At pH 7.0 this gives a charge density of 0.54 $\mu C/cm^2$ for the control and 0.71 $\mu C/cm^2$ for the thylakoids supplemented with linolenic acid. The charge density of the control thylakoids and its dependence upon

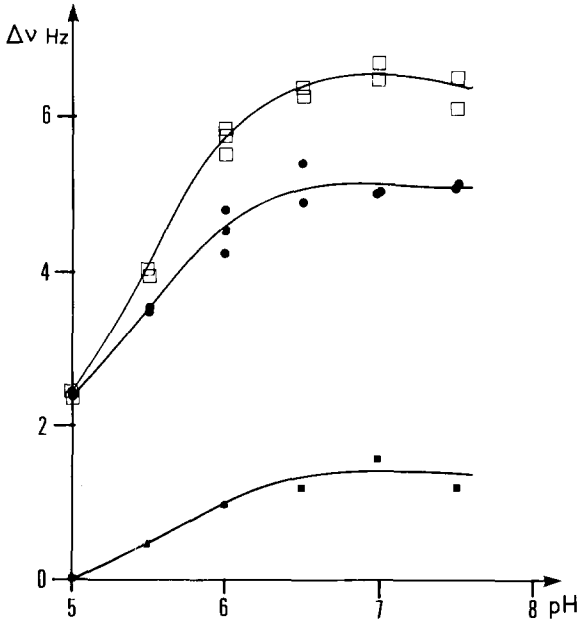

Fig. 3. Electrophoretic mobility as a function of the pH of the resuspension medium, with (□) 100 μM linolenic acid or without (●). The third curve (■) represents the difference between the two first curves.

TABLE I

REVERSIBILITY OF LINOLENIC ACID-INDUCED EFFECTS

Chloroplasts were incubated in 10 mM $MgCl_2$.

Initial pH	Successive additions	F_0/F_m
7.5	0	0.34
6.0	0	0.36
7.5	100 μM linolenic acid	1.0
6.0	100 μM linolenic acid	0.36
7.5	100 μM linolenic acid, then Mes to reach pH 6.0	1.0
6.0	100 μM linolenic acid, then Tricine to reach pH 7.5	1.0

pH are quite similar to those found by Schapendonk et al. [10] and Nakatani et al. [11].

The difference between the mobilities of chloroplasts 100 μM linolenic acid incubated with and control chloroplasts gives a pK around 5.7. The same pK_a was found for addition of 50 μM linolenic acid.

Thirdly, we tested the reversibility of the fatty acid-induced F_0 rise when the pH is varied. Table I shows that when the linolenic acid addition takes place at pH 7.5, F_0 remains at the F_m level after decreasing the pH to 6.0. In contrast, when linolenic acid is added to the suspension at pH 6.0, raising the pH to 7.5 induces an increase in the F_0 level.

Then, from the results with the methyl ester, we can conclude that only the dissociated form ($RCOO^-$) increases F_0. The electrophoresis measurement gave a pK_a for linolenic acid included in the membranes of about 5.7 : thus, there should be enough of the $RCOO^-$ form at pH 6 to produce an F_0 rise, but the latter was not seen. But, if the F_0 increase is due to the incorporation of 1 linolenic acid molecule on the PS II protein center, it means that it represents 1 linolenic acid molecule per about 500–600 Chl, and the following calculations may explain the discrepancy. There is approx. 1 Chl per 200–400 Å^2; for the control 0.54 μC/cm^2 corresponds to one charge per 7–15 Chl. When linolenic acid has been added, the charge density at pH 7.0 becomes 0.71 μC/cm^2, that represents supplementary charges equivalent to one charge per 23–47 Chl. This is 10–80-times more than the value of 1 per 500–600 Chl. Then, there are probably a multiplicity of sites for incorporation of linolenic acid, and what we observe by electrophoresis is a mean pK_a.

To explain the nonreversibility of the F_0 rise induced at pH 7.5 when the pH is decreased to 6.0 (Table I), we have to suppose that the $RCOO^-$ form either cannot be reprotonated, or that it induces an irreversible conformational change of the PS II center.

Effect of linolenic acid on Q to B electron transfer

At acidic pH, linolenic acid addition does not produce the F_0 increase, but it induces a DCMU-like effect, blocking between Q and B the primary and secondary electron acceptors of PS II, increasing the rate of the fluorescence induction. As shown in Fig. 4, 100 μM linolenic acid is equivalent to 10 μM DCMU.

This block of electron transfer after Q may be due to a modification of the protein complex bearing B, and which is the site of DCMU fixation, but the linolenic acid concentrations needed to block electron transfer are much higher than the DCMU concentration and, maybe, the site of fixation is not as specific as the DCMU one.

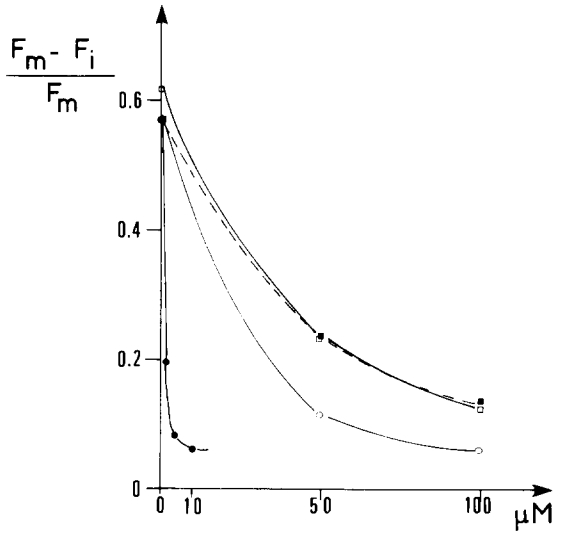

Fig. 4. Effect of DCMU, linolenic acid and its methyl ester on the fluorescence induction. F_m, maximum stationnary level of fluorescence; F_i, fluorescence level at the end of the rapid phase of induction (see Material and Methods). (●) DCMU, (○) linolenic acid at pH 6, (■) methyl ester at pH 6, (□) methyl ester at pH 7.5.

At alkaline pH, the block between Q and B cannot be detected because no variable fluorescence remains. Thus, we tested the effect of linolenic acid methyl ester on the Q to B electron transfer. Fig. 4 shows that it is effective at pH 6.0 and 7.5. Furthermore, we measured another phenomenon, the luminescence induced in darkness by DCMU addition [4]. In dark-adapted chloroplasts there always remain some charges on the donor and acceptor sides of PS II. DCMU not only blocks the electron transfer between Q and B but also promotes the passage of electrons remaining on B to Q and then the recombination of charges of the donor side and Q^- gives rise to luminescence. We observed that at both pH 7.5 and 6.0, addition of linolenic acid produces the same luminescence as DCMU.

Effect of linolenic acid on the cation-induced increase of PS II fluorescence

It has been postulated (see Refs. 12 and 13) that the addition of screening cations to the negatively charged thylakoids produces diffusion of the various protein complexes (PS I and PS II) in such a way as to decrease energy transfer between them, and consequently increase the chlorophyll fluorescence yield of PS II. There is further creation of domains on the membrane surface having either low (PS II) and high (PS I) net surface charge densities, and stacking at low-charge regions between adjacent membranes is induced.

Scoufflaire et al. [2] observed that treating thylakoids at pH 7.5 with linolenic acid produces a loss of salt-induced stacking and chlorophyll fluorescence increase, without changes in membrane fluidity. Furthermore, they observed that the treatment was accompanied by an increase in the surface charge density of the thylakoid membrane as estimated either by 9-aminoacridine fluorescence quenching or electrophoretic mobility measurements. They concluded that the loss in ability of cations to induce stacking, chlorophyll fluorescence changes and probably domain formation is due to changes in the density of electrical charges on the membrane surface.

Fig. 5 shows that the linolenic acid-induced inhibition of the cation effects is not really an inhibition, in the sense that the PS II fluorescence

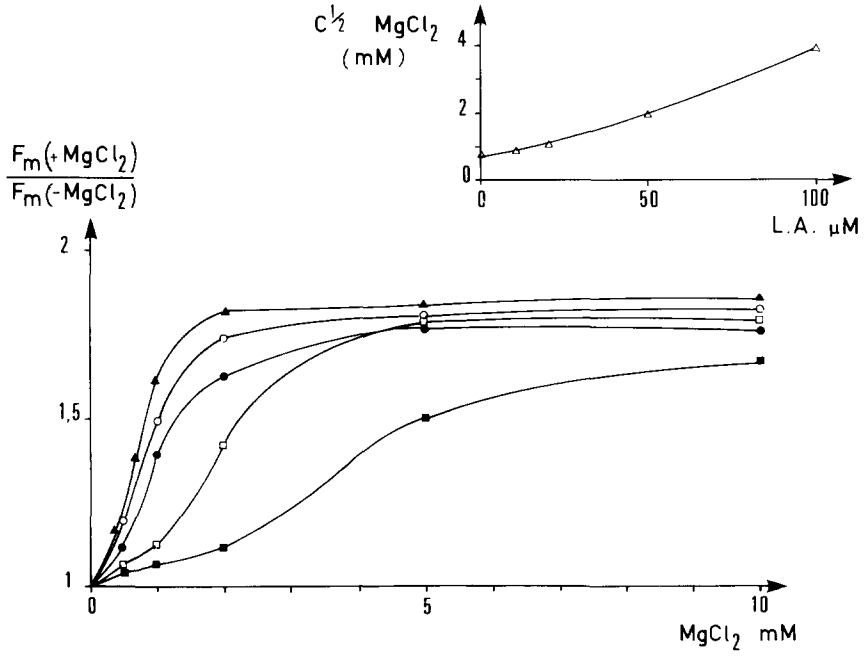

Fig. 5. Fluorescence increase as a function of $MgCl_2$ concentrations in the presence of various concentrations of linolenic acid, at pH 7.5. (▲) No addition , (○) 10 µM, (●) 20 µM, (□) 50 µM, (■) 100 µM linolenic acid. (inset) $MgCl_2$ concentration giving half of the fluorescence increase, versus the linolenic acid (L.A.) concentration.

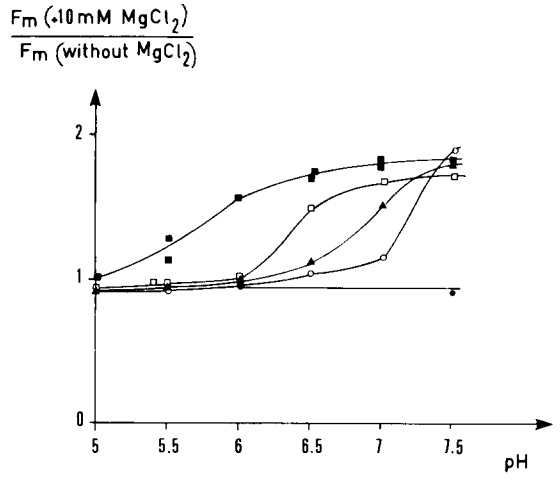

Fig. 6. Cation-induced fluorescence increase at various pH values in the presence or absence of linolenic acid or its methyl ester at various concentrations. (■) No addition, (□) 25 μM, (▲) 50 μM, (○) 100 μM linolenic acid, (●) 20 μM methyl ester.

increase can be obtained in the linolenic acid-treated thylakoids exactly as in the nontreated ones by adding a higher cation concentration. A striking effect (Briantais and Vernotte, unpublished data) is that treatment with linolenic acid accelerates considerably the fluorescence increase and, as no fluidity increase has been detected [2], the mechanism of protein complex segregation may be questioned.

Nevertheless, the higher $MgCl_2$ concentration is probably needed by the higher negative charge density of the linolenic acid-treated thylakoids and these supplementary negative charges must be due to dissociated linolenic acid ($RCOO^-$ form). Then we attempted to verify the pK_a of linolenic acid calculated from electrophoresis experiments by measuring the $MgCl_2$ concentration needed at each pH to obtain the same maximum fluorescence as in the control.

Surprisingly at pH 6.0 we observed that linolenic acid addition determines a block of the fluorescence increase induced by cations that is not overcome by an increase of the $MgCl_2$ concentration (at least up to 75 mM). The pH dependency of this 'real' linolenic acid-induced block of the fluorescence increase is shown Fig. 6; it depends upon the linolenic acid concentration. The block of the cation-induced increase of fluorescence observed at pH 6.0 is quickly reversed by shifting the pH to 7.5. Conversely, if linolenic acid is added at pH 7.5 and then the pH decreased to 6.0, the addition of $MgCl_2$ does not increase the fluorescence yield. Thus, the pH dependency is reversible in both directions.

We suppose that it is the RCOOH form that inhibits the cation effect on fluorescence, and to test this hypothesis, we checked the effect of linolenic acid methyl ester over the pH range 5–7.5. As shown in Fig. 6 the methyl ester inhibits the cation-induced fluorescence increase.

Consequently, the various curves of Fig. 6 can simply be explained if it is the RCOOH form that inhibits the fluorescence increase. We have found, for linolenic acid incorporated in the membrane, a pK_a of 5.7 (Fig. 3) irrespective of its concentration. Then, at each pH the RCOOH form is proportional to the linolenic acid concentration, for example, at pH 7.0, 25 or 50 μM linolenic acid does not produce inhibition whereas 100 μM does so because in the latter case, the RCOOH form is still sufficient to promote the block.

This proposition, that RCOOH but not $RCOO^-$ can prevent the effect of $MgCl_2$ on the fluorescence, can be understood if we assume a different position within the membrane of the protonated and unprotonated forms of the fatty acid. Indeed, Sanson and Ptak [14], using spin-labeled fatty acids included in lecithin multilayers, have shown that according to the ionisation or methylation of the fatty acid, the hydrophilic head groups could be located in different parts of the lipid/water interface, the lipophilic portion being consequently more or less deeply immersed in the lipid phase. We can imagine that protonated and methylated linolenic acids are positioned in such a manner that the diffusion of the PS I and PS II protein complexes is impeded, whereas the $RCOO^-$ form does not prevent the protein movements.

Specificity of linolenic acid

We tested 18 : 3, 18 : 2, 18 : 1 and 18 : 0 acids for the various effects described in this paper. The F_0 increase and the DCMU-like effect are produced approximately in the same manner by all the unsaturated fatty acids tested, but not by the saturated one (stearic acid).

All unsaturated fatty acids are similar to lino-

lenic acid with respect to modifications of the cation-induced increase of fluorescence, with the same effects of pH on the phenomenon. The saturated one, in contrast, blocks not only at acidic pH but also at alkaline pH; perhaps its pK_a is different, or, due to its rigid configuration it increases the viscosity of the membrane, impeding the diffusion of the proteins within the membrane.

Conclusion

The pH dependence of the linolenic acid-induced increase of the F_0 level has been characterised. The transition, at pH 6.5, is probably due to the ionisation of the fatty acid incorporated near or into the PS II center; but we cannot rule out a conformational change of the PS II itself making it sensitive to unprotonated linolenic acid. Whatever the case may be, our experiments allow us to characterize better the inhibition mechanism. In the presence of linolenic acid at alkaline pH, F_0 is equal to F_m: Golbeck et al. [1], excluding the hypothesis of a dark reduction of Q by linolenic acid, proposed that "the L.A. functions either by inhibiting the transfer of energy from antennae chlorophylls to the trap or by inhibiting the charge separation within the PS II trap".

Our measurements of the fluorescence lifetime allow us to choose between these hypotheses.

The PS II electron-transfer chain may be represented as:

$$\text{OEC} \rightarrow \text{Z} \rightarrow \text{P-680} \rightarrow \text{I} \rightarrow \text{Q} \rightarrow \text{B} \rightarrow \text{plastoquinone pool}$$

where P-680 is the chlorophyll center, Z an electron donor to this chlorophyll, I a pheophytin primary acceptor, Q the primary stable acceptor (all of these compounds are supposed to be linked to the same protein); B the secondary acceptor (linked to another protein, supposed to be the DCMU site), and OEC the oxygen-evolving complex.

The lifetime of the fluorescence F_m presents the same characteristics in the linolenic acid-treated and the nontreated chloroplasts; then it is likely that the same mechanism for fluorescence emission is involved in both cases. According to Haehnel et al. [15], the variable part of the fluorescence reflects the kinetics of charge recombination in the reaction center, as also proposed by Shuvalov et al. [6]. In the frame of that model, we can conclude that linolenic acid does not inhibit the energy transfer between the antenna and the PS II trap, and does not inhibit the charge separation, but does inhibit the charge stabilization on Q, blocking between I and Q.

We have shown that, even at pH 7.5, luminescence of dark-adapted thylakoids can be triggered by linolenic acid addition. The appearance of luminescence when DCMU is added to dark-adapted thylakoids has been attributed by Etienne and Lavorel [4] to recombination between the charges remaining on the donor and acceptor sides, the electron stabilized on B^- being transferred to Q by DCMU addition, then to I and P. Therefore, the appearance of linolenic acid-triggered luminescence implies that the electron transfer is operating from B to P, whereas it does not work from P to Q. Then linolenic acid may act between I and Q like DCMU between Q and B, i.e., displacing the equilibrium between the electron carriers toward the reverse direction."

The DCMU-like effect of linolenic acid is produced at every pH, and also produced by the methyl ester of the fatty acids. The inability of stearic acid to induce this electron-transfer inhibition may be explained either by the necessity of a double bond or by steric hindrance due to the saturated fatty acid.

The cation-induced increase of fluorescence is dependent on both the pH and the concentration of the fatty acid present in the thylakoids and perhaps this explains the variability of the effects observed during ageing in vitro of chloroplasts where free fatty acid release has been observed.

Acknowledgement

This research was partly supported by P.I.R.S.E.M. ATP contract 1109 (Approches Pluridisciplinaires de la Photosynthèse)

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