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CHANGE IN pH DEPENDENCE AND SEQUENTIAL INHIBITION OF PHOTOSYNTHETIC ACTIVITY IN CHLOROPLASTS BY UNSATURATED FATTY ACIDS

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SUMMARY

1. The effects of unsaturated fatty acids on photochemical activities and volume changes in isolated spinach chloroplasts were investigated at various pH.

2. In control experiments, maximum rates of electron transport activity occurred at pH 8.5 in Photosystem II (from H_2O to $Fe(CN)_6^{3-}$) and at 8.9 in Photosystem I (from reduced *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (reduced TMPD) to methyl viologen). The addition of unsaturated fatty acids (linolenic, linoleic and oleic) caused a sequential inhibition of Photosystem II and Photosystem I electron transports and of the associated photophosphorylations.

3. Unsaturated fatty acids caused a shift of the pH optimum for electron flow toward acidity. Up to a fatty acid chlorophyll molar ratio of 9, linolenic acid stimulated electron flow in Photosystem I, inhibited it in Photosystem II, and simultaneously caused an acid shift of the pH optimum only in Photosystem II. Higher concentrations of the acid were needed to inhibit electron flow and cause a shift of the pH optimum in Photosystem I. Coupling the systems with ADP and P_i did not significantly change the results.

4. Low concentrations of linolenic acid which had an uncoupling effect did not shift the pH optimum for both electron flow systems. Higher concentrations of linolenic acid which had no uncoupling effect did shift the pH optimum.

5. Unsaturated fatty acids induced chloroplast swelling in the dark without shifting its pH optimum which remained in the range of 8–9. However, these acids shifted the pH optimum of light-induced shrinkage and diminished the extent of the shrinkage.

6. These results indicate that there is not necessarily a stringent relationship between uncoupling and the acid shift of the pH optimum of electron flow.

7. My interpretation is that the acid shift of the pH optimum of electron flow and its inhibition by fatty acids are due to a deterioration of the thylakoid membrane, combined with an inhibition of the light-induced proton uptake mechanism and an increase in the permeability of the membrane to water and protons.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; PMS, phenazine methosulfate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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INTRODUCTION

Fatty acids were shown to influence several structural parameters and photochemical functions of chloroplasts *in vitro*. It was for instance experimentally verified that fatty acids enhanced chloroplast swelling[1–4] and inhibited light-induced shrinkage[1]. Fatty acids were also shown to dissociate electron flow activity from photophosphorylation thereby acting as uncouplers[5]. Other uncoupling agents as for instance NH_4Cl and EDTA[6–8], detergents[7] or sonication[7,9,10] were known to induce a shift in the pH optimum of the electron flow reaction. The question, therefore, was as to whether fatty acids would as well induce a shift in the pH optimum, what one would expect according to the current interpretation which links the shift in pH optimum directly to the uncoupling event[7,9,10].

To answer this question, the electron flow activity and the photophosphorylation of Photosystem I and Photosystem II were measured as a function of fatty acid concentrations at various pH. In order to get further insight into the mechanism of fatty acid interaction with the thylakoid membrane, we also measured the changes of chloroplast swelling and light-induced shrinkage at various pH.

This investigation shows that the uncoupling event does not necessarily result in a shift of the pH optimum of electron transport.

MATERIALS AND METHODS

Materials

Class II spinach chloroplasts were prepared in a medium containing 100 mM Tris–HCl (pH 8) and 175 mM NaCl as described previously[11] and were generally used unwashed. Chlorophyll was determined by the method of Bruinsma[12]. The chloroplast suspension was then diluted in the same medium to obtain 1 mg chlorophyll per ml and kept at 0–4 °C before use.

Since the action of fatty acids on chloroplast volume changes and photochemical activities depended on the molar ratio of fatty acids/chlorophyll, the same chlorophyll concentration was used in all reaction mixtures, *i.e.* 20 μg chlorophyll per ml. Therefore, all the results presented in this paper refer to this amount of chlorophyll and any change in fatty acids or chlorophyll concentrations must be evaluated in terms of the fatty acids/chlorophyll ratio.

Measurements of volume changes of chloroplasts

Dark swelling of chloroplast suspensions was estimated by the decrease in absorbance at 540 nm ($-\Delta A_{540nm}$) (see ref. 13) in the following reaction mixture: 175 mM NaCl, 50 mM of buffers at various pH, chloroplasts (20 μg chlorophyll per ml) and, where indicated, fatty acids. Since fatty acids were dissolved in ethanol, all reaction mixtures contained 0.5% ethanol.

Light-induced shrinkage was evaluated by measurement of the light-scattering increase at 90° (546 nm) with a modified[11] photovolt fluorimeter (Model 540). A primary interference filter (Balzers B-40/539) isolated the green light used for the scattering measurements whereas a secondary interference filter (Baird Atomic) prevented interference by actinic light (> 600 nm, Kodak filter, wratten N_{26}) emitted from one of the cuvette sides. The temperature was maintained at 20 °C by circulating water

around a jacketed 1-cm cuvette, containing the following reaction mixture: 50 mM of buffer at various pH values, 35 mM NaCl, 5 mM $MgCl_2$, 20 μM phenazine methosulfate (PMS) or 1 mM $Fe(CN)_6^{3-}$, 0.5% ethanol, chloroplasts (20 μg chlorophyll per ml), and where indicated, fatty acids. Increases in scattering intensity following treatment with red light are expressed as percent changes of the initial scattering level (100% light scattering).

Measurements of photochemical activities

Electron transport for Photosystem II (from H_2O to $Fe(CN)_6^{3-}$) was measured spectrophotometrically at 420 nm by the photoreduction of $Fe(CN)_6^{3-}$ as the oxidant, in the following reaction mixture: 50 mM of buffer at various pH, 35 mM NaCl, 5 mM $MgCl_2$, 1 mM $K_3Fe(CN)_6$, 0.5% ethanol, chloroplasts (20 μg chlorophyll per ml), and where indicated, fatty acids.

Electron transport for Photosystem I (from reduced *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (reduced TMPD) to methyl viologen) was measured polarographically (O_2 electrode, Clark type) by the consumption of oxygen in the presence of methyl viologen as the electron acceptor which is oxidized by oxygen to form H_2O_2 (trapped by inhibiting chloroplast catalase with azide) in the following reaction mixture: 50 mM of buffer at various pH, 35 mM NaCl, 5 mM $MgCl_2$, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 20 μM TMPD, 4 mM sodium ascorbate, 150 μM methyl viologen, 0.5% ethanol, 2 mM NaN_3, chloroplasts (20 μg chlorophyll per ml) and, where indicated, fatty acids. When the systems were coupled, ADP and P_i were added as indicated in each case in the legends to figures. Light-induced O_2 consumption was measured for 3 min without methyl viologen. This latter compound was then added in the light so that the weak background reaction due to TMPD alone could be deducted in the final results.

ATP synthesis was determined by measuring the uptake of P_i (Horwitt's[14] technique) in the following basic reaction mixture: 50 mM of *N*-tris(hydroxymethyl)-methylglycine (Tricine) buffer at pH 7.7 or 8.5, 35 mM NaCl, 5 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM ADP, 20 μM PMS or 1 mM $K_3Fe(CN)_6$, 0.5% ethanol, fatty acids where indicated and chloroplasts (20 μg chlorophyll per ml) over an 8-min period during which photophosphorylation was linear.

Light intensity was approximately $5 \cdot 10^5$ ergs$\cdot$cm$^{-2}\cdot$s^{-1} and the temperature was maintained constant at 20 °C for all these reactions.

Chemicals

Fatty acids, TMPD and all other chemicals (purest form) were purchased from Fluka, except oleic acid and ascorbate purchased from Merck, 2-(*N*-morpholino)-ethanesulfonic acid (MES) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Sigma, DCMU from DuPont de Nemours, methyl viologen from Mann Research Labs, and PMS from Calbiochem.

RESULTS

Fig. 1A shows the effects of increasing linolenic acid concentrations at various pH on the electron transport activity of Photosystem II. As we can see (non-coupled systems), inhibition of the electron flow at high pH was larger than at low pH. In fact, at pH 6.3, the activity was independent of the fatty acid concentrations and

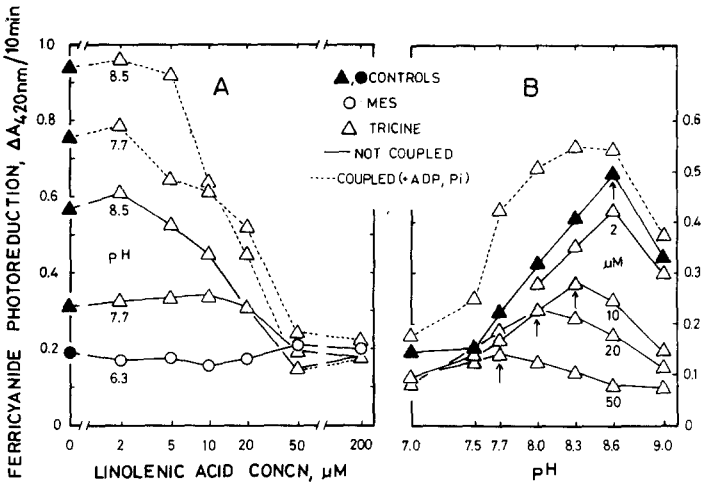

Fig. 1. Effect of linolenic acid on Photosystem II electron flow (from H_2O to $Fe(CN_6^{3-})$ as a function of fatty acid concentration (A) and pH (B). Where indicated, 50 mM of buffers (Tricine or MES) were used and 2 and 4 mM of ADP and P_i, respectively. Other conditions and reaction mixtures are described in Material and Methods. 0.1 $\Delta A_{420\,nm}$/10 min corresponded to 18 μmoles $Fe(CN)_6^{3-}$ reduced per mg chlorophyll per h. In B, arrows show the shift of optimal pH toward acidity.

stayed at the level of a remnant electron flow activity which could not be suppressed, even at high concentrations such as 200 μM of linolenic acid. Coupling the system increased the overall electron flow activity (pH 7.7 and 8.5) which was also inhibited by increasing concentration of fatty acids. In addition, this stimulation became less prominent with increasing fatty acid concentrations. Similar curves were obtained with oleic and linoleic acids under otherwise identical conditions.

In Fig. 1B, it is shown that the pH optimum of the electron flow rate, which was 8.6 under control conditions (no fatty acids), shifted towards the acidic side with increasing concentrations of fatty acids (see arrows). As an example, going from 0 to 50 μM of linolenic acid caused a ΔpH of 0.9 unit (from 8.6 to 7.7). As seen before (Fig. 1A), the overall activity of electron flow decreased with increasing fatty acid concentration.

Obviously, the pH curve shifted approximately 0.5 unit by coupling the system in absence of fatty acids (Fig. 1B). This shift was observed within a pH range of 8.6–7.5. Such a shift due to coupling was also observed to about the same extent and in the same direction in the presence of fatty acids (not shown).

The effect of linolenic acid on the electron transport activity of Photosystem I is illustrated in Fig. 2. In the control experiments, the pH optimum was found to be 8.9 (Fig. 2B). As for Photosystem II, the inhibition caused by fatty acids (oleic, linoleic and linolenic acids) was much greater at high pH than at low pH (Fig. 2A). But one striking difference was that much higher concentrations of fatty acids were needed to inhibit Photosystem I activity. For instance, 50–100 μM of linolenic acid, which were sufficient to inhibit Photosystem II almost completely, even stimulated Photosystem I activity. As a rule, the electron flow rate was not impaired by fatty acid concentrations

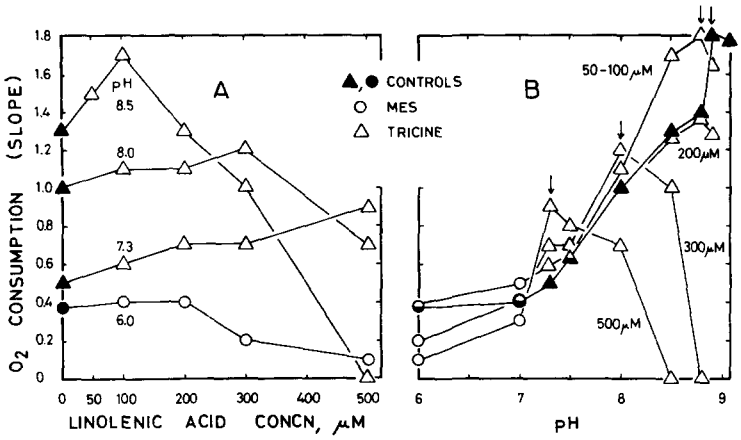

Fig. 2. Effect of linolenic acid on Photosystem I electron flow (from reduced TMPD to methyl viologen) as a function of fatty acid concentration (A) and pH (B). The buffers used were MES from pH 6 to 7 and Tricine from 7.5 to 9.1. Other conditions and reaction mixtures are described in Material and Methods. Slope 1 corresponded to 225 μmoles O_2 consumed per mg chlorophyll per h. In B, arrows show the shift of optimal pH toward acidity.

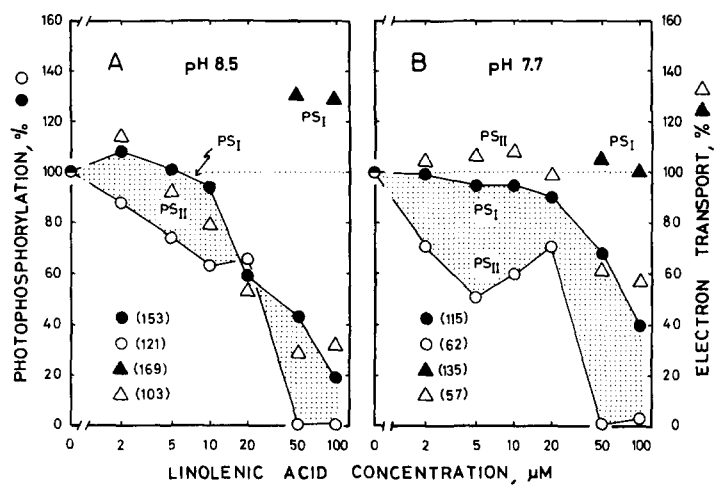

Fig. 3. Effect of linolenic acid on photophosphorylation in Photosystems I (Photosystem I; PMS, ●) and II (Photosystem II; $Fe(CN)_6^{3-}$, ○) at pH 8.5 (A) and 7.7 (B). Triangles refer to electron transport activities obtained in separate experiments reported in Figs 1A and 2A: ◭, Photosystem II ($Fe(CN)_6^{3-}$ photoreduction) and ▲, Photosystem I (methyl viologen photoreduction). Numbers in parentheses correspond to values of the controls (100%) expressed as μmoles P_i esterified per mg chlorophyll per h for photophosphorylation and, as μmoles $Fe(CN)_6^{3-}$ reduced per mg chlorophyll per h (Photosystem II, △) and as μmoles O_2 consumed per mg chlorophyll per h (Photosystem I, ▲). Conditions and reaction mixtures are described in Materials and Methods.

below 200 μM, however, above 200 μM, strong attenuation of the flow rate occurred along with an acid shift of the pH optimum.

When the system was coupled, the pH profiles, the attenuation of electron flow activity and the shift of its pH optimum did not change neither in the absence nor in the presence of fatty acids (not shown).

Since it was thought that the shift of the pH optimum was due to the separation of electron flow from photophosphorylation by fatty acids, the effect of linolenic acid on ATP synthesis was tested in both photosystems. Figs 3A and 3B show that photophosphorylation in Photosystem II ($Fe(CN)_6^{3-}$) was much more inhibited by linolenic acid than in Photosystem I (PMS or from reduced TMPD to methyl viologen) at pH 7.7 as well as pH 8.5. This difference in fatty acids sensitivity was larger at pH 7.7

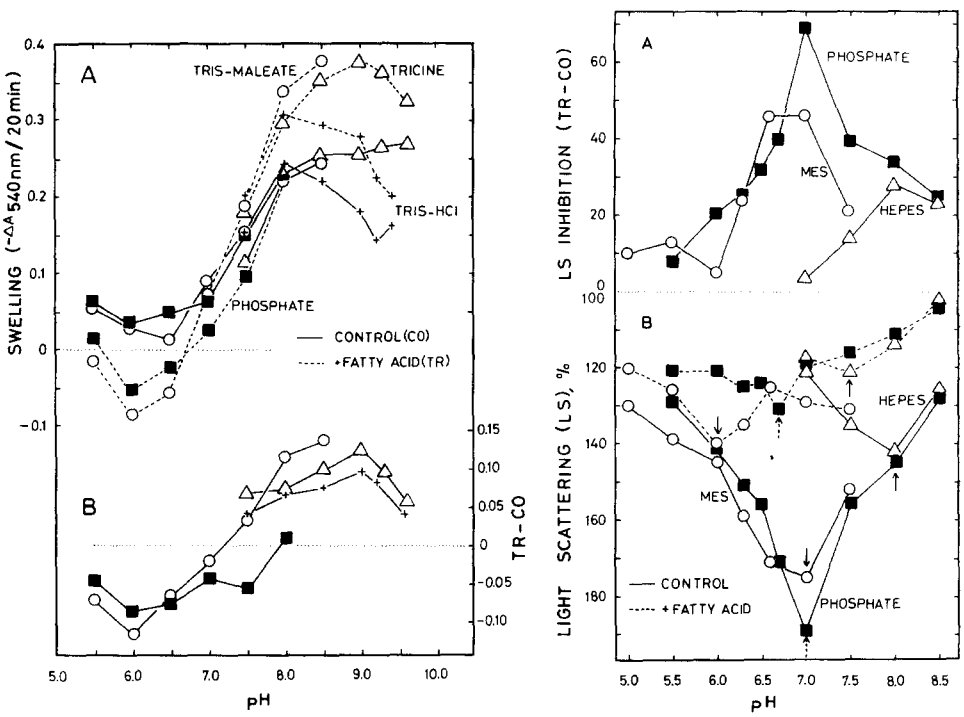

Fig. 4. pH dependence of chloroplast swelling in the presence and absence of oleic acid. Reaction mixtures contained: 175 mM NaCl, 50 mM of buffer (○, Tris–maleate; ■, phosphate; +, Tris–HCl; △, Tricine), chloroplasts (20 μg chlorophyll per ml), 0.5% ethanol, and where indicated, 200 μM oleic acid. In A, swelling is expressed as $-\Delta A_{540\,nm}$ 20 min. In B, swelling stimulation by oleic acid is expressed as treated *minus* control values (TR − CO).

Fig. 5. pH dependence of light-induced chloroplast shrinkage measured by light scattering in the presence and absence of linolenic acid. Reaction mixtures contained: 50 mM of buffer (■, phosphate; ○, MES or △, HEPES), 20 μM PMS, 0.5% ethanol, chloroplasts (20 μg chlorophyll per ml) and where indicated 100 μM linolenic acid. Incubation time in the presence of fatty acid before turning on the actinic light was 3 min. Results corresponded to the extent of the reactions. In A, the inhibition of light-induced scattered light is expressed as treated *minus* control values (TR − CO). In B, arrows show the shifts of optimal pH toward acidity for the three buffers used, following fatty acid addition.

than at pH 8.5 (compare the size of the respective dotted surfaces). Out of the two curves, the one for Photosystem II is biphasic, in the sense that within the range of 5–20 μM, the inhibitory action of fatty acid was reversed into a stimulatory action, followed by a sharp drop in activity. The stimulatory effect which is particularly pronounced at pH 7.7 remains an unexplained but reproducible fact.

In order to compare the fatty acid sensitivity of the electron flow activity with that of photophosphorylation, I have replotted some of the electron flow activities in Figs 3A and B. It can be seen that 2–5 μM of linolenic acid (Photosystem II) and 50–100 μM (Photosystem I) did uncouple whereas 50–100 μM (Photosystem II) and 200–500 μM (Photosystem I) did not uncouple the systems. These concentrations ranges relate to the earlier data concerning the shift of the pH optimum of electron flow rates in the following way: (a) uncoupling ranges (Photosystem I and Photosystem II) lead to no shift of pH optimum; (b) no uncoupling ranges (Photosystem I and Photosystem II) lead to a shift of pH optimum.

In a next experiment, the swelling of chloroplasts as a function of pH was measured without (control) and with 200 μM of fatty acid. Fig. 4B shows that the fatty acid-induced swelling had a broad pH optimum (pH 8–9) but according to Fig. 4A, no displacement of the pH optimum (control *versus* fatty acid) could be discerned.

Light-induced shrinkage was measured in absence or presence of 100 μM linolenic acid, as a function of pH (Figs 5A and B). Again, the shrinkage was pH dependent. The pH optima were 7.0 with phosphate and MES buffer and 8.0 with HEPES buffer in the control. In the presence of fatty acids, an acid shift of the pH optimum of the light-induced shrinkage occurred and the activity was decreased. The pH optimum shifted from pH 8.0 to 7.5 (HEPES), from 7.0 to 6.7 (phosphate) and from 7.0 to 6.0 (MES) (see arrows in Fig. 4B). We know from further experiments that the extent of the peak shift was a function of the linolenic acid concentration. For example, with MES buffers, the data were as follows: 50, 100, 200 μM of linolenic acid generated a shift of the pH optimum from 7.0 to 6.5, from 7.0 to 6.0 and from 7.0 to 5.5, respectively.

In the presence of $Fe(CN)_6^{3-}$ as the electron acceptor, light-induced shrinkage had a pH optimum at 6.3. The addition of linolenic acid (60 μM) inhibited the reaction but did not cause any significant shift of the pH optimum.

DISCUSSION

As mentioned in the introduction, it was postulated that free fatty acids are instrumental in regulatory processes of chloroplast structure[1–4] and photosynthetic activities[4,5,15–17]. Our data further support this postulate and indicate that both, Photosystem I and Photosystem II are affected by C_{18}-unsaturated fatty acids in a sequential manner.

The specificity of fatty acid interaction

The electron flow activities of Photosystem II and Photosystem I were affected by fatty acids but at different concentration ranges. The effective concentration range was lower for Photosystem II than for Photosystem I. In fact, 22 μM of linolenic acid (*i.e.* for a fatty acid/chlorophyll molar ratio of 1.0) at optimal pH depressed the electron flow activity by 50% while the Photosystem I electron flow activity was even

slightly stimulated. The Photosystem I system was inhibited to 50%, only by 370 μM of fatty acid (for a fatty acid/chlorophyll molar ratio of 16.5). These results are in line with earlier reports about inhibitory effects of exogenous[2,5,15–17] or endogenous[18,19] fatty acids on electron transport of Photosystem II. There is also agreement about the sequential nature of the electron flow inhibition [from Photosystem II to Photosystem I] by linolenic acid, as earlier found by fluorescence[4] and absorption[20] measurements.

A similar sequential inhibition was also found for Photosystem II and Photosystem I associated photophosphorylation (see Fig. 3). However, when comparing the fatty acid concentrations which inhibited photophosphorylation with those inhibiting electron transport, we realized that ATP synthesis was more sensitive than the electron transport activity in Photosystem I. For instance, at pH 8.5, 50% inhibition of ATP synthesis or electron flow activity were achieved with 20 or 370 μM of fatty acids, respectively. In Photosystem II, the two photochemical activities were about equally inhibited at pH 8.5 (see Fig. 3A). However, at pH 7.7, the profile for ATP synthesis did not parallel that of the electron flow activity but showed the so far unexplained biphasic character (see Fig. 3B). As far as known, none of these phenomena concerning the selective action of fatty acids on ATP formation have been reported.

For the chloroplast swelling as a function of fatty acid concentrations, no shift in the pH optimum was found. This suggests that the swelling mechanism is not directly related to the electron transport or photophosphorylation[13,21] reactions but is rather a sign of membrane deterioration[1,11]. Contrarily, light-induced thylakoid shrinkage (light-scattering effect) shows a shift in the pH optimum indicating a relationship to the photochemical reaction[13,21].

Uncoupling effect of fatty acids and shift in pH optimum

Fatty acids are known to be uncoupling agents[5]. Our data indicate that Photosystem II at low fatty acid concentrations did uncouple, but the pH optimum remained unchanged. It is only at higher fatty acid concentrations that a shift in the pH optimum became discernible but this shift occurred both under coupled and not coupled conditions. Therefore, the statements[7,9,10] that the shift in pH optimum is a direct function of uncoupling are generally not valid.

The shift of the pH optimum can be explained in a better way by assuming that the rate of electron flow is controlled not only by the degree of energy coupling but also by the internal pH of the thylakoid compartment[22–24]. In fresh chloroplasts, light initiates the proton pump mechanism which results in a lowering of the inner compartment pH. At optimal external pH (8.0–8.5 for electron flow), this light-induced mechanism produces an internal pH of approx. 6 which is precisely the optimal internal pH for electron transport[22]. In the presence of fatty acids, the membrane gets deteriorated and becomes leaky to water[1] and supposedly to protons (change in the diffusion constant). Simultaneously, the light-induced proton uptake gets impaired (unpublished results). At external pH of 8.5, the active uptake of protons is insufficient to lower the internal pH to 6 and, therefore, the electron transport is inhibited (see Figs 1 and 2). On the other hand, by lowering the external pH and thereby increasing the proton concentration, proton diffusion into the thylakoid compartment is accelerated, which restores the favorable conditions for electron transport. This, in essence, would explain the shift in pH optimum.

The physiological significance

The above results can be related to the known effects of aging on the structure and photochemical activities in chloroplasts[1,11,17,25,26]. A similarity between the effects of fatty acids and aging was postulated[1,11], on the basis that during aging *in vitro* the amount of endogenous free fatty acids (especially unsaturated acids) increased in chloroplasts, consequently to membrane lipids hydrolysis[4,5,18,19]. These acids were shown to interact with the structure and functions of the thylakoid membrane. For instance, fatty acids induced swelling and inhibited light-induced shrinkage[1], both effects being a characteristic of aging *in vitro*[11]. Also, at appropriate conditions of concentration and incubation time, fatty acids and aging were shown to uncouple[5,25] and inhibit Photosystem II electron transport activity[4,5,15–19,25]. If aging causes the same sequential inhibition of electron flow as fatty acids, one might expect that, during aging of chloroplasts, the Photosystem II related activities (O_2 evolution, non-cyclic electron transport and ATP synthesis) disappear progressively in behalf of Photosystem I associated reactions. Therefore, the source of reducing power would be provided through Photosystem I only, from a natural donor other than water and ATP supplemented through cyclic photophosphorylation. Cyclic ATP synthesis would then play a direct role in photosynthetic CO_2 assimilation, as confirmed recently[27].

Thus, these results confirm that the study of the effects of fatty acids on the structure and function of chloroplasts is a valuable approach to gain some insight in aging processes.

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