Biochimica et Biophysica Acta, 548 (1979) 128—138

BBA 47742

A QUANTITATIVE STUDY OF THE SLOW DECLINE OF CHLOROPHYLL *a* FLUORESCENCE IN ISOLATED CHLOROPLASTS

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(Received March 2nd, 1979)

Key words: Chlorophyll; Fluorescence quenching; Proton uptake; Cation effect; (Chloroplast)

Summary

A detailed study of the photo-induced decline in chlorophyll *a* fluorescence intensity (Kautsky phenomenon) in coupled isolated chloroplasts from a high level (P) to a low stationary level (S) is presented.

1. A linear relationship between P → S quenching and intrathylakoid H^+ concentration was found. When the light-induced proton gradient was abolished by uncoupling, the fluorescence emission at room temperature was lowered proportionally to increased H^+ concentration in the medium.

2. Fluorescence spectra at −196°C of samples frozen at the P and S states showed no significant differences in the Photosystem I/Photosystem II ratio of fluorescence emission. Furthermore, freezing to −196°C reversed the P → S quenching. This indicates that the P → S quenching is not related to an increase of spillover of excitation energy from Photosystem II to Photosystem I.

3. When Mg^{2+} was added to thylakoids suspended in a medium free of divalent cations, the inhibition of spillover required lower Mg^{2+} concentrations (half saturation at 0.6 mM) than the restoration of the P → S quenching (half saturation at 2 mM Mg^{2+}). Increased proton concentration in the medium also inhibited spillover.

4. The results are interpreted in terms of two sites of Mg^{2+} and H^+ effects on excitation deactivation in Photosystem II. One site is located on the outer face of the thylakoid membrane; action of both Mg^{2+} and H^+ at this side diminishes spillover. The second site is located on the inner face of the membrane; as Mg^{2+} is displaced there by protons, a non-photochemical quenching of Photosystem II fluorescence is induced, which is manifested by the P → S decline.

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Mes, morpholinoethanesulfonic acid.

Introduction

In numerous studies of intact cells and intact and broken chloroplasts the slow chlorophyll *a* fluorescence decline from the initial peak (P) to a steady state (S) of relatively low fluorescence emission has been investigated (see Ref. 1 for a recent review and Refs. 2—4). It has been suggested that this photo-induced quenching, generally called P → S phase, involves structural changes associated with the movement of Mg^{2+} which is correlated to proton uptake into the thylakoids. It was attractive to identify P and S levels, respectively, with the high and low fluorescence yield corresponding to low and high 'spillover' of excitation energy from Photosystem II to Photosystem I or, in more general terms, to changes in the distribution of excitation energy between the two photosystems. Such 'spillover changes' are obtained either by variation of Mg^{2+} concentration in suspensions of thylakoid membranes or by preillumination of intact cells (see Ref. 1).

To test the possible relationship between P → S quenching and changes in energy distribution, we determined the amplitude of spillover at the states of high and low fluorescence emission in the P → S phase of coupled broken chloroplasts and compared this to the spillover changes induced by $MgCl_2$ addition to broken chloroplasts initially suspended in a low salt medium (10 mM NaCl).

As the P → S quenching is correlated to proton uptake, it may depend on the acidification of the inside compartment of the thylakoids or on the energized state of the membrane. These possibilities have been discussed for whole cells by Papageorgiou and Govindjee [5] and by Papageorgiou [6]. Besides, Wraight et al. [7] showed that in isolated chloroplasts acidification decreases Photosystem II fluorescence. In the present study, we compared the effects on fluorescence of acidification of the intrathylakoid compartment (by the photo-induced proton uptake) and acidification of both sides of the thylakoid membrane (incubating gramicidin-treated chloroplasts at various pH).

Materials and Methods

Broken chloroplasts were isolated from pea leaves according to a procedure previously described [8] except that ascorbate was omitted in the grinding medium. The final resuspension medium contained, unless indicated otherwise, 0.4 M sorbitol, 10 mM NaCl, 20 mM sodium ascorbate and 10 mM tricine, pH 7.5. Chlorophyll *a* fluorescence was measured at room temperature using an exciting light beam at 480 ± 10 nm. The intensity was such that 30 photons were absorbed per reaction center and per second (as determined by the half time of fluorescence rise in the presence of DCMU). The fluorescence was detected through a Corning CS 2-64 filter, or at 685 ± 3 nm through a monochromator. Chloroplast samples (10 μg chlorophyll per ml) were placed in a 1-cm cuvette and stirred during measurements.

Fluorescence spectra at liquid nitrogen temperature were determined using a device similar to the appatus described by Cho and Govindjee [9]. A 0.5-ml sample of the chloroplast suspension containing 50 μg chlorophyll per ml was adsorbed on one layer of cheese-cloth which covered the bottom of the trans-

parent Dewar flask. The half band width for fluorescence analysis was 3 nm.

Light-induced proton uptake was measured by the fluorescence quenching of 9-aminoacridine (final concentration $2 \cdot 10^{-6}$ M). Simultaneously, chlorophyll fluorescence was also recorded. Experimental conditions were the same as for chlorophyll fluorescence measurement at room temperature (above) except that the wavelength of the exciting light was changed to 420 ± 15 nm, keeping an intensity corresponding to 30 photons absorbed per reaction center and per second. The fluorescence of 9-aminoacridine was detected at 500 ± 5 nm. The H^+ concentration inside the thylakoid was calculated using the following formula:

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \left(\frac{F_{max}}{F_{sta}} - 1\right) \frac{V_e}{V_i},$$

where $[H^+]_{in}$ and $[H^+]_{out}$ are the H^+ concentrations inside and outside the thylakoid, respectively, F_{max} is the maximum fluorescence of 9-aminoacridine of the dark adapted sample and F_{sta} is the steady state of 9-aminoacridine fluorescence in the light. V_e and V_i are the volumes of the outer and inner space of the thylakoid suspension, respectively. V_e/V_i is determined according to Nobel [10]:

$$\frac{V_e}{V_i} = \frac{\text{osmolarity of the medium}}{2 \cdot \text{chlorophyll molar concentration}}$$

Results

Fig. 1 (left traces) shows that in the absence of ascorbate broken chloroplasts do not exhibit a slow photo-induced P → S quenching even when $MgCl_2$ is present. The addition of ascorbate (right trace), azide or $NADP^+$ plus ferredoxin (not shown) restalishes the characteristic P → S quenching in the suspension which contains $MgCl_2$. The presence of ascorbate, azide or $NADP^+$ + ferredoxin slightly lowers the maximum fluorescence level. In gramicidin-treated chloroplasts, where no photo-induced quenching occurs, the same diminution is observed. With the technique described in Ref. 11, we can show that addition of ascorbate or azide to gramicidin-poisoned chloroplasts causes an enhancement of Photosystem I electron transport. Ascorbate (10 mM) stimulated the rate of Photosystem I by a factor of 2.3, sodium azide (0.25 mM) by a factor of 2.5. (For comparison, $2.5 \cdot 10^{-6}$ M methyl viologen increased the rate by a factor of 8.4).) The slight decrease of the P fluorescence level seen in Fig. 1 upon addition of ascorbate thus can be attributed to partial oxidation of Q, the primary acceptor of Photosystem II.

All following experiments were performed in the presence of ascorbate and in saturating light, corresponding to 30 photons/center per s. Under these conditions the photo-induced fluorescence quenching is fully reversible, as is the P → S quenching of intact chloroplasts (cf. Ref. 4). In the dark the half time of reversion was 20 s. Uncoupling with gramicidin (10^{-6} M) accelerated the reversion ($t_{1/2}$ = 3 s). The addition of ADP (2 mM) reversed about half of the P → S decline ($t_{1/2}$ = 60 s). As previously observed by Mills and Barber [12] in intact chloroplasts, the reversion of the quenching upon DCMU addition

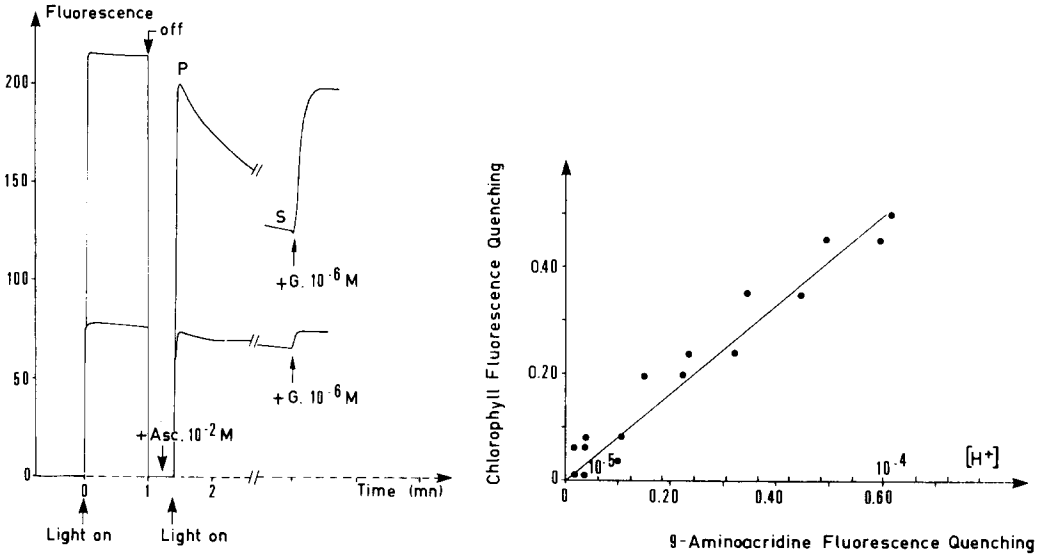

Fig. 1. Chlorophyll *a* fluorescence as a function of time of illumination under various conditions. Chloroplasts were isolated and resuspended in ascorbate-free media. The lower traces are obtained with a chloroplast suspension which does not contain $MgCl_2$, the upper ones with a suspension containing 10 mM $MgCl_2$ added to the resuspension medium. The addition of 10 mM ascorbate in the dark is indicated by +Asc, the addition of 10^{-6} M gramicidin by +G.

Fig. 2. Relationship between P → S chlorophyll fluorescence quenching and proton concentration inside the thylakoid created by light-induced proton uptake. The extent of the photoinduced P → S quenching, defined as (P/S) − 1, is plotted versus the photoinduced quenching of 9-aminoacridine fluorescence in chloroplast suspensions containing 10 mM $MgCl_2$. The different points are obtained by adding DCMU in various concentrations (from zero to 10^{-6} M).

(10^{-6} M) at the S level in broken chloroplasts shows two phases. In our experiments the amplitude of the faster one (less than 1 s) represented only about 2% of P minus S fluorescence levels. This indicates that the quencher Q is in S, as in P, almost totally reduced.

The addition of increasing amounts of DCMU from 10^{-8} to 10^{-6} M to samples containing 10 mM $MgCl_2$ in the resuspension medium gradually inhibits the formation of the light-induced ΔpH and the P → S quenching. Fig. 2 is a plot of the P → S fluorescence quenching amplitude versus the internal proton concentration calculated from the 9-aminoacridine signal in the presence of various DCMU concentrations. It appears that the P → S quenching is directly proportional to the H^+ concentration in the intrathylakoid space.

A similar linear relationship between chlorophyll fluorescence quenching and proton concentration is observed in chloroplasts uncoupled with gramicidin, in the presence of 10 mM $MgCl_2$ (Fig. 3a). In this case, the pH is imposed on the thylakoids using 2-(*N*-morpholino)ethanesulfonic acid (Mes). Due to the action of gramicidin, the pH in the intrathylakoid space should be close to that of the medium. It should be noted that a comparison of Figs. 2 and 3a reveals a large difference in the H^+ concentration required in the two types of experiments to achieve the same extent of chlorophyll fluorescence quenching. From Fig. 3b, showing the fluorescence intensities of uncoupled chloroplasts as a function of pH, it can be seen that the pH also affects the chlorophyll fluorescence ampli-

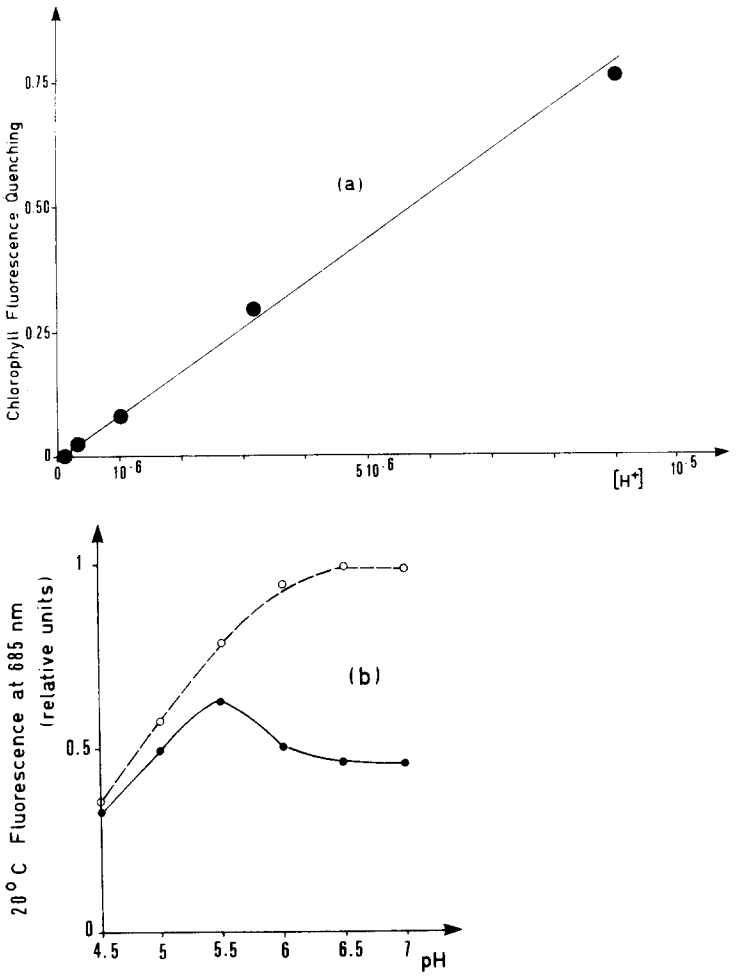

Fig. 3. Effect of pH on the fluorescence intensity of uncoupled broken chloroplasts. The basic resuspension medium contained 0.4 M sorbitol, 10 mM NaCl, 10^{-6} M gramicidin and 10 mM Mes, the pH of which was adjusted to the different values. (a) Quenching of chlorophyll fluorescence as a function of the proton concentration imposed on chloroplast suspensions containing 10 mM $MgCl_2$. The quenching is defined by the equation:

$$\frac{\text{Fluorescence at pH 7.0}}{\text{Fluorescence at pH x}} - 1$$

and was calculated from the data given in (b) (○). (b) Steady states of fluorescence intensity of chloroplast suspensions. ●———●, minus $MgCl_2$; ○- - - - -○, plus 10 mM $MgCl_2$.

tude of the chloroplast suspension which does not contain $MgCl_2$ (solid points). An optimal amplitude of fluorescence is observed near pH 5.5.

Fig. 4 demonstrates the effects of cations (Mg^{2+} and Na^+) on the P → S quenching (a), the maximum fluorescence yield P (b), and on the ΔpH (c). The ΔpH is independent of both Na^+ and Mg^{2+} concentrations, if 10 mM NaCl is present in the basic resuspending medium.

Curves (a) show that the cation requirement for the P → S quenching is larger for Na^+ than for Mg^{2+} as has been observed previously [13]. The comparison of

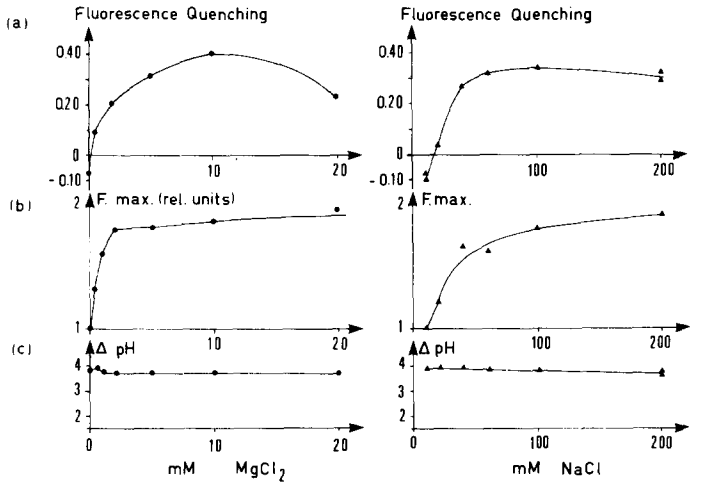

Fig. 4. Effect of $MgCl_2$ and NaCl concentrations on (a) P → S chlorophyll fluorescence quenching, (b) on maximum chlorophyll fluorescence, P, and (c) on the light-induced proton gradient. ΔpH values (C) were calculated from photo-induced quenching of 9-aminoacridine fluorescence.

curves (a) and (b) indicates that spillover inhibition, represented by the increased fluorescence emission at the P level (b), is more sensitive to Mg^{2+} than the P → S decay (a); the half concentrations for saturation of the effects are 0.6 and 2 mM, respectively. The difference in saturation between Fig. 4(a) and (b) is not obvious for Na^+.

In order to detect possible changes in spillover occurring in relation to the P → S decline, chloroplast samples were frozen in liquid nitrogen in the P and S states. As a measure of spillover the ratio of the 735/685 nm fluorescence at −196°C was determined. Table I shows the relative fluorescence intensities of chloroplasts at 20°C (685 nm) and at −196°C (685 and 735 nm). At 685 nm, the quenching observed in chloroplasts devoid of $MgCl_2$ is also seen at −196°C. In contrast, the freezing of chloroplasts in the S state reestablishes a high level of fluorescence, identical with the P level. Noticeable are also the similar values of 735 nm fluorescence in chloroplasts frozen in P and S states. In contrast, chloroplasts plus and minus $MgCl_2$ show the typical variations of their Photo-

TABLE I

RELATIVE FLUORESCENCE INTENSITIES OF BROKEN CHLOREPLAST SUSPENSIONS AT 20 AND −196°C.

Chlorophyll fluorescence chloroplasts uncoupled by gramicidin, with or without 10 mM $MgCl_2$ and of coupled chloroplasts was recorded at 20 and −196°C after fast freezing.

		20°C fluorescence	−196°C fluorescence	
		685 nm	685 nm	735 nm
Uncoupled chloroplasts	$-Mg^{2+}$	0.50	0.65	1.18
	$+Mg^{2+}$	1.00	1.00	0.86
Coupled chloroplasts + Mg^{2+}	P	1.00	1.00	1.03
	S	0.60	1.00	1.04

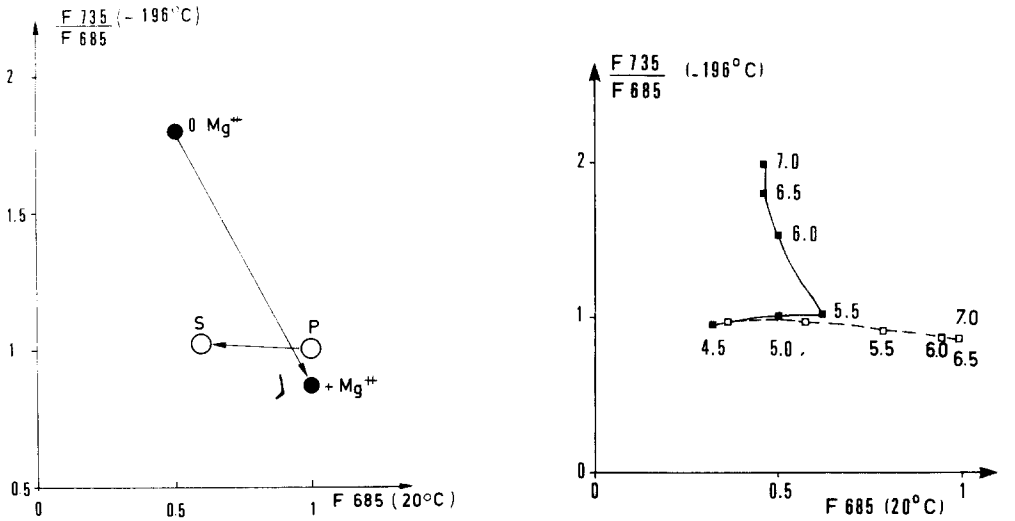

Fig. 5. Relationships between the ratio of 735/685 nm fluorescence emission at —196°C and the corresponding 685 nm fluorescence intensity at 20°C. Measurements at 20 and —196°C were made in the same Dewar flask. After the 685 nm fluorescence was measured at 20°C, liquid nitrogen was added to the sample and the low-temperature fluorescence spectrum recorded. • → •, uncoupled chloroplasts (10^{-6} M gramicidin) without and with $MgCl_2$; ○ ← ○, coupled chloroplasts in the presence of 10 mM $MgCl_2$, frozen in the P and S states.

Fig. 6. Effect of pH on the ratio of 735/685 nm fluorescence emission at —196°C. The ratio F_{735}/F_{685} is plotted versus the 685 nm fluorescence intensity at 20°C, as in Fig. 5. Broken chloroplasts were suspended in a medium buffered by Mes (containing 10^{-6} gramicidin and 20 mM ascorbate), without $MgCl_2$ (■———■), and with 10 mM $MgCl_2$ (□— — —□). The pH of the suspension was varied between 7 and 4.5 (see Fig. 3). Numbers denote pH values.

system I fluorescence. Although the room temperature Photosystem II fluorescence of coupled chloroplasts decreases close to the value of the fluorescence measured in broken uncoupled chloroplasts resuspended in a Mg^{2+}-free medium, it is obvious that the state of the chloroplast corresponding to the S level is different from that of chloroplasts in a Mg^{2+}-free medium. Fig. 5, derived from Table I, indicates that no significant spillover change takes place during the P → S decline.

Fig. 6 shows the changes of the 735/685 nm emission ratio which accompany the 20°C fluorescence yield variations produced by acidification using Mes buffer (see Fig. 3b). The acidification of the Mg^{2+}-free suspension has two distinctive effects: one initiated at lower H^+ concentration, is a decrease of spillover which is accompanied by an increase of Photosystem II fluorescence at room temperature. This effect overlaps with the second phenomenon, the chlorophyll fluorescence quenching (see Horizontal line, solid squares). This second phenomenon is the only one occurring in the suspension containing $MgCl_2$ (open squares).

Fig. 7 indicates that illumination of coupled chloroplasts without $MgCl_2$ does not change the spillover (A and B). The addition of $MgCl_2$ in the light does not significantly increase the Photosystem II fluorescence yield at 20°C but produces a large diminution of spillover (C and D). This decrease has about the

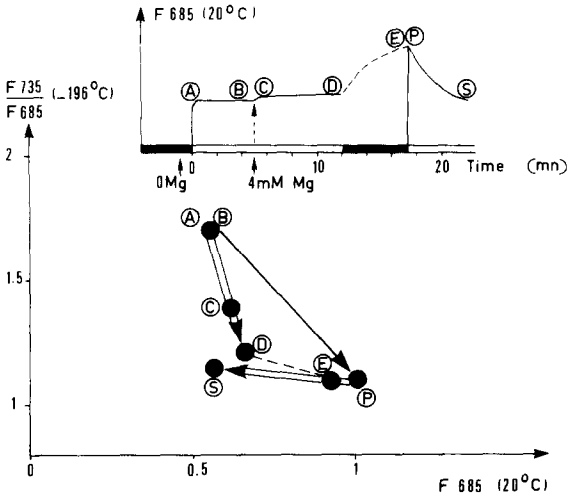

Fig. 7. The ratio of 735/685 nm fluorescence emission at −196° C in various states of broken chloroplasts. The upper part of the figure is a diagram of the variations of chlorophyll *a* fluorescence at 20° C. Letters indicate places where liquid nitrogen was added; closed bars denote darkness. In the lower graph the results of fluorescence spectroscopy at −196° C are depicted in the same manner as in Figs. 5 and 6.

same amplitude as when $MgCl_2$ is added to chloroplasts in the dark (E). Again, the P → S decay shows no significant change in the spillover.

Discussion

Ascorbate (Fig. 1), NADP + ferredoxin or azide promote the fluorescence quenching. Presumably, this is based on increased ΔpH values due to stimulated Photosystem I electron transport observed in the presence of these reagents. The results shown in Fig. 2 indicate that the light-induced quenching of chlorophyll fluorescence in isolated chloroplasts is proportional to the proton concentration reached inside the thylakoid. Such a linear relationship is obtained also by replotting the data published by Garlaschi et al. [14]. The comparison of their data with ours is shown in Fig. 8.

If P → S decay involves the same modification of the thylakoid membrane as the quenching of chlorophyll fluorescence by acidification using buffers, it must be concluded from a comparison of Figs. 2 and 3a that the photo-induced acidification of the intrathylakoid space, measured by the quenching of 9-aminoacridine fluorescence is overestimated by a factor of about 15. This conclusion is qualitatively in agreement with De Benedetti and Garlaschi [15]. Moreover, if the inside pH could reach a value of 4, the donor side of Photosystem II should be irreversibly damaged [16,17] and the P → S decay should be irreversible. This is not the case.

The data reported above are consistent with the requirement of a Mg^{2+} release to obtain the P → S quenching. An exit of Mg^{2+} as a counter ion of H^+ uptake has been observed in broken [18—20] and intact [21,22] chloroplasts. Release of H^+ from thylakoid in the dark by addition of Mg^{2+} has been described by Bose and Hoch [23]. Ben-Hayyim [24] who studied effects of ionophore A23187, suggested that internal Mg^{2+} is bound to proteins and that

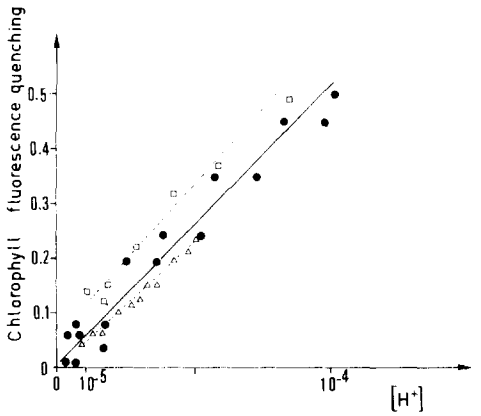

Fig. 8. Relationship between P → S fluorescence quenching and intrathylakoid H^+ concentration; comparison of our data with those of Garlaschi et al. [14]. Values of $[H^+]$ are deduced from 9-aminoacridine fluorescence quenching; •, our data from Fig. 2 of this communication; □, data from Garlaschi et al., Fig. 4, curve 2; inhibition by NH_4Cl at various concentrations; △, data from Garlaschi et al., Fig. 5; inhibition by gramicidin at various concentrations.

the strength of this binding is a function of proton concentration inside the thylakoid. Presumably, when internal magnesium is displaced by protons transported to the intrathylakoid space, a conformational change of the proteins concerned will induce a change of the fluorescence deactivation pathway. The rate of this conformational change is, at least, as fast as the relaxation of the P → S quenching upon gramicidin addition (half time less than 3 s).

The experiments depicted in Figs. 5 and 7 demonstrate clearly that along the P → S quenching, only a very weak or no increase of spillover takes place, although the room temperature fluorescence amplitude of Photosystem II is lowered drastically. Furthermore, there is strong evidence that the P → S quenching is not due to a reoxidation of Q. (The case of P → S quenching in intact cells or whole leaves may, however, be more complex; see Refs. 6, 25, 26). The P → S fluorescence quenching, in contrast to the quenching by spillover, and also in contrast to the photo-induced quenching developed in the presence of phenazine methosulphate [27,1], is reversed by a decrease of temperature to —196°C (Table I). Thus the P → S quenching is probably caused by an increase of thermal deactivation of excited pigment molecules related to Photosystem II. Such thermal deactivation does not seem to compete substantially with photochemistry, since a large and stable ΔpH, produced by noncyclic electron flow, is maintained along the P → S quenching. The maximum amplitude of the supposedly thermal quenching from P to S is equivalent to the spillover in chloroplasts suspended in a Mg^{2+}-free medium (with 10 mM NaCl), since the S level is close to the maximum fluorescence yield obtained in the Mg^{2+}-free suspension.

The comparison of the Mg^{2+} concentration curves for P → S quenching and spillover decrease (Fig. 4) points out a significant difference in sensitivity to Mg^{2+}. A similar relationship, although with much higher values for half-saturation, has been described by Jennings et al. [2]. However, the phenomenon referred to as slow fluorescence quenching by Jennings et al. was, in fact, the composite of two processes, a reversible quenching corresponding to the P → S

phase, and a irreversible quenching, probably reflecting a photoinhibition [3,4]. Based on differential effects of Mg^{2+} on light scattering and fluorescence of broken chloroplasts [28] and on efffects of various cations and ionophores on fluorescence [29] two sites of Mg^{2+} effects on thylakoid membranes were postulated. The results presented here suggest that one site, presumably located on the outer thylakoid face, is responsible for spillover changes. The second site apparently is located on the inner face; exchange of Mg^{2+} for proton at this site leads to the P → S fluorescence deciine.

The study of the relationship between spillover and Photosystem II fluorescence amplitude at various pH in Mg^{2+}-free chloroplast suspensions (Fig. 6) indicate that the outside proton concentration can affect spillover. The increase of H^+ concentration decreases spillover as Mg^{2+} does. This may indicate that protons act at the outer binding site in a similar manner as cations. Fig. 3b points out that below pH 5.5 a quenching which is Mg^{2+}-independent is induced by acidification. This quenching may be the same as previously described [7,16,17] as due to the inactivation of the donor side of System II. This process is not related to the P → S decline. We propose that both H^+ and Mg^{2+} decrease spillover when acting on the outside face of the thylakoid and that H^+ can displace Mg^{2+} linked to the proteins of the inner face. This ion-exchange then determines conformational changes of the membrane which are responsible for the non-photochemical (P → S) quenching but do not affect spillover.

Presumably, in intact isolated chloroplasts the fluorescence emission also depends upon the H^+ concentration in the thylakoid compartment. Preliminary data do not indicate a significant spillover increase correlated to the P → S decline. If the P → S quenching in intact cells follows the same relationship as in isolated chloroplasts, the extent of quenching would be an indirect measure of the photo-induced acidification of the intrathylakoid space in vivo. Experiments are in progress to verify this assumption.

Acknowledgements

We thank Drs. Y. de Kouchkovsky, P. Pasquier and F. Haraux for helpful discussions concerning the technique of 9-aminoacridine fluorescence measurement and Dr. C.J. Arntzen and Dr. Govindjee for general discussions. This work was supported in part by a grant ATP No. A 13100 from PIRDES, and in part by a NATO grant No. SA 5-2-05/XVI (Ext.) 77 (II) MDL.

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