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CHARACTERIZATION OF CHLOROPHYLL FLUORESCENCE QUENCHING IN CHLOROPLASTS BY FLUORESCENCE SPECTROSCOPY AT 77 K

I. ΔpH-DEPENDENT QUENCHING

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The nature of the light-induced ΔpH-dependent decline of chlorophyll *a* fluorescence in intact and broken spinach chloroplasts was investigated. Fluorescence spectra at 77 K of chloroplasts frozen in the low-fluorescent (high ΔpH) state showed increased ratios of the band peak at 735 nm (Photosystem (PS) I fluorescence) to the peak at 695 nm (PS II fluorescence). The increase in the F_{735}/F_{695} ratio at 77 K was related to the extent of fluorescence quenching at room temperature. Normalization of low-temperature spectra with fluorescein as an internal standard revealed a lowering of F_{695} that was not accompanied by an increase in F_{735}: preillumination before freezing decreased both F_{695} and, to a lesser extent, F_{735} in the spectra recorded at 77 K. Fluorescence induction of chloroplasts frozen in the low-fluorescent state showed a markedly decreased variable fluorescence (F_v) of PS II, but no concomitant increase in initial fluorescence (F_0) of PS I. Thus, the buildup of a proton gradient at the thylakoid membrane, as reflected by fluorescence quenching at room temperature, affects low-temperature fluorecence emission in a manner entirely different from the effect of removal of Mg^{2+}, which is thought to alter the distribution of excitation energy in favor of PS I. The ΔpH-dependent quenching therefore cannot be caused by such change in energy distribution and is suggested to reflect increased thermal deactivation.

Introduction

Chl *a* fluorescence emitted by chloroplasts has frequently been used as a tool to detect changes in the distribution of excitation energy between the two photosystems. A transition from 'State 1' to 'State 2' that may occur during the induction phase of photosynthesis [1,2] should lead – due to increasing energy transfer to PS I – to quenching of fluorescence at room temperature, most of which is emitted by PS II. Recently, an ATP-dependent light-induced fluorescence quenching has been reported to be caused by phosphorylation of the light-harvesting Chl *a/b* complex [3–8]. This is thought to be related to the State 1–State 2 transition and presumably is regulated by the redox state of the plastoquinone pool [5,9–11] or, alternatively, by the adenylate energy charge [12]. However, matters are complicated, as there are several other possible ways of fluorescence quenching. In leaves lacking the light-harvesting complex and therefore not showing a State 1–State 2 transition, Lieberman et al. [13] observed normal

Abbreviations: Chl, chlorophyll; PS, photosystem. *F*, fluorescence emission; P, peak, S, steady state of fluorescence emission; Q, primary electron acceptor of PS II. Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

P-to-S fluorescence quenching. We have recently shown [14–16] that in light exciting both photosystems about equally, the P-to-S decline seen in intact isolated chloroplasts, as well as in *Chlorella* cells, consists of two major components: photochemical quenching by reoxidation of Q and non-photochemical quenching due to the buildup of a ΔpH across the thylakoid membrane. PS II fluorescence is also drastically lowered when chloroplasts are depleted of Mg^{2+}. This well studied Mg^{2+} effect of fluorescence (see Refs. 17 and 18 for reviews, and Refs. 19 and 20) is viewed as being based on changes in energy distribution, however, it does not seem to be involved in light-induced fluorescence quenching under physiological conditions [16]. Furthermore, some irreversible quenching might be caused by destructive effects of light [21].

In view of these various ways of fluorescence quenching, it is of interest to elucidate their different mechanisms. In the present study we have tried to characterize the ΔpH-dependent quenching, particularly by using low-temperature fluorescence spectroscopy. There is ample evidence [17,22] that at 77 K the fluorescence bands observed at 735, 695, and 685 nm can be attributed to emission by mainly PS I, PS II and the light-harvesting complex, respectively. We have shown before [14,16,23] that the energy (ΔpH)-dependent fluorescence quenching in chloroplasts, as compared to fluorescence lowering due to Mg^{2+} depletion, is accompanied by only a small increase in the ratio of the fluorescence bands F_{735}/F_{695} (or F_{735}/F_{685}) at 77 K. From this followed that ΔpH-dependent quenching is different from the Mg^{2+} effect and does not involve a major shift in energy distribution. As will be shown here, chloroplasts frozen with liquid nitrogen when a light-induced proton gradient across the thylakoid membrane exists are indeed characterized by an increased F_{735}/F_{695} ratio at 77 K. However, this does not result from an increase in PS I and equivalent decrease in PS II fluorescence. Rather, both PS II fluorescence and – to a lesser degree – PS I fluorescence at 77 K are lowered. In the following paper [24], we provide evidence that, in contrast, ATP-dependent quenching is related to changes in the low-temperature spectra that indicate increased energy transfer to PS I.

Materials and Methods

Intact chloroplasts were isolated from leaves of spinach (*Spinacia oleracea* L.) according to the method of Jensen and Bassham [25], as modified by Heber [26].

For fluorescence measurements, the intact chloroplasts were suspended in 'solution C' [25], containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 10 mM NaCl, 0.5 mM KH_2PO_4 and 40 mM Hepes; pH 7.6 (NaOH). The final chlorophyll content was 5 or 10 μg/ml. Broken chloroplasts were obtained by osmotic rupture of intact chloroplasts in water or 5 mM $MgCl_2$ and subsequent addition of an equal volume of double-strength assay medium (final concentrations: 0.33 M sorbitol, 10 mM KCl, 0 or 5 mM $MgCl_2$, 40 mM Hepes; pH 7.6 with KOH). To normalize the low-temperature fluorescence spectra, 2 μM fluorescein (sodium salt) was added as an internal standard to the medium. At this concentration the fluorescein did not inhibit CO_2-dependent O_2 evolution by the intact chloroplasts, nor did it interfere with the fluorescence emission. The chloroplast suspension was filled to 1 cm height into a cylindrical glass tube (diameter 1.8 mm) that could be cooled with liquid nitrogen in a translucent Dewar vessel.

Chlorophyll fluorescence at room temperature and at 77 K was measured with a Farrand KM1 spectrofluorometer. The exciting light beam of 480 nm had a band-pass of 20 nm and an intensity of 4–15 $W \cdot m^{-2}$. Emission spectra at 77 K were recorded from 800 to 500 nm and normalized at 535 nm (fluorescein emission); the band-pass was 1 nm, and the spectra were corrected for the wavelength sensitivity of the multiplier. A 1 mm infrared-absorbing filter (Calflex, C, Balzers) and blue filters (9782 and 5030, Corning) were placed between the exciting light source and sample. The entrance slit of the emission monochromator was shielded by a cutoff filter (3486, Corning). For induction measurements at 77 K, the emission band-pass was 5 nm.

Results

When fluorescence was quenched at room temperature by light-induced buildup of a proton

gradient, fluorescence emission spectra at 77 K of intact and broken chloroplast samples that were frozen rapidly with liquid nitrogen exhibited increased ratios of the bands at 735 and 695 nm (Table I). The presence of methyl viologen during the preillumination period, which stimulates ΔpH-dependent quenching [14,16], significantly enlarges the change in the F_{735}/F_{695} ratio. This is in contrast to ATP-dependent quenching, which is inhibited by methyl viologen [5,24]. To avoid possible interference with ATP-dependent quenching, most of the experiments reported here have been carried out in media containing 25 μM methyl viologen.

As expected from the effect of uncouplers on ΔpH-dependent quenching [27,28], the increase in the F_{735}/F_{695} ratio is eliminated when an uncoupler is present during preillumination (Table II). Also, the increase in F_{735}/F_{695} is fully reversed when a dark period is allowed after preillumination, before the samples are frozen. The kinetics of this dark reversion, shown in Fig. 1, is similar to that of reversion of the ΔpH-dependent quenching upon darkening or addition of DCMU [14]. The reversion is much faster than that of ATP-dependent quenching [5].

Table III demonstrates that the increase in the F_{735}/F_{695} ratio we observe after preillumination does not represent a State 1–State 2 transition, as it is induced by light of $\lambda > 695$ nm which preferentially excites PS I, as well as by light exciting both photosystems (half-bandwidth 630–680 nm).

To compare fluorescence emission at 77 K from different samples, spectra were normalized at 535 nm using fluorescein as an internal standard (see Materials and Methods). In Fig. 2a and b, normalized spectra from broken chloroplasts frozen after dark incubation with or without $MgCl_2$ and after light adaptation in the presence of Mg^{2+} are depicted. Fig. 2a shows the known Mg^{2+} effect: removal of Mg^{2+} leads to strongly increased PS I and decreased PS II fluorescence. In contrast, light adaptation (Fig. 2b) lowers both PS II and PS I fluorescence at 77 K. The same effect is observed in light-adapted intact chloroplasts (Fig. 2c). The kinetics of these changes for intact chloroplasts is shown in Fig. 3a. Lowering of F_{695} and F_{735}, and increase in the F_{735}/F_{695} ratio are correlated to room-temperature P-to-S quenching. (Most of this is ΔpH-dependent quenching, although a Q-de-

TABLE I

EFFECT OF METHYL VIOLOGEN ON FLUORESCENCE EMISSION AT 77 K OF LIGHT-ADAPTED CHLOROPLASTS (F_{735}/F_{695})

Conditions: Preincubation at 20°C; 3 min in dark or light (480 nm, 5 $W \cdot m^{-2}$) with/without 25 μM methyl viologen.

		No addition	+ methyl viologen
Intact chloroplasts,	dark	1.66	1.74
	light	2.15	2.40
Broken chloroplasts,	dark	1.60	1.61
(+5 mM $MgCl_2$)	light	1.90	2.10

TABLE II

RATIOS F_{735}/F_{695} AT 77 K OF INTACT CHLOROPLASTS INCUBATED IN LIGHT OR DARKNESS: EFFECT OF UNCOUPLING WITH NH_4Cl

Conditions: Preincubation at 20°C; 3 min in dark or light (480 nm, 9 $W \cdot m^{-2}$) with/without 10 mM NH_4Cl. Samples contained 25 μM methyl viologen. The integrity of chloroplasts was 87%.

	No addition	+ 10 mM NH_4Cl
Dark	1.60	1.77
Light	2.67	1.69

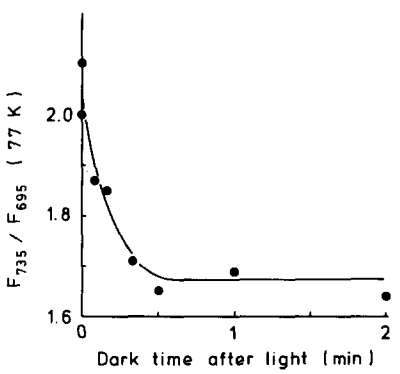

Fig. 1. Dark reversibility of the light-induced change in the F_{735}/F_{695} ratio at 77 K. Intact chloroplasts (integrity 88%) were suspended in solution C containing 2 mM $KHCO_3$ and 20 μg·ml^{-1} catalase, and preilluminated at room temperature for 3 min with 15 $W \cdot m^{-2}$ 650 nm light (band-pass 20 nm). Subsequently, samples were frozen after dark periods as given in the graph and spectra recorded at 77 K. F_{735}/F_{695} ratios of three dark-adapted controls were 1.63, 1.63 and 1.65.

TABLE III

FLUORESCENCE OF INTACT CHLOROPLASTS AT 77 K AFTER ILLUMINATION AT 20°C WITH LIGHT OF DIFFERENT WAVELENGTHS

Conditions: Preillumination; 3 min; intensity, 50 $W \cdot m^{-2}$. Samples contained 2 mM $KHCO_3$. Chloroplasts were 78% intact. The following filter combinations were used: half-bandwidth 630–680 nm; infrared-absorbing filter (Calflex C, Balzers), cutoff filter (RG 630, Schott), interference filter (K65, Balzers); $\lambda > 695$ nm: Calflex C, RG 695 (Schott); half-bandwidth 713–733 nm; Calflex C, RG 715 (Schott), interference filter 720 nm (Baird Atomic); $\lambda > 715$ nm: Calflex C, RG 715.

Wavelength of preillumination (nm)	Fluorescence ratio (F_{735}/F_{695})
- (dark control)	1.50
630–680	2.12
> 695	2.11
713–733	2.09
> 715	2.05

pendent component is present, see Ref. 16.) In broken chloroplasts preilluminated in the presence of 5 mM $MgCl_2$, quenching at room temperature is less and proceeds more slowly, but otherwise very similar data were obtained (Fig. 3b).

Fluorescence induction at 77 K shows pronounced differences between the effect of light incubation and the Mg^{2+} effect on dark-adapted chloroplasts (Table IV). In this case, after preillumination in the presence of methyl viologen, chloroplasts were kept for 1 s in darkness before freezing. This dark period allowed for reoxidation of Q without significant decay of the high-energy state. At 77 K, dark-adapted broken chloroplasts showed in the presence of $MgCl_2$ an F_v/F_m ratio of about 0.8 at 690 nm and of about 0.3 at 735 nm. Very similar ratios were found with intact chloroplasts. In both types of chloroplasts, preillumination leads to a strong lowering of F_v at 690 nm, whereas F_0 is not changed significantly. This

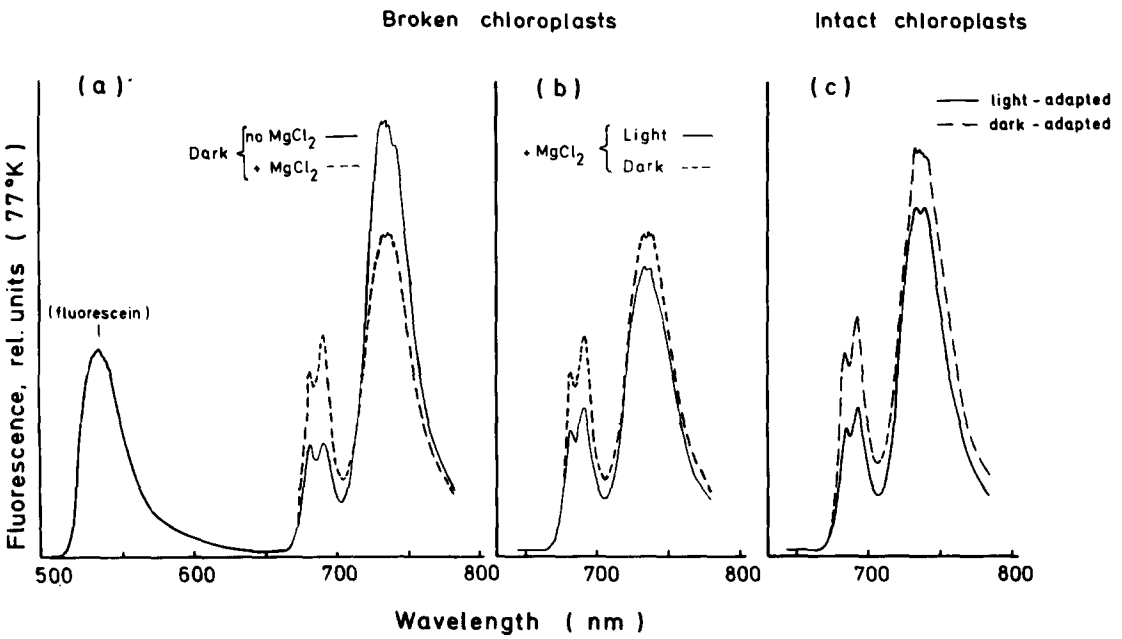

Fig. 2. Normalized fluorescence emission spectra at 77 K of broken and intact chloroplasts. Before freezing with liquid N_2, samples of broken chloroplasts were incubated at room temperature either for 4 min in the dark in the absence and presence of 5 mM $MgCl_2$ (a) or for 1 min in the dark followed by 3 min illumination with 4 $W \cdot m^{-2}$ blue light (480 nm, band-pass 20 nm) in the presence of $MgCl_2$ (b). In panels a and b, the same spectrum of chloroplasts incubated in the dark with $MgCl_2$ is depicted (broken lines). Intact chloroplasts (c) were preincubated in the dark or light. The integrity of the chloroplasts was 95%. The media (see Materials and Methods) contained 25 μM methyl viologen and 2 μM fluorescein (sodium salt) as an internal standard. The spectra are normalized at the fluorescein emission of 535 nm shown only in panel a.

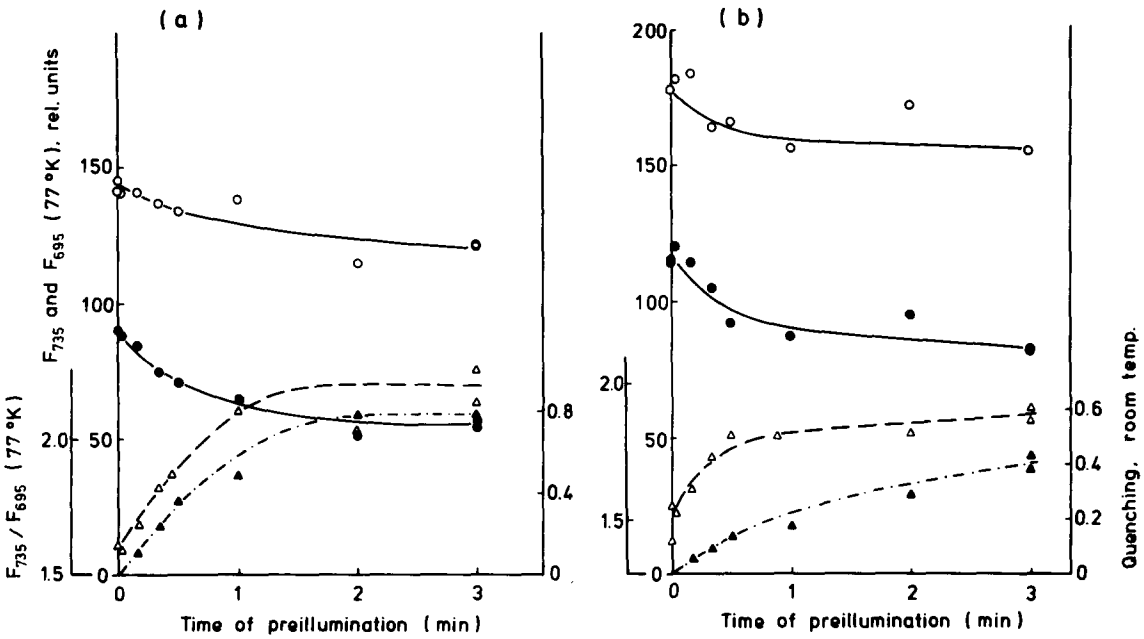

Fig. 3. Changes in the fluorescence spectra of intact (a) and broken (b) chhloroplasts at 77 K and related quenching at room temperature as functions of time of illumination. 90% intact chloroplasts were suspended in solution C, or osmotically broken chloroplasts were suspended in sorbitol medium containing 5 mM $MgCl_2$. Both media contained 2 μM fluorescein as internal standard and 25 μM methyl viologen. The samples were illuminated at room temperature (blue light of 480 nm, half-bandwidth 20 nm, intensity about 3 $W \cdot m^{-2}$) for the times given in the graph, before they were frozen with liquid nitrogen. During this light incubation, the slow decline of fluorescence emission was recorded at 685 nm (band-pass 1 nm). Values of F_{735} and F_{695} taken from the spectra recorded after freezing were normalized at 535 nm (fluorescein emission). (○) F_{735}; (●) F_{695}; (△) F_{735}/F_{695}; (▲) fluorescence quenching at room temperature, defined as $(F_p - F_t)/F_t$, where F_p is the fluorescence emission in the peak and F_t the fluorescence level after the given time of preillumination.

lowers the F_v/F_m ratio to about 0.7. At 735 nm the change in F_v/F_m is less pronounced; although there is some uncertainty in the data, it appears that both F_0 and F_v are lowered during light adaptation. F_m at 735 nm was always decreased cf. Figs. 2 and 3). Regarding the Mg^{2+} effect on

TABLE IV

FLUORESCENCE INDUCTION OF ISOLATED CHLOROPLASTS AT 77 K

Conditions: Preincubation at 20°C; 3 min in dark or light (480 nm, 4 $W \cdot m^{-2}$). Samples of 85% intact or osmotically broken chloroplasts contained 25 μM methyl viologen and 0.5 μM fluorescein (sodium, salt). After light incubation, 1 s darkness was allowed before freezing. F_0, initial, F_v variable and $F_m = F_0 + F_v$ maximum fluorescence emission, respectively. Values are normalized at 535 nm (fluorescein emission).

Preincubation		690 nm			735 nm			$F_{m(735\ nm)}/F_{m(690\ nm)}$
		F_0	F_v	F_v/F_m	F_0	F_v	F_v/F_m	
Intact chloroplasts	dark	18	67	0.79	114	46	0.29	1.88
	light	19	41	0.68	110	33	0.23	2.38
Broken chloroplasts	$-MgCl_2$, dark	18	35	0.66	200	39	0.16	4.51
	$+MgCl_2$, dark	19	67	0.78	114	42	0.27	1.81
	$+MgCl_2$, light	19	49	0.72	102	41	0.29	2.10

broken chloroplasts, the data in Table IV are similar to those published by Butler and Kitajima [29]. At 690 nm, Mg^{2+} removal – like preillumination – decreases F_v. However, in contrast to light adaptation, Mg^{2+} depletion leads to a strong increase in the F_0 level at 735 nm, which is mainly responsible for the large increase in the F_{735}/F_{690} ratio. The Mg^{2+} effect on chlorophyll fluorescence and the fluorescence quenching related to the ΔpH must therefore be based on different mechanisms.

Discussion

Nonphotochemical quenching of chlorophyll fluorescence caused by the light-induced proton gradient is the predominant quenching process under conditions that limit utilization of the ΔpH by photophosphorylation (e.g., in intact chloroplasts during the lag phase of CO_2 fixation, or in the absence of CO_2; see Refs. 14 and 16). Our data from fluorescence emission at 77 K of isolated chloroplasts frozen in the quenched state show that in contrast to suggestions made in several papers (see Refs. 17 and 30), the ΔpH-dependent quenching is not related to increased excitation energy transfer to PS I. Low-temperature spectra do exhibit an increased ratio of F_{735}/F_{695}, as expected for altered excitation energy transfer. Although the increase in the F_{735}/F_{695} ratio is small (cf. Refs. 16 and 23), it is well reproducible and, according to our experiments, certainly is a property of ΔpH-dependent quenching: Like the latter, the increase in F_{735}/F_{695} is reversed in the dark, abolished by uncoupling and stimulated by methyl viologen. Fluorescence spectra normalized at 535 nm with fluorescein as an internal standard indicate that the ΔpH-dependent quenching is correlated to a significant decrease in both PS II and PS I fluorescence at 77 K. The increase in the F_{735}/F_{695} ratio results from the fact that F_{735} is lowered somewhat less than F_{695}. Fluorescence induction at 77 K is characterized in particular by strongly lowered variable fluorescence of PS II correlated to ΔpH-dependent quenching, whereas F_0 is not significantly altered. In contrast to the effect of Mg^{2+} removal, there is no corresponding increase in the F_0 level at 735 nm. Thus, with respect to both spectra and induction at 77 K, the ΔpH-dependent quenching is distinctly different from the Mg^{2+} effect. We assume that ΔpH-dependent quenching is caused by increased thermal deactivation of excited states. This might be caused by structural membrane alterations due to H^+-Mg^{2+} exchange at the inner thylakoid surface. On the other hand, it is generally accepted that Mg^{2+} added to broken chloroplasts causes altered energy distribution by action on the outer surface, particularly on the light-harvesting complex.

As will be shown in the following paper [24], ATP-dependent fluorescence quenching, which is supposed to be related to phosphorylation of the light-harvesting complex, appears to follow a third mechanism different from both ΔpH-dependent quenching and the Mg^{2+} effect.

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