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Aging of the photosynthetic apparatus VI. Changes in pH dependence of ΔpH, thylakoid internal pH and proton uptake and relationships to electron transport

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The pH difference generated across the chloroplast membrane upon illumination (ΔpH) and the internal pH (pHi) were analyzed in aged spinach chloroplasts and in fresh chloroplasts supplemented with linolenate. In electron-flow conditions where both photosystems or either photosystem alone were functional, the ΔpH dropped and their optima shifted toward more acidic external pH (pHo) with a simultaneous increase in pHi. Upon aging or addition of linolenate, a decrease of pHo was therefore required to maintain the pHi in the range of 5–5.5 for maximum electron-flow activity. Moreover, aging like linolenate, diminished the proton pump activity and shifted its optimum (pH 6.7 in the controls) toward higher pHo. Although ΔpH and pHi changes were similar in all electron-flow conditions, the sensitivity of ΔpH toward aging and linolenate was eventually higher under photosystem II than photosystem I conditions.

In conclusion, the electron-flow activity seems to be delicately controlled by the proton pump, ΔpH, pHi and pHo. Unsaturated fatty acids which are released during chloroplast aging damage the membrane integrity in such a way that the subtle equilibrium between these factors is disturbed.

Free fatty acids control the structure (*5, 15, 16, 22, 27*) and function (*3, 13, 14, 28–31*) of chloroplast membranes. For instance, the inhibition of photosynthetic electron flow by unsaturated fatty acids is pH-dependent: linolenic acid causes a shift in the pH optimum toward acidity (*28, 29, 31*). This phenomenon was shown to be related to the thylakoid membrane integrity as suggested by the action of exogenous linolenic acid on ΔpH, pHi and proton uptake (*29*).

Aging of isolated spinach chloroplasts results also in an acidic shift of the pH optimum for electron flow through both photosystems or through either photosystem alone (*14, 31*). This was correlated with the release of unsaturated fatty acids, predominantly linolenate, which takes place in the membrane environment during aging (*31*).

Using the same conditions as those adopted in the electron-flow studies (*31*), we verified that aging and linolenic acid have similar effects on light-induced ΔpH, pHi and proton uptake in thylakoid membranes. These results confirm that the factors which do regulate the aging process are mainly due to the level of free fatty acids in the thylakoid membranes.

Material and methods

Spinach (*Spinacia oleracea* var. Nobel) was grown in a growth chamber (*29*). Thylakoid membranes were prepared as described previously (*29*) and resuspended in 25 mM HEPES (pH 7.6) and 0.35 M sucrose to 2 mg chlorophyll/ml or in the aging medium (25 mM HEPES, pH 7 and 175 mM NaCl) to 1 mg chlorophyll/ml. Thylakoid membranes were dark-aged at 20°C and portions were removed at various times for ΔpH and proton pump measurements. All isolation operations were carried out at 4°C and in dim light. Chlorophyll was determined spectrophotometrically (*4*).

The ΔpH in chloroplast membrane systems was estimated from the extent of the fluorescence quenching of 9-aminoacridine, employing the technique originally proposed by Schuldiner et al. (*21*). The fluorescence was measured with a spectrofluorimeter adapted for illumination on one side of the cuvette. Actinic light (approximately 5×10^5 ergs·cm^{-2}·sec^{-1} provided by a halogen lamp, was passed through two Calflex and a Corning CS-260 filters. Monochromatic exciting light (390 nm), provided by a Bausch and Lomb high-intensity monochromator (xenon light source combined with a visible grating), was passed through a PAR light chopper (Model 125 A, 48 aperture wheel) before reaching the 1-cm cuvette. The fluorescence emission was detected at a 90° angle with an EMI photomultiplier tube (quartz window) monitored by a Bausch and Lomb monochromator at 460 nm. The chopper and the photomultiplier tube were connected to a PAR lock-in amplifier (Model 128 A) and the fluorescence signal was recorded with a W+W recorder (Model 1200). The osmotic volume (V) of the chloroplast preparation was estimated from the data provided by Rottenberg et al. (*19*) and was found to be 32.5 μl/mg of chlorophyll in our experimental conditions. The ΔpH was obtained from the relationship $\Delta\text{pH} = \log [Q/(1-Q) \times 1/V]$ (Q=fraction of the total fluorescence that was quenched) and the internal pH (pHi) calculated by subtracting the ΔpH from the external pH (pHo). The basic reaction mixture contained: 30 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) or N-tris (hydroxymethyl) methylglycine at various pH, 40 mM NaCl, 0.8 μM 9-aminoacridine, 0.5% ethanol or linolenic acid, and chloroplasts (20 μg chlorophyll/ml). According to the type of electron flow studied, various electron donors and acceptors were added as indicated in the figure legends. When linolenic acid, DCMU, diaminodurol (dissolved in ethanol) and DBMIB (dissolved in methanol) were added to these reaction mixtures, the appropriate controls were made with ethanol (0.5%) and methanol. At the concentrations used, ethanol and methanol had no detectable effect on electron flow, ΔpH, pHi and proton uptake.

To measure the proton pump, chloroplasts were isolated as indicated previously but resuspended in 175 mM NaCl (pH 8) without buffer. The pH changes were measured with a Metrohm pH-meter (Type E 300 B) and recorded continuously (*17*). The reaction mixture (5 ml) contained 35 mM NaCl, 20 μM phenazine methosulfate, 0.5% ethanol and chloroplasts (40 μg chlorophyll/ml). Light intensity of the actinic light was approximately 5×10^5 ergs·cm^{-2}·sec^{-1} and the temperature was 20°C. The μ equiv. H^+ taken up per mg chlorophyll was estimated from the data provided by Walz et al. [see Fig. 1A in (*32*)].

9-Aminoacridine hydrochloride monohydrate and linolenic acid were obtained

from Fluka. Dibromothymoquinone was kindly provided by Drs H. Baltscheffsky and A. Trebst.

Results

The influence of chloroplast aging on light-induced ΔpH is illustrated in Fig. 1 for three types of electron flows which were previously studied (*31*). The ΔpH of the controls increased as a function of pHo with a maximum around 9 for PS II+I and PS I (Fig. 1A and B) and around 8–8.5 for PS II (Fig. 1C) conditions. The ΔpH values of the PS II+I controls agree with those reported by Avron's group (*2, 19–21*). However, at acid pHo, the ΔpH values for the PS II controls were generally lower than those for the two other electron-flow systems. When the chloroplasts were aged, the ΔpH dropped for all three electron flow conditions. For example (Fig. 1A), at high pHo (9.0), the ΔpH decreased from 3.1 to 0 after 6 hr of chloroplast aging. At lower pHo, the extents of the ΔpH drop were smaller. This implies that aging caused a shift of the ΔpH optimum toward more acidic pHo and around pHo 7.5, had a much smaller effect on the ΔpH. Although the ΔpH changes were similar in the three types of electron-flow conditions, it is evident that the sensitivity of the proton concentration gradient to aging was eventually higher under PS II than PS I conditions (see for example the effect of 4-hr incubation in Fig. 1B and 1C).

Fig. 2 shows that the effects of linolenic acid on ΔpH were similar to those initiated by chloroplast aging. The basic features described in Fig. 1 were again observed upon the addition of linolenic acid: (a) a ΔpH drop which was greater at

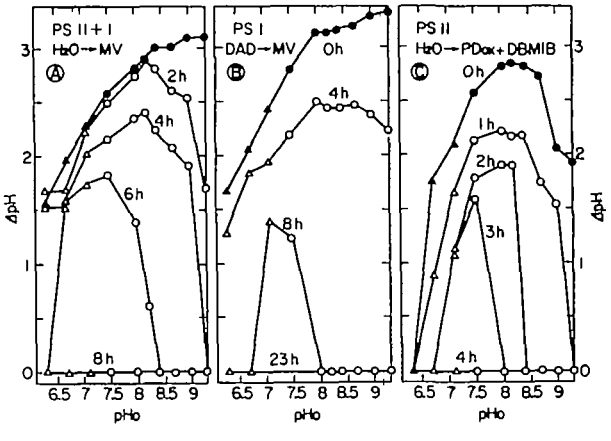

Fig. 1. *ΔpH Dependence on pHo and chloroplast aging expressed in hours (h) in three different electron-flow systems.* (A) In photosystems II+I (from H_2O to methylviologen): the basic reaction mixture was supplemented with 0.15 mM methylviologen and 2 mM NaN_3. (B) In photosystem I only (from diaminodurene to methylviologen): the basic reaction mixture was supplemented with 10 μM DCMU, 300 μM diaminodurene, 2 mM sodium ascorbate, 0.15 mM methylviologen and 2 mM NaN_3. (C) In photosystem II only (from H_2O to oxidized *p*-phenylene diamine): the basic reaction mixture was supplemented with 0.2 mM *p*-phenylene diamine, 1.2 mM $K_3Fe(CN)_6$ and 1 μM DBMIB. ▲, ●, controls; △, ▲, HEPES (pH 6.3–7.5); ○, ●, tricine-glycine (pH 7.5–9.3).

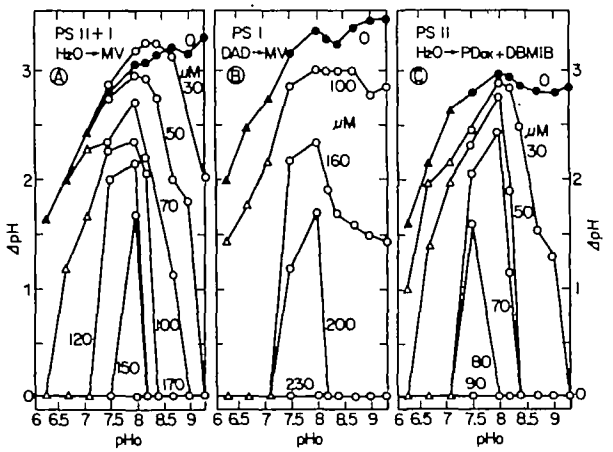

Fig. 2. *ΔpH Dependence on pHo and linolenic acid concentration in the three different electron-flow systems.* (A) Photosystems II+I. (B) Photosystem I alone. (C) Photosystem II alone. Conditions were as described in **Material and methods** and Fig. 1.

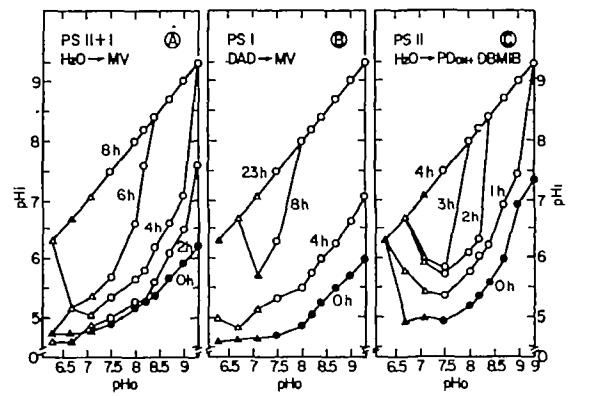

Fig. 3. *pHi Dependence on pHo and chloroplast aging expressed in hours (h).* The electron-flow systems and conditions were the same as in Fig. 1.

high than at low pHo, (b) a shift of the ΔpH optimum toward acidity, (c) a greater sensitivity of ΔpH under PS II (and PS II+I) than under PS I conditions, (d) a smaller inhibition of ΔpH in the pHo 7.5–8.0 region.

Since the rate of electron transport seems to be controlled not only by ΔpH but also by pHi (*2*), we investigated the dependence of pHi on various pHo during the aging process (Fig. 3) and compared it with the pHi of fresh thylakoids supplemented with linolenic acid (Fig. 4). In control experiments (closed symbols), pHi increased when pHo was raised. The maximum electron flow is known to be around pHo 8.7 in the H_2O/methylviologen, 8.5 in the diaminodurol/methylviologen and 7.8 in the H_2O/oxidized *p*-phenylenediamine systems (*31*). The corresponding internal pH values were around 5.5, 5.3 and 5.1, respectively. These pHi values

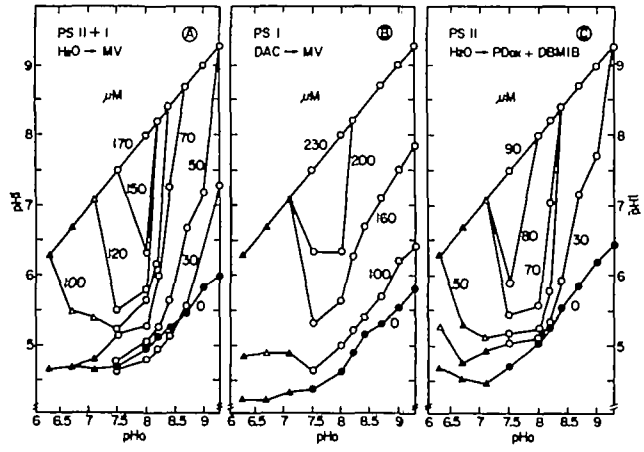

Fig. 4. *pHi Dependence on pHo and linolenic acid concentrations* (μM). The electron-flow systems and conditions were the same as in Fig. 1.

agree well with those reported for other electron flow conditions (*2*, *19–21*). Fig. 3 and 4 show also that below pHo 8, the pHi values were generally constant, probably indicating the existence of a natural buffering capacity in the acidic pHi range as suggested by Schuldiner et al. (*21*). Both aging (Fig. 3) and addition of increasing concentrations of linolenic acid (Fig. 4) displaced the curves toward higher pHi in the same way. For example, at pHo 8.5 in the PS I system alone, the pHi were respectively 5.3, 6.1, 8.5 after 0 (control), 4 and 8 hr of aging (Fig. 3B) and 5.2, 5.5, 6.8 and 8.5 in the presence of 0 (control), 100, 160 and 200 μM of linolenic acid (Fig. 4B). At pHo 7.5, the pHi were respectively 4.7, 5.3, 6.3 and 7.5 after 0, 4, 8 and 23 hr of aging (Fig. 3B) and 4.4, 4.7, 5.3, 6.3 and 7.5 in the presence of 0 (control), 100, 160, 200 and 230 μM of linolenic acid, respectively (Fig. 4B). Since a pHi around 5.2 was the optimum for photosystem I electron flow, in the course of chloroplast aging or in the presence of increasing concentration of linolenic acid, a decrease of pHo would be required to maintain pHi in the proper range (pHi 5.2) for maximum activity. This is also true for the two other types of electron-flow conditions, i.e., PS II+I (Fig. 3A and 4A) and PS II alone (Fig. 3C and 4C). Moreover, Fig. 3 and 4 show that at pHo 7.5±0.5, aging and linolenic acid had smaller effects on the pHi, maybe due to a buffering capacity which was more effective in this pH range.

In order to test this buffering capacity, the extent of proton uptake, as well as ΔpH and pHi were plotted as a function of pHo and compared for fresh and aged chloroplasts. Fig. 5 shows that in the controls, the pHo optimum of proton uptake (6.7) was two to three units lower than the pHo optimum of ΔpH (9.0). After three hr of aging, both the proton uptake and ΔpH diminished and the optima were displaced toward one another (see arrows in Fig. 5). If the extent of the proton pump activity is basically a measure of the internal buffer capacity (*19*), one may infer that aging, like linolenic acid (*29*), decreases the buffering capacity. After three hr of aging, all conditions (proton uptake, ΔpH, pHi and pHo) were optimal for electron-flow activity, only around pHo 7.4, as found previously in the presence of linolenic acid (*29*).

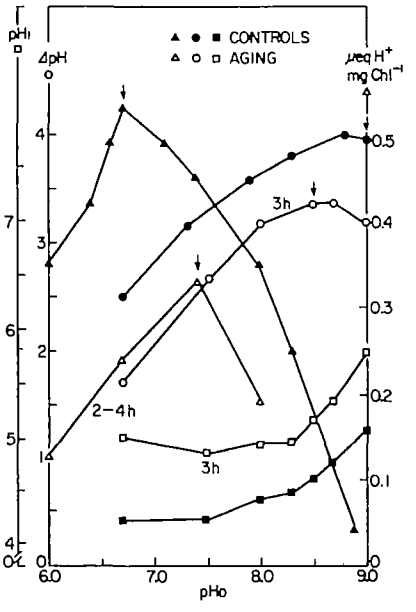

Fig. 5. *Effect of chloroplast aging (h) on proton uptake (▲, △) ΔpH (○, ●) and pHi (□, ■) as a function of pHo.* The experiments were carried out under photosystem I conditions (20 μM phenazine methosulphate). The μequiv. H^+ taken up per mg chlorophyll was estimated as described previously (*29*). According to Walz et al. (*32*), the proton binding of chloroplasts in the light is a linear function of pH in the range of 6 to 9, which was the range we used.

Discussion

We have recently reported that both aging of isolated thylakoids and addition of linolenate to fresh thylakoids result in a similar acid shift of the pH optima for electron flows through both photosystems or either photosystem alone (*31*). This phenomenon can now be explained or at least correlated with the integrity of the thylakoid membrane and its diminishing ability to create a proton gradient between the inner and outer spaces of the membrane during the aging process.

In thylakoids aged in vitro or supplemented with linolenate, the membrane deteriorates what is accompanied by decreases in the proton uptake (see Fig. 5 and ref. *29*) and ΔpH; the extent of the decreases depends upon aging time (Fig. 1) or linolenate concentration (Fig. 2). As a consequence, pHi increases eventually reaching pHo (Fig. 3 and 4). As these pH values are no longer optimal for electron flow, the activity is inhibited (*31*). Since the size of ΔpH is lowered, the pHo optimum for electron flow has to be shifted toward the acidic side in both thylakoids aged in vitro or supplemented with linolenate (*28, 29, 31*). Under these conditions, one can postulate that the fluxes of cations and anions are altered in such a way that swelling occurs (*12, 23, 27*) and light-induced shrinkage decreases (*23, 27*). Although light-induced ΔpH through both electron-flow conditions were affected by aging and linolenate, the ΔpH through PS II were much more sensitive than those through PS I conditions (Fig. 1 and 2). This finding is similar to and can be nicely correlated with the observations of the electron-flow activities themselves (*24, 28, 31*). Moreover, the overall extent of proton uptake (internal buffering capacity) is diminished in aged thylakoids and its optimum shifts from pHo 6.7 to higher pHo as suggested by the results in Fig. 1, 3 and 5. Thus, in addition to swelling (*18, 23, 27*), light-induced shrinkage (*23, 27*), *o*-diphenol oxidase activity (*25, 26*), photo-

phosphorylation (*24*, *28*) and electron transport activities (*3*, *5*, *6*, *9*, *10*, *13–15*, *22*, *24*, *28–31*, *34*), the ΔpH, pHi and proton uptake are affected in the same way by aging and added linolenate. Therefore, as reported previously (*27*), one can simulate the aging process in thylakoids by using linolenic acid.

The factors which do regulate the aging process, especially the accumulation of free fatty acids in the thylakoid membranes, are still unknown. However, the present investigation suggests that aging is controlled, at least in part, by a pH mechanism. Galactolipases which are bound to thylakoid membranes (*1*) are known to hydrolyze both mono- and digalactosyldiglyceride, the pH optima for the two substrates being respectively 7.5 and 5.9 for the spinach enzyme (*11*). We can consider that in intact thylakoids, light generates a pHi of approximately 4.5 to 5.0 (Fig. 3 and 4) when pHo are respectively 7.4 and 7.9 [these values being the pH of the stroma in darkness and light (*33*)]. In this pHi range, galactolipases (their localization within the membrane remain unknown) are probably almost inactive and no free fatty acids are released. On the contrary, in aged chloroplasts, the pHi increases thus creating conditions favorable for lipid hydrolysis.

Finally, we would like to mention that the fluorescence quenching of 9-aminoacridine as an indicator of the pH difference generated across the chloroplast membrane upon illumination has been questioned seriously by Fiolet et al. (*7*, *8*). Although not incompatible with the energization theory proposed by these authors, our results fit well with the theory of Schuldiner et al. (*21*).

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