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

II. ATP-DEPENDENT QUENCHING

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In intact, uncoupled type B chloroplasts from spinach, added ATP causes a slow light-induced decline ($t_{1/2} \approx 3$ min) of chlorophyll *a* fluorescence at room temperature. Fluorescence spectra were recorded after fast cooling to 77 K and normalized with fluorescein as an internal standard. Related to the fluorescence quenching at room temperature, an increase in Photosystem (PS) I fluorescence (F_{735}) and a decrease in PS II fluorescence (F_{695}) were observed in the low-temperature spectra. The change in the F_{735}/F_{695} ratio was abolished by the presence of methyl viologen. Fluorescence induction at 77 K of chloroplasts frozen in the quenched state showed lowered variable (F_v) and initial (F_0) fluorescence at 690 nm and an increase in F_0 at 735 nm. The results are interpreted as indicating an ATP-dependent change of the initial distribution of excitation energy in favor of PS I, which is controlled by the redox state of the electron-transport chain and, according to current theories, is caused by phosphorylation of the light-harvesting complex.

Introduction

In isolated uncoupled chloroplasts, where ΔpH-dependent chlorophyll fluorescence quenching is absent [1], exogenous ATP causes a slow fluorescence decline that is induced by light. This phenomenon was first observed by Bennett et al. [2] and Horton and Black [3]. These and subsequent studies (see Ref. 4) have provided ample evidence that ATP-dependent quenching is caused by phosphorylation of the light-harvesting Chl *a/b* complex. The quenching is supposed to indicate increased excitation energy transfer to PS I. It was proposed that an imbalance of excitation energy distribution in favor of PS II results in reduction of plastoquinone that enhances phosphorylation of the light-harvesting complex and consequently results in increased excitation of PS I. Low-temperature fluorescence spectra have, in fact, shown that ATP-dependent quenching is related to an increase in the ratio of the fluorescence band peaks at 735 and 685 or 695 nm [2,3,5,6]. However, a change in this ratio does not give conclusive proof of changed energy distribution. As shown in the preceding paper [4], ΔpH-dependent quenching is also related to such a change in fluorescence emission at 77 K, but this is not based on increased energy transfer to PS I. Therefore, a study of ATP-dependent quenching was undertaken in which low-temperature fluorescence spectra were normalized using fluorescein as an internal stan-

Abbreviations: F, level of fluorescence emission; F_0, initial fluorescence; F_v, variable fluorescence; $F_m = F_0 + F_v$, maximum fluorescence level; PS, photosystem; Q, primary electron acceptor of PS II-Chl; chlorophyll; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid; DCMV, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

dard [4]. The results indicate that ATP-dependent fluorescence quenching does indeed represent an altered energy transfer and thus differs from ΔpH-dependent quenching. It is also shown that ATP-dependent quenching is not identical with the Mg^{2+} effect on fluorescence.

Materials and Methods

Isolation of intact spinach chloroplasts and recording of chlorophyll fluorescence spectra at 77 K in the presence of fluorescein as an internal standard was carried out as described in the foregoing paper [4]. The intactness of chloroplasts in the preparations was 85–95%. Chloroplasts were suspended in a reaction medium (10 μg Chl/ml), which contained 0.1 M sorbitol, 20 mM KCl, 40 mM Hepes buffer (pH 7.6, with KOH) and additions as specified in the legends.

Chlorophyll fluorescence emission at 686 nm, excited by blue light, was measured at 20°C as described previously [7,8]. In this case, samples, placed into a stirred temperature-controlled cuvette, contained 50 μg Chl/3 ml reaction medium.

Results

It seems difficult to detect light-induced ATP-dependent fluorescence quenching in coupled intact chloroplasts suspended in an isotonic medium. In this case, endogenous ATP is provided by photophosphorylation, and due to the low permeability of the chloroplast envelope towards adenylates (see Ref. 9), should be contained in the stroma. However, strong ΔpH-dependent quenching occurs in these chloroplasts, which might mask or suppress ATP-dependent quenching. As has been done in other investigations [2,3,5,6], we used uncoupled chloroplasts, in which ΔpH-dependent quenching is absent, with ATP added to the medium. In osmotically broken chloroplasts from spinach, ATP-dependent quenching was not well reproducible and seemed to be easily lost due to aging of preparations. We observed reproducible quenching using intact spinach chloroplasts suspended in a moderately hypotonic medium (containing 0.1 M sorbitol). Such chloroplasts should, according to the definition given by Hall [10], be grouped in type B. They possess an intact envelope as judged by phase-contrast microscopy; however, the envelope allows permeation of added adenylates [11]. Fig. 1a indicates that these chloroplasts, even if suspended in a medium of low strength of divalent cations, maintain a high level of variable fluorescence. In the presence of DCMU and of the uncoupler nigericin (+KCl), addition of EDTA lowers the fluorescence level only when also ionophore A23187 is added, which facilitates transport of divalent cations. For comparison, the fluorescence level and effect of $MgCl_2$ in osmotically broken chloroplasts suspended in an isotonic (0.33 M sorbitol) but otherwise identical medium are depicted in Fig. 1b. This suggests that the envelope of the type B chloroplasts, although permitting penetration of ATP, restricts leakage of divalent cations. Thus, the possibility can be excluded that part of the quenching observed in the presence of ATP is caused by loss of Mg^{2+}.

Type B chloroplasts suspended in a 0.1 M sorbitol medium show normal energy (ΔpH)-dependent quenching (Fig. 2), which is eliminated by uncoupling. If ATP is added to the uncoupled

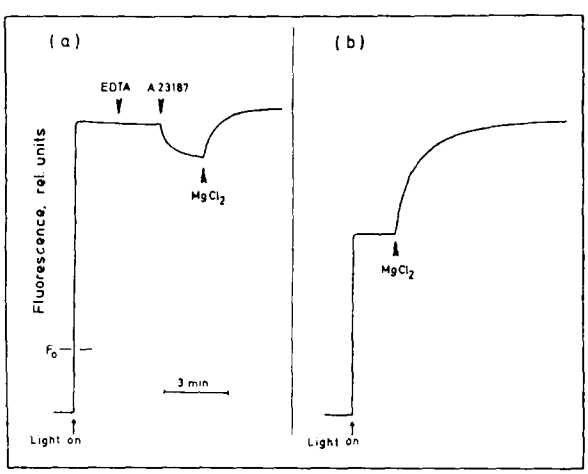

Fig. 1. Fluorescence of intact type B chloroplasts at 20°C suspended in a hypotonic medium (a) and of osmotically broken chloroplasts suspended in an isotonic medium (b). The medium composition is given in Materials and Methods; for panel b the final sorbitol concentration was 0.33 M. Additions as indicated in the figure were: nigericin, $2 \cdot 10^{-8}$ M; DCMU, $2 \cdot 10^{-5}$ M (both added in the dark); EDTA, $2 \cdot 10^{-4}$ M; ionophore A23187, 8 μM; $MgCl_2$, 3 mM. Chloroplasts were excited with 10 $W \cdot m^{-2}$ blue light (band-pass 405–490 nm); fluorescence was recorded at 686 nm.

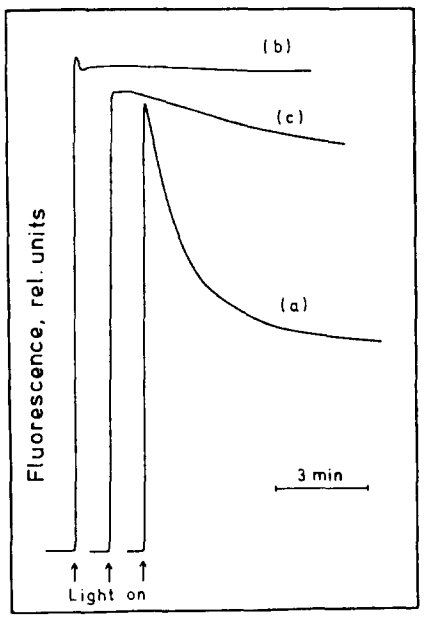

Fig. 2. Fluorescence of intact type B chloroplasts at 20°C. To the reaction medium were added: (a) 1 mM $MgCl_2$; (b) 1 mM $MgCl_2$ and $2 \cdot 10^{-8}$ M nigericin; (c) 0.2 mM ATP, 1.2 mM $MgCl_2$ and $2 \cdot 10^{-8}$ M nigericin. Experimental conditions as for Fig. 1.

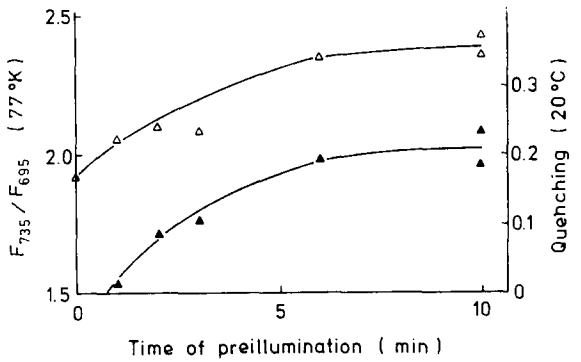

Fig. 3. ATP- and light-dependent fluorescence changes in uncoupled intact type B chloroplasts at room temperature and 77 K as a function of time. To the reaction medium 0.2 mM ATP, $2 \cdot 10^{-8}$ M nigericin and 1.2 mM $MgCl_2$ were added. Samples were illuminated with about 2 $W \cdot m^{-2}$ of blue light (480 nm, band-pass 20 nm) and frozen after the times given. (△) F_{735}/F_{695} at 77 K; (▲) quenching at room temperature, defined as $(F_p - F_t)/F_t$ with F_p denoting the fluorescence peak and F_t the fluorescence level at the time of freezing. For fluorescence measurement at 77 K the same actinic light was used. Fluorescence spectra were recorded with a band-pass of 1 nm and are corrected for the sensitivity of the photomultiplier.

chloroplasts, we observe a distinct slow quenching with a half-time, as reported by Horton and Black [5], of about 3 min. The quenching at room temperature is correlated to an increase in the ratio of fluorescence bands at 77 K, F_{735}/F_{695} (Fig. 3). Although the extent of this quenching is much smaller than the ΔpH -dependent fluorescence decline, the increase in the F_{735}/F_{695} ratio is of similar magnitude (cf. Ref. 4). If preillumination occurs in the presence of methyl viologen, this increase is abolished (Table I), as is the quenching at room temperature [5].

Low-temperature fluorescence spectra normalized at 535 nm of uncoupled type B chloroplasts are given in Fig. 4. Preillumination in the presence of ATP, as compared to its absence, results in lowered PS II and increased PS I fluorescence at 77 K (Fig. 4a). A similar difference is seen between dark- and light-adapted samples in the presence of ATP (Fig. 4b). No appreciable difference is seen between dark- and light-adapted chloroplasts in the absence of ATP (not shown). Thus, ATP-dependent and ΔpH-dependent quenching are clearly distinguishable by the low-temperature fluorescence spectra of chloroplasts frozen in the quenched state.

Contrary to former studies [2,5], the ATP-dependent quenching observed in our experiments was not readily reversible in the dark, and in some cases even progressed in the dark following a 10 min light period (cf. Ref. 5). The reason for this discrepancy is not clear at present. Due to the slow reversion, the quenched state could be frozen after

TABLE I

RATIOS F_{735}/F_{695} AT 77 K OF UNCOUPLED INTACT (TYPE B) CHLOROPLASTS AFTER PREINCUBATION WITH ATP:EFFECT OF METHYL VIOLOGEN

Conditions: Preincubation at room temperature; 10 min in dark or light (480 nm, 2 $W \cdot m^{-2}$), with/without 25 μM methyl viologen. Additions to the reaction medium: ATP, 0.2 mM; nigericin, $2 \cdot 10^{-8}$ M; $MgCl_2$, 1.2 mM.

	Without methyl viologen	With methyl viologen
Dark	1.87	1.89
Light	2.20	1.82

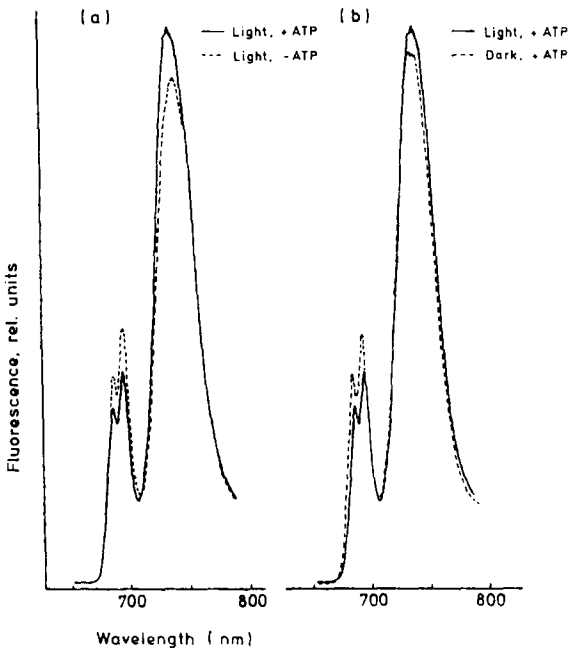

Fig. 4. Normalized fluorescence spectra (77 K) of uncoupled intact type B chloroplasts. To the reaction medium were added: 2 μM fluorescein (sodium salt), $2 \cdot 10^{-8}$ M nigericin and either 1 mM $MgCl_2$ or 1.2 mM $MgCl_2$ plus 0.2 mM ATP. (a) Samples were frozen after 10 min in the light (475 nm, band-pass 20 nm, intensity 14 $W \cdot m^{-2}$) in the presence and absence of ATP. (b) Samples were frozen after 10 min in the light or dark in the presence of ATP. Spectra were recorded as for Fig. 3 and normalized at 535 nm (fluorescein emission).

a dark period that was sufficiently long to allow reoxidation of Q to occur after preillumination. Addition of the phosphatase inhibitor NaF, which prevents reversion of ATP-dependent quenching in pea chloroplasts [2,5], was not necessary. Table II shows data of fluorescence induction of chloroplasts cooled to 77 K after preincubation at room temperature for 10 min in the light followed by 10 min in the dark. The incubation occurred in the absence and presence of ATP. As in the spectra of Fig. 4, the presence of ATP causes a decrease at 690 nm and an increase at 735 nm in total fluorescence, resulting in an increased F_{735}/F_{690} ratio. Remarkably, the F_v/F_m ratio at 690 nm is not altered, i.e., F_0 and F_v are lowered to the same extent. In this respect, ATP-dependent quenching not only differs from the ΔpH-dependent fluorescence decline, but also from the Mg^{2+} effect on fluorescence (cf. Ref. 4). Table II shows, in addition, that the higher emission at 735 nm observed after incubation in the presence of ATP is based on an increased F_0 level.

Discussion

Our results demonstrate the occurrence of a reproducible ATP-dependent light-induced fluorescence quenching in intact type B spinach chloroplasts suspended in a hypotonic medium. The quenching at room temperature is correlated to increased fluorescence ratios F_{735}/F_{695} at 77 K. Addition of methyl viologen inhibits the change at 77 K in accordance with its effect at room temperature [5]. Since methyl viologen is an efficient electron acceptor of PS I, it enhances reoxidation of the electron-transport chain [12]. The inhibition of ATP-dependent changes of fluorescence emission at 77 K supports the view that these are controlled by the redox state of the plastoquinone pool. It should be noted that methyl viologen exerts the opposite effect on ΔpH-dependent quenching [4,12], as it increases the proton gradi-

TABLE II

FLUORESCENCE INDUCTION AT 77 K OF UNCOUPLED (TYPE B) CHLOROPLASTS

Conditions: Preincubation at room temperature; 10 min light (475 nm, 14 $W \cdot m^{-2}$), followed by 10 min dark, with/without 0.2 mM ATP. Additions to the reaction medium were: fluorescein (sodium salt), 2 μM; nigericin, $2 \cdot 10^{-8}$ M; $MgCl_2$, 1 mM (in samples without ATP) or 1.2 mM (in samples with ATP). Values are normalized at 535 nm (fluorescein emission).

Preincubation	690 nm			735 nm			$F_{m(735nm)}/F_{m(690nm)}$
	F_0	F_v	F_v/F_m	F_0	F_v	F_v/F_m	
−ATP	17	54	0.76	87	36	0.29	1.73
+ATP	14	45	0.76	112	34	0.24	2.47

ent by stimulation of electron transport. The reason for the slow (or absent) dark reversion of ATP-dependent quenching in our spinach chloroplast preparations was not investigated. It might be due to inhibited phosphatase action or to progressing quenching in the dark following a light period [5] that balances the phosphatase reaction. As only low light intensities were applied during illumination periods, irreversible quenching due to destructive photooxidation can be excluded.

Normalized spectra at 77 K clearly indicate that the change in the F_{735}/F_{695} ratio is caused by decreased PS II and increased PS I fluorescence, as expected of increased excitation energy distribution to PS I. In this respect, ATP-dependent quenching resembles the effect of Mg^{2+} removal from broken chloroplasts [13]. Fluorescence induction at 77 K, however, shows a significant difference between the effects of ATP and Mg^{2+}. In our experiments the ATP-dependent quenching is characterized by lowering of both F_v and F_0 of PS II emission to the same extent (F_v/F_m at 690 nm being constant), whereas Mg^{2+} affects F_v relatively more than F_0. The increase in PS I fluorescence (F_{735}) in both cases seems to result mostly from an increased F_0 level. In terms of the model of Butler and Kitajima [13], Mg^{2+} affects energy transfer from PS II to PS I (spillover) more strongly than the initial energy distribution between the antenna pigments of the two photosystems. In the presence of ATP, the equal lowering of F_v and F_0 at 690 nm may then be due to a changed initial distribution of excited states in favor of PS I; this may be caused by an increase in the distance between PS II and the light-harvesting complex as a result of phosphorylation. As has been reported by Horton and Black [5], the F_v/F_m ratio measured during ATP-dependent quenching at room temperature also remained constant. These results are in contrast to the studies of Bennett et al. [2], Kyle et al. [14] and Haworth et al. [15] who found that both at room temperature and at 77 K values of F_v (at 695 nm) were altered relatively more than F_0 which resulted in lowered F_v/F_m ratios. At 77 K there was, nevertheless, an appreciable decrease in F_0 at 695 nm upon thylakoid membrane phosphorylation, whereas dephosphorylation did not cause a significant change in F_0 [15]. In contrast, F_0 measured at room temperature was increased by phosphorylation [14]. These discrepancies remain to be resolved. However, it should be noted that the data of Refs. 14 and 15 also indicate clear differences between the effects of cation depletion and of membrane phosphorylation; and an increase in α, the fraction of absorbed quanta distributed to PS I, is viewed by the authors as a major result of phosphorylation.

The present study supports the view that the State 1–State 2 transition observed in algae and plant leaves is related to ATP-dependent quenching. Thus, under physiological conditions, in addition to photochemical fluorescence quenching, two different mechanisms of nonphotochemical quenching in photosynthetic systems have to be considered: ΔpH-dependent quenching that probably represents increased thermal deactivation, and ATP-dependent quenching indicating altered energy distribution. Both mechanisms are distinguished from the effect of Mg^{2+} on chloroplast fluorescence. As has been pointed out previously [12,16], the effect of Mg^{2+} depletion probably does not contribute to the fluorescence changes observed in vivo. Thylakoid protein phosphorylation has been observed in coupled, intact chloroplasts [17], but is largely inhibited by the onset of CO_2 fixation. Thus, the protein phosphorylation might, particularly during the lag phase of CO_2 fixation, contribute to the overall fluorescence decline at room temperature. However, in comparison to effects on fluorescence exerted by the redox state and by the proton gradient across the thylakoid membrane [8], ATP-dependent quenching appears to be a minor component.

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