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CHLOROPHYLL FLUORESCENCE AS A PROBE FOR THE DETERMINATION OF THE PHOTO-INDUCED PROTON GRADIENT IN ISOLATED CHLOROPLASTS

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Summary

Gramicidin D-treated chloroplasts show an acid-induced quenching of the chlorophyll fluorescence, which is composed of a reversible and irreversible part. The reversible quenching is analogous to the photo-induced quenching in coupled chloroplasts and can be taken to determine the light induced ΔpH.

Recently it has been shown [1,2] that the slow photo-induced quenching of chlorophyll fluorescence in isolated chloroplasts is proportional to the photo-induced increase of $[H^+]$ in the intrathylakoid space, measured by the quenching of the fluorescence of 9-amino-acridine. It has been shown [1] that chemical acidification of gramicidin-treated chloroplasts induces a quenching of chlorophyll fluorescence which is also proportional to the H^+ concentration imposed in the resuspending medium. (As shown previously by Wraight et al. [3] acidification affects essentially the variable fluorescence.) A large difference between the slopes of these two linear relationships was obtained (see Fig. 4): the slope obtained in the case of the chemical acidification (curve 2) is approx. 15 times larger than in the case of the photo-induced acidification (curve 1). This difference was interpreted mainly as due to a large overestimation of the inside $[H^+]$ calculated from the 9-amino-acridine quenching. The main uncertainty of this technique

Abbreviation: Mes, 2-(*N*-morpholino)ethanesulfonic acid.

is the determination of the internal volume of the thylakoids and the knowledge of the exact mechanism of the quenching of 9-amino-acridine fluorescence. However recent studies of Haraux and de Kouchkovsky (personal communication) have shown that 9-amino-acridine quenching is a valid tool for ΔpH measurements. In order to solve this problem we checked with more accuracy the quenching of chlorophyll fluorescence induced by chemical acidification and found that it is heterogeneous. We have looked at the kinetics of the decay of chlorophyll fluorescence in gramicidin treated chloroplasts upon acidic buffer addition. We also checked the reversibility of the quenching by restoration of an alkaline pH. Here is reported that the acid induced decay of chlorophyll fluorescence includes two phases: a fast one ($\leqslant$ 1 s) which is partly reversible as is the photo-induced quenching and an irreversible one. The two slopes: first, of the reversible quenching versus H^+ concentration imposed and second, of the photo-induced quenching in coupled chloroplasts versus H^+ concentration estimated by 9-amino-acridine, are close together.

Broken pea chloroplasts are isolated according to a procedure previously described [4]. The final suspension contains 0.2 M Sorbitol, 10^{-2} M NaCl, $5 \cdot 10^{-3}$ M $MgCl_2$, $2 \cdot 10^{-4}$ M Tricine, pH 7.5, Gramicidin D 10^{-6} M, and chloroplasts equivalent to 10 μg chlorophylls per ml.

Chlorophyll *a* fluorescence intensity is measured at room temperature using an exciting beam at 480 ± 10 nm, the intensity is such that 30 photons are absorbed per reaction center and per second. The fluorescence is detected through a Corning CS 2-64 filter.

2.5 ml of chloroplasts suspension in a 1-cm cuvette are stirred during measurement. Various amounts of Mes (2-(*N*-morpholino)ethanesulfonic acid) buffer, 1 molar, adjusted to various pH values are added to the sample in order to acidify it; 40—50 μl Tricine buffer, pH 8.5, 1 molar, are subsequently added in order to reestablish the initial pH.

The kinetics of the fluorescence quenching induced by acidification is shown in Fig. 1. Mes addition induces a biphasic decrease of fluorescence. The limiting step of the fast phase can be due to the time required for the

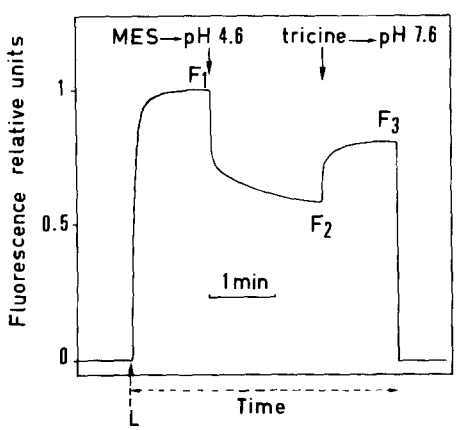


Fig. 1. Time course of the chlorophyll *a* fluorescence decay induced by acidification of gramicidin-treated chloroplasts and of its reversion by subsequent alkalinization.

mixing of the Mes buffer with the sample. A steady state is reached after 30 min.

As shown in Table I, both fast and slow phases are dark processes. Tricine addition, which restores the initial pH (7.6), partially reverses the quenching.

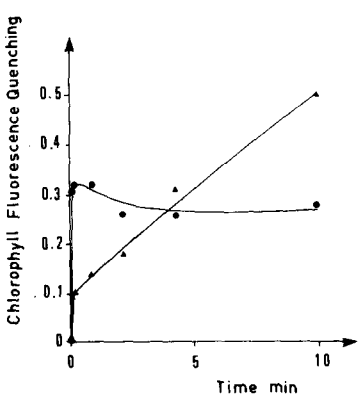
Fig. 2 is obtained from an experiment identical to the one presented in Fig. 1 but varying the time at pH 4.6; the reversible quenching is given by $((F_3/F_2) - 1)$, the irreversible one by $((F_1/F_3) - 1)$. This figure shows that both irreversible and reversible quenchings participate to the rapid decline of fluorescence whereas the slow phase is only made by the irreversible quenching. This is in agreement with the experiment shown in Fig. 3. If Gramicidin D is omitted it is observed upon acidification that a part of the fast phase is slowed down.

The pH dependence of the different types of acid induced fluorescence quenchings is exhibited in Fig. 4. In addition, we show the relationship between photo-induced chlorophyll fluorescence quenching and intrathylakoid H^+ concentration determined by the photo-induced quenching of 9-amino-acridine fluorescence (from Fig. 2 of Ref. 1). A linear relationship also exists for the two types of acid-induced quenching of chlorophyll

TABLE I

EFFECT OF LIGHT OR DARKNESS UPON CHLOROPHYLL *a* FLUORESCENCE QUENCHING OF GRAMICIDIN-TREATED CHLOROPLASTS UPON ACIDIFICATION

Time of incubation at pH 4.7	Quenching	
	Incubation in the dark	Incubation in the light
15	0.21	0.18
30	0.24	0.21
240	0.31	0.34



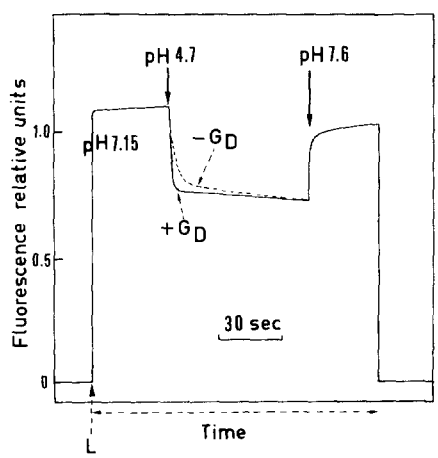


Fig. 2. Kinetics of chlorophyll *a* fluorescence quenching upon acidification of gramicidin-treated chloroplasts varying the time of pH 4.6 exposure. ●—●, reversible quenching $((F_3/F_2) - 1)$ see Fig. 1. ▲—▲, irreversible quenching $((F_1/F_3) - 1)$ see Fig. 1.

Fig. 3. Effect of Gramicidin D (G_D) on the kinetics of the acid-induced decay of fluorescence.

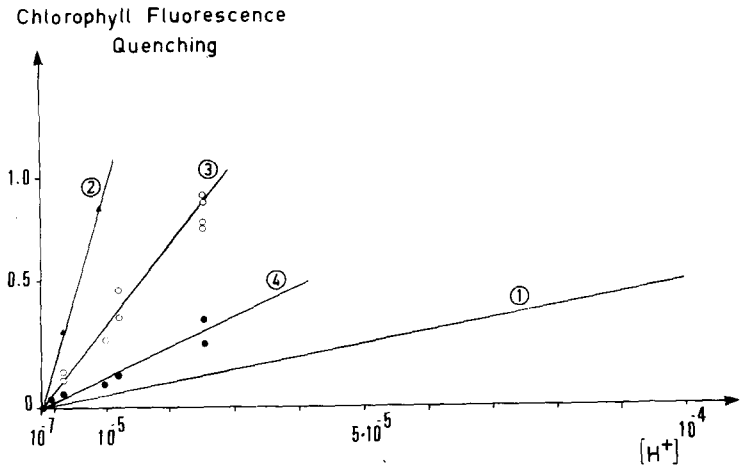


Fig. 4. Relationship between the chlorophyll *a* fluorescence quenching and H^+ concentration. (1, —) Photoinduced quenching in coupled chloroplasts, H^+ concentration is calculated from 9-amino-acridine fluorescence quenching (from Fig. 2 of Ref. 1). (2, ▲—▲) Quenching induced by incubation of Gramicidin D treated chloroplasts at various pH for 30 min. (3, ○—○) Same as (2) but after 2 min of incubation. (4, ●—●) Reversible quenching after 2 min incubation, calculated as in Fig. 2.

fluorescence. The slope of reversible quenching, but not that of the total quenching, is close to the slope of the photo-induced quenching.

The experiments presented here point out the heterogeneity of the quenching of chlorophyll fluorescence induced by chemical acidification. A part of the faster phase is due to the acidification of the intra-thylakoid space, as shown by the change of kinetics induced by the omission of Gramicidin D. This part is reversible, whereas the part of the fast phase that is not affected by gramicidin is irreversible and must be attributed to the acidification of the outer thylakoid face. The slope of the linear relationship between the reversible quenching and H^+ concentration is twice the slope of the photo-induced quenching versus H^+ concentration calculated from the quenching of 9-amino-acridine; this discrepancy can be due to the uncertainty on the internal volume estimation used for proton concentration calculation from 9-amino-acridine quenching. All these aspects allow us to identify the fast and reversible quenching as the same phenomenon as the photo-induced fluorescence decline in coupled chloroplasts. Then, in contrast to one of the conclusions of our previous paper, we consider that the photo-induced quenching of 9-amino-acridine fluorescence can be used to determine the photo-induced gradient of H^+, within the limits of the error due to the measurement of the internal volume. Note that the photo-induced quenching of chlorophyll fluorescence does not require such a volume measurement, so we propose that it is an easier way of determining the acidification of the inside. Surprisingly the inside face of the thylakoid is able, without irreversible damage (at least during short periods) to endure the presence of a high H^+ concentration (pH 4.6). In contrast, the outside face is damaged by such low pH.

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