PROTON TRANSLOCATION AND ENERGY DEPENDENT QUENCHING OF CHLOROPHYLL *a* FLUORESCENCE

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1. Introduction

A light-induced, energy dependent, quenching of chlorophyll *a* fluorescence in the presence of DCMU and an activator of cyclic electron flow, e.g. PMS or DAD, has been described recently by two groups of workers [1, 2]. This quenching differs from the type associated with changes in the redox state of primary acceptor "Q" of photosystem II, in being reversed on the addition of uncoupling agents. The reversal of the quenching by uncouplers, such as dianemycin and amines, which presumably act by destroying a pH gradient formed in the light led to the suggestion that the quenching is a reflection of the high-energy state associated with proton translocation [2].

Using different methods, i.e. subchloroplast particles with diminished proton uptake, and treating chloroplasts with agents which inhibit light-induced proton uptake in such a fashion that the inhibition can be readily reversed, we have provided additional evidence for a direct relationship between light-induced proton uptake and the degree of quenching of fluorescence.

Abbreviations:

CCCP : carbonyl cyanide *m*-chlorophenylhydrazone
DAD : 2,3,5,6-tetramethyl-*p*-phenylene diamine
DCCD : dicylohexylcarbodiimide
DCMU: 3-(3,4-dichlorophenyl)-1, 1-dimethylurea
EDTA : ethylene diamine tetracetic acid
PMS : phenazine methosulfate

2. Methods

Chloroplasts were prepared from greenhouse spinach. Leaves were homogenized at 4° in a medium containing 0.8 M sucrose, 0.02 M tricine-NaOH, pH 7.8, and 0.01 M NaCl (STN solution). The chloroplast pellet was resuspended either in the grinding buffer, in 10 mM NaCl, or in 10 mN Na-polygalacturonate (NaPG), washed once (10,000 *g* for 10 min), and resuspended in the same medium at a final concentration of 1 mg/ml chlorophyll. The chlorophyll concentration was determined according to Arnon [3].

EDTA-treated chloroplasts were prepared according to McCarty and Racker [4]. Subchloroplast particles prepared by sonic oscillation of chloroplasts [5] or by treatment of chloroplasts with digitonin [6] were gifts from Drs. R.E. McCarty and N. Nelson, respectively.

The fluorimeter used for the measurement of the light-induced changes in fluorescence yield has been described previously [7]. The light source was a 650 W tungsten-iodine lamp (Sylvania Sun Gun), whose output was collimated and passed through 2 cm of water as a heat filter and a Corning blue glass filter (CS 4-96). The light intensity incident on the sample was 1.9×10^5 erg/cm^2 sec. The fluorescence was detected by a RCA C70007A photomultiplier tube (S-1 response; cooled with solid CO_2) in conjunction with a 680 nm interference filter and a Corning red glass filter (CS 2-64). The photomultiplier signal was amplified with a Philbrick 25 C operational amplifier serving an electrometer function,

Table 1
Comparison of fluorescence quenching by DAD and light-induced proton uptake in chloroplasts and subchloroplast particles.

	% quenching by DAD	μeq H^+ accum./mg Chl
Chloroplasts	37.5	1.18
Sonicated subchloroplast particles	16.0	0.55
Digitonin subchloroplast particles	9.0	0.24

Table 2
Effect of NH_4Cl on fluorescence quenching by DAD in chloroplasts and subchloroplast particles.

	Additions	% quenching by DAD
Chloroplasts	none	68
	6.6 mM NH_4Cl	7
Sonicated subchloroplast particles	none	20
	6.6 mM NH_4Cl	0
Digitonin subchloroplast particles	none	10
	6.6 mM NH_4Cl	0

and recorded with a Bausch and Lomb VOM 5 recorder or a Hewlett-Packard 141A oscilloscope. The reaction mixture for fluorescence measurements, unless otherwise noted, contained (in 3 ml) either 10 mN NaPG, 10 mM NaCl, or 50 mM NaCl; 15 mM tricine-NaOH, pH 7.8; 15 μM DCMU and chloroplasts or subchloroplast particles equivalent to 10-14 μg of chlorophyll. Where indicated, DAD was present at a final concentration of 0.33 mM.

The per cent quenching in the presence of DAD is defined in the following way: $\% q$ = steady level of fluorescence after 1–2 min of illumination with DAD present divided by the maximal fluorescence level prior to the addition of DAD.

Light-induced proton uptake was measured as described elsewhere [8]. The cyclic co-factors employed in these studies were PMS (0.025 mM), pyocyanine (0.05 mM) or DAD (0.33 mM); they all gave similar results. The reaction mixture (in 3.5 ml) contained one of the above co-factors, together with either 10 mN NaPG, 10 mM NaCl or 50 mM NaCl, and chloroplasts or subchloroplast particles equivalent to 100–140 μg of chlorophyll. The starting pH was adjusted to pH 6.4.

Diaminodurene was generously supplied by Dr. R.E. McCarty. CCCP and DCCD were purchased from Calbiochem. Polygalacturonic acid obtained from Nutritional Biochemicals Inc.

3. Results

The experiments performed here demonstrate a series of quantitative correlations between the amount of proton uptake as measured by the pH of the medium, and the degree of quenching of fluorescence by DAD. A comparison was made, for instance, between "whole" chloroplasts and subchloroplast particles which retain system II activity but which have a diminished ability to translocate protons in the light (table 1). It is evident that the amount of fluorescence quenching by DAD is fairly well correlated with the amount of protons accumulated. In each case the reversibility of the quenching was checked by the subsequent addition of 10 μM CCCP.

The well known uncoupler, ammonium chloride, inhibits both ATP synthesis and light-induced proton uptake in chloroplasts in a parallel fashion [6]. In subchloroplast particles, ammonium chloride inhibits proton uptake at concentrations which have little effect on ATP synthesis [5]. In agreement with the results of Wraight and Crofts [2], addition of ammonium chloride to chloroplasts reversed over 90% of the DAD-dependent quenching (table 2). In addition, it completely abolished quenching in the subchloroplast particles. The latter result strongly suggests that the quenching is intimately associated with the pH gradient produced by proton uptake.

Another approach was to inhibit proton uptake by (a) swelling and resuspending chloroplasts in the presence of NaPG [9] or (b) treating chloroplasts with EDTA to remove the chloroplast coupling factor

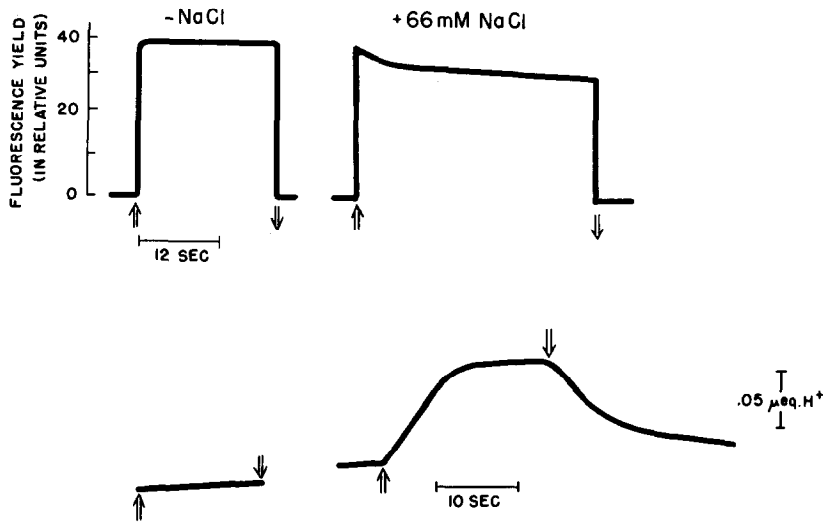

Fig. 1. Restoration by NaCl of quenching and proton uptake in NaPG chloroplasts. The reaction mixture for fluorescence measurements contained 10 mN NaPG, 15 mM tricine-NaOH, pH 7.8, 15 μM DCMU, and chloroplasts equivalent to 10 μg of chlorophyll. The reaction mixture for proton uptake contained 10 mN NaPG, 0.05 mM pyocyanine, and chloroplasts equivalent to 120 μg of chlorophyll; the starting pH was 6.4. The fluorescence of the control chloroplasts was quenched 33% by DAD, and the control pH shift was 1.23 μeq H^+/mg Chl. Light on, upward arrows; light off, downward arrows.

Table 3
Effect of EDTA treatment on fluorescence quenching by DAD and on proton accumulation.

	Additions	% quenching by DAD*	μeq H^+ accum. mg Chl
Experiment 1			
Control chloroplasts	none	55	1.02
EDTA-treated chloroplasts	none	22	0.51
Experiment 2			
Control chloroplasts	none	52	0.83
EDTA-treated chloroplasts	none	7	0.08
Experiment 3			
Control chloroplasts	none	59	1.18
	CCCP	7	0.24
EDTA-treated chloroplasts	none	0	0.10
	DCCD	19	0.69
	CCCP	3	0.00

Where indicated the final concentration of CCCP, 0.01 mM, DCCD, 0.1 mM.
* Measurement of DAD quenching at pH 6.5 yielded similar results.

[10, 11]. Both of these treatments are reversible to some extent; the former on the subsequent addition of salts (e.g., NaCl or KCl), and the latter on the addition of DCCD [12].

The correlation between the degree of fluorescence quenching by DAD and amount of light-induced proton uptake of chloroplasts in NaPG before and after adding 66 mM NaCl is seen in fig. 1. Note that both the quenching by DAD and pH shift are initially absent; addition of NaCl restored both the quenching and the proton uptake to about 50% of the control value.

A similar experiment was performed with EDTA-treated chloroplasts in the presence and absence of DCCD. The results, similar to those above, are shown in table 3. We also observed a restoration of DAD quenching on the addition of a crude coupling factor preparation (EDTA extract) to coupling factor-depleted chloroplasts (data not shown).

The quenching of chlorophyll *a* fluorescence by DAD (in the presence of DCMU) could be caused by an increased amount of energy transfer from pigment system II (which is the source of most of the fluorescence at room temperature) to the relatively non-fluorescent pigment system I. A treatment which

Table 4
Effect of magnesium ions on the quenching of fluorescence by DAD. Chloroplasts were prepared according to Murata [14]. The reaction mixture (in 3 ml) contained 10 mM KCl, 1.7 mM tricine-NaOH, pH 7.8, 15 μM DCMU, and chloroplasts equivalent to 10 μg of chlorophyll.

	Rel. fluorescence yield	% quenching by DAD
Experiment 1 ($I = 1.9 \times 10^5$ erg/cm^2 sec		
Control chloroplasts	1.00	31
Control chloroplasts + 2.5 mM $MgCl_2$*	1.09	29
Experiment 2 ($I = 0.12 \times 10^5$ erg/cm^2 sec		
Control chloroplasts	1.00	13
Control chloroplasts + 2.5 mM $MgCl_2$	1.17	14

* Chloroplasts were illuminated for 5 min in the presence of $MgCl_2$ [14], then DAD was added and the per cent quenching determined.

diminishes the amount of this "spillover" to system I pigments might, therefore, reduce the amount of quenching. Previous work [13–15] showed that spillover of energy from photosystem II to photosystem I could be partially suppressed by addition of divalent cations (e.g., Mg^{2+}); this results in an increased level of photosystem II fluorescence. Accordingly, DAD additions were made in the presence and absence of 2.5 mM $MgCl_2$ (table 4); but the extent of the DAD attenuation of fluorescence was the same in both cases.

4. Discussion

Wraight and Crofts [2], extending the observations of Murata and Sugahara [1], suggested that the light-induced DAD (or PMS) quenching of chlorophyll *a* fluorescence in the presence of DCMU may be an indicator or monitor of the chemical concentration component (ΔpH) of the electro-chemical activity gradient. Our data, showing further quantitative correlations between the amount of proton uptake (varied in six different ways) and the extent of fluorescence quenching (tables 1–3; fig. 1), provide further support for the hypothesis of Wraight and Crofts.

The results in table 2 should be contrasted with those of McEvey and Lynn [16], who found that ammonium chloride had no effect on the internal H^+ activity of subchloroplast particles. They used absorbance changes of the dye, neutral red, to monitor changes within the thylakoids. It is possible that the discrepancy between their data and ours is due to the fact that the quenching of fluorescence by DAD and the neutral red absorbance changes are not measures of the same component.

The detailed mechanism of the light-induced quenching of chlorophyll *a* fluorescence in the presence of DAD remains to be elucidated. It does not appear to involve a redistribution of excitation energy from pigment system II to I, since the degree of quenching was not affected by changes in the extent of "spillover" caused by adding Mg^{2+} ions to chloroplasts (table 4). Wraight and Crofts suggest that the quenching results from a higher proportion of excited states undergoing thermal degradation, but the reasons for this are not yet established.

Acknowledgements

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