Biochimica et Biophysica Acta, 459 (1977) 27–35

BBA 47226

SLOW FLUORESCENCE QUENCHING OF TYPE A CHLOROPLASTS RESOLUTION INTO TWO COMPONENTS

PATRICIA M. SOKOLOVE and T. V. MARSHO

Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, Md. 21228 (U.S.A.)

(Received July 20th, 1976)

SUMMARY

The divalent-cation-specific ionophore A23187 is used to define two components of the slow fluorescence quenching of type *a* spinach chloroplasts: ionophore-reversible and ionophore-resistant quenching. Ionophore-reversible quenching predominates at relatively low light intensities and approaches saturation as light levels are increased. It is sensitive to uncouplers and to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and is dark reversible. At high light intensities the bulk ($> 80\%$) of slow fluorescence quenching is ionophore-resistant. Ionophore-resistant quenching is stimulated by carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) at pH 7.6 and by both CCCP and methylamine at pH 9.0. It is insensitive to DCMU and is not reversed in subsequent darkness. Taken together, the two components account for all quenching observed in Type A chloroplasts.

Ionophore-reversible quenching is identified with the Mg^{2+}-mediated fluorescence quenching described by Krause (Biochim. Biophys. Acta (1974) 333, 301–313) and by Barber and Telfer (in Membrane Transport in Plants (Dainty, J., and Zimmermann, U., eds.), pp. 281–288, Springer-Verlag, Berlin, 1974). Ionophore-resistant quenching, a first-order process requiring high light, resembles the quenching reported by Jennings et al. (Biochim. Biophys. Acta (1976) 423, 264–274).

The resolution of the fluorescence quenching phenomenon into two distinct components reconciles the apparently contradictory observations of these earlier investigations.

INTRODUCTION

Slow, light-induced fluorescence quenching in intact (Type A) [1] spinach chloroplasts was first observed by Krause [2, 3] and by Barber and Telfer [4]. The quenching was eliminated in osmotically shocked chloroplasts and to some extent restored by the addition of Mg^{2+}. In addition, slow quenching was sensitive to

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

uncouplers. The phenomenon was therefore attributed to Mg^{2+}-movement from the thylakoids to the stroma in response to light-induced thylakoid acidification. The demonstration that the divalent-cation-specific ionophore A23187 reversed the quenching [5] provided further evidence for mediation of quenching by divalent cation movement.

More recently, Jennings et al. [6], using osmotically shocked (Type D) [1] chloroplasts supplemented with Mg^{2+}, have observed that uncouplers stimulate slow fluorescence quenching, whereas subjection of the chloroplasts to an acid-base transition is inhibitory. They conclude that the light-induced proton gradient does not drive fluorescence quenching.

In light of this apparent contradiction, and because our own initial attempts to repeat the work of Krause did not meet with success, we report here a re-examination of the slow-fluorescence quenching of Type A spinach chloroplasts.

METHODS

Type A chloroplasts were prepared, as described previously [7], from spinach. Chloroplasts fixed CO_2 at rates in excess of 50 μmol/mg chlorophyll per h, measured polarographically [8] in the presence of bicarbonate [9], and were more than 60 % intact as determined by the ferricyanide reduction method [10, 11].

Fluorescence was measured as described previously [7], with the exception that broad-band blue actinic illumination, at an intensity of 930 kerg $\cdot$ $cm^{-2} \cdot s^{-1}$, was provided by a 1000 watt projector lamp shielded by a 3-cm water filter and a Schott KG-3, Corning 4–96 filter combination.

Neutral density screens were used to vary light intensity. Fluorescence was measured at 20 °C for chloroplasts suspended at a concentration of approx. 7.5 μg chlorophyll/ml in 0.33 M sorbitol, 50 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.6.

RESULTS

The role of Mg^{2+}-movement in slow fluorescence quenching represents a central unresolved issue. The divalent-cation-specific ionophore A23187, which promotes H^+/divalent cation exchange [12], was therefore used to define two classes of quenching. Quenching that is reversed by A23187 addition (c.f. 5) is termed ionophore-reversible and is considered to be the result of divalent cation movement. (Evidence suggests that Mg^{2+} is the major cation involved [13, 14].) Quenching that occurs in the presence of A23187 is termed ionophore-resistant and is presumed to originate in some manner that does not require divalent cation movement through membranes. Fig. 1 contains examples of the two classes of quenching and delineates the regime used to measure each. Under appropriate conditions, i.e., high light, substantial ionophore-resistant slow quenching is observed (Fig. 1B). Further, the kinetics of the two classes of slow quenching clearly differ. In contrast to ionophore-reversible quenching, ionophore-resistant quenching commences without a lag and is first-order throughout.

Comparison of the work of Krause [2, 3] with that of Jennings et al. [6] revealed substantial differences in both the quality and intensity of actinic light

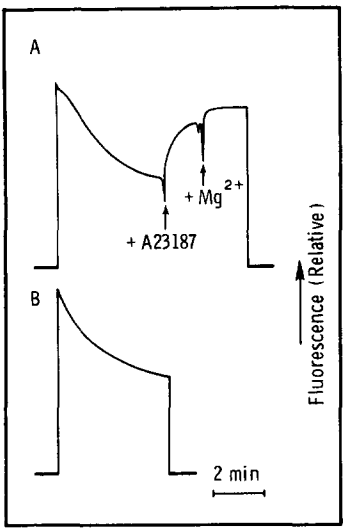

Fig. 1. Slow fluorescence quenching in Type A spinach chloroplasts. (A) Ionophore-reversible quenching. Chloroplasts are illuminated for 4 min, at which point A23187 (1.25 μg/ml) and $MgCl_2$ (7.5 mM) are added. Ionophore-reversible quenching is taken as the sum of the fluorescence increases produced by the ionophore and $MgCl_2$. Further additions of ionophore or $MgCl_2$ fail to increase fluorescence. Light intensity is 10.5 %. (B) Ionophore-resistant quenching. Chloroplasts supplemented with A23187 (1.25 μg/ml) are illuminated for 4 min. Light intensity is 100 %. Onset and termination of illumination are indicated respectively by the rapid upward and downward deflections of the trace. Note that the recorder sensitivity was adjusted to give similar initial deflections.

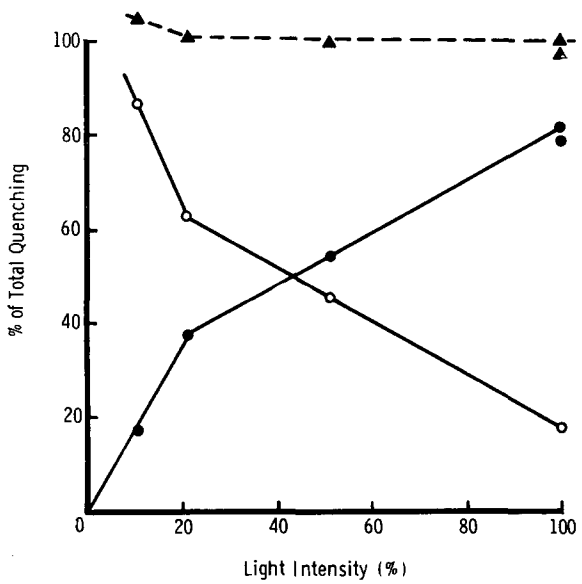

Fig. 2. The relative contributions of ionophore-reversible (○) and ionophore-resistant (●) fluorescence quenching to the total observed slow fluorescence quenching as a function of light intensity. The two types of quenching were measured independently as described in Fig. 1. Values are expressed as a percent of the total quenching observed in the absence of ionophore. The sum of the two separate measurements is indicated by the dotted line.

employed. The light intensities used by Jennings et al. [6] were more than twice those used by Krause [2, 3] (100 kerg · cm^{-2} · s^{-1}, presumably narrow-band blue (440 nm) light vs. 38 kerg · cm^{-2} · s^{-1}, broad-band red). Consideration of the absorption spectrum of chloroplasts indicates an even greater disparity (5–10 fold) in absorbed radiation between these experiments.

The relative contributions of ionophore-reversible and ionophore-resistant quenching to total slow fluorescence quenching as a function of actinic light intensity (broad-band blue) are shown in Fig. 2. It can be seen that the relative magnitudes of the two components vary significantly with light intensity. At low light, ionophore-reversible quenching predominates, while at high light, almost all quenching is ionophore-resistant. (The total percent quenching observed was relatively constant over the range of intensities employed.)

We estimate that absorbed radiation in the experiments of Krause (refs. 2 and 3; see also the experiments of Barber and coworkers, refs. 5, 13 and 14) was equivalent to approx. 10 % on the scale shown in Fig. 2, whereas absorbed radiation in the experiments of Jennings et al. [6] was equivalent to 50–100 % intensity on the same scale.

It should be noted that the sum of ionophore-reversible and ionophore-resistant quenching is approximately equal to the total observed quenching for Type A chloroplasts at all light intensities. This suggests that all major forms of slow quenching are included in the two classes as defined.

At low light intensity, the sum of the two component forms of quenching often slightly exceeded the total measured quenching. This is probably attributable to an overestimation of the ionophore-reversible component. Both A23187 and Mg^{2+} are added in determinations of ionophore-reversible quenching. This procedure is used

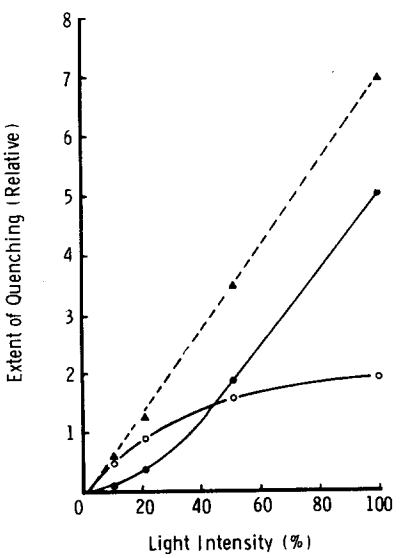

Fig. 3. Extent of ionophore-reversible (○) and ionophore-resistant quenching (●) as a function of light intensity. The changes in fluorescence observed under the regimes outlined in Fig. 1 are normalized and plotted, along with their sum (▲), as a function of light intensity.

because the relative fluorescence reversal observed with A23187 alone varies from day to day in an unsystematic fashion. (There is no correlation with percent intactness of the chloroplast preparation.) In the presence of A23187, Mg^{2+}-stimulation of the fluorescence of any broken chloroplasts in the preparation [3, 15, 16] will be included in the quantity defined as ionophore-reversible quenching, thus leading to an overestimation of that quantity. Mg^{2+}-stimulation of the fluorescence of osmotically shocked, uncoupled chloroplasts is substantial at the lower light intensities employed here, but is relatively attenuated at the higher intensities (Sokolove, unpublished observation). Thus, overestimation of ionophore-reversible quenching is most likely at low intensities.

In Fig. 3, the extents of ionophore-reversible and ionophore-resistant quenching, as well as their sum, are plotted as a function of actinic light intensity. The total slow quenching observed is a linear function of light intensity, indicating that a fixed fraction of fluorescence is quenched. However, neither of the components of slow fluorescence quenching increases in a linear fashion with light intensity: ionophore-reversible quenching appears to approach saturation as light level is increased, whereas ionophore-resistant quenching behaves in a complementary fashion, increasing progressively more rapidly as light intensity is increased. These data suggest that ionophore-reversible and ionophore-resistant quenching represent alternative fates for some fraction of the excitation initially re-emitted as fluorescence under these experimental conditions.

The effects of CCCP and methylamine on ionophore-reversible and ionophore-resistant quenching at pH 7.6 and 9.0 are shown in Fig. 4. (Each component of the slow fluorescence quenching is followed at a light intensity at which it predominates: the former at low light, the latter at high.) Ionophore-reversible quenching is completely inhibited by CCCP ($5 \cdot 10^{-6}$ M) and methylamine (40 mM) at both pH 7.6 and pH 9.0. Ionophore-resistant quenching is stimulated by CCCP at both pH values; methylamine stimulates strongly at pH 9.0, but has little effect at pH 7.6.

It should be noted that the ionophore-resistant quenching is qualitatively unaffected by the difference in pH (compare Fig. 4, B and D). In contrast, slow quenching in low light (predominantly ionophore-reversible quenching) is more extensive and considerably slower at pH 7.6 (Fig. 4A) than at pH 9.0 (Fig. 4C).

The dark reversibility and sensitivity to DCMU of the two classes of slow fluorescence quenching are illustrated in Fig. 5. Ionophore-resistant quenching is essentially irreversible in the dark (Fig. 5B), in contrast to ionophore-reversible quenching (Fig. 5A). In fact, it would appear that difficulties in obtaining complete dark reversal of slow fluorescence quenching (as between the first two illuminations in trace A, Fig. 5; see also ref. 13) arise from ionophore-resistant fluorescence quenching. When this component of slow fluorescence quenching has been allowed to run essentially to completion (i.e., during the first illumination), quenching appears to be completely dark reversible. (Compare the second and third illuminations of the sample.) Furthermore, the remaining quenching is completely ionophore-reversible. At all light levels, dark-irreversible quenching equivalent in extent to that measured in the presence of A23187 is also seen in its absence. Thus ionophore-resistant quenching is not dependent upon the presence of ionophore. A substantial portion ($> 87\,\%$) of the ionophore-resistant quenching persists in the presence of 10^{-5} M DCMU (Fig. 5D); ionophore-reversible quenching is completely eliminated (Fig. 5C).

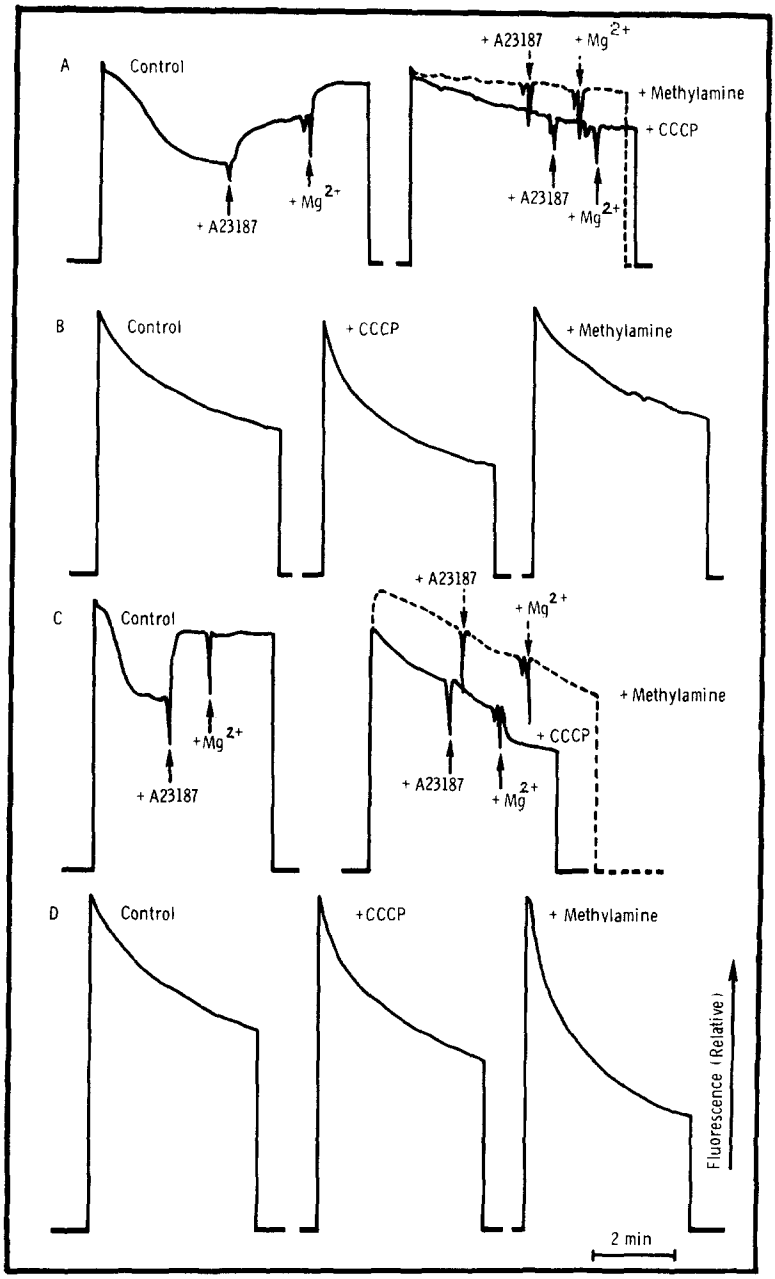

Fig. 4. Sensitivity of ionophore-reversible and ionophore-resistant fluorescence quenching to uncouplers. Traces in A and B are for chloroplasts at pH 7.6; traces in C and D were made at pH 9.0. In A and C, light intensity is 10.5 %, and A23187 (1.25 μg/ml) and $MgCl_2$ (7.5 mM) are added to determine ionophore-reversibility. In B and D, light intensity is 100 % and A23187 (1.25 μg/ml) is present from the outset. The final concentrations of uncouplers are CCCP, $5 \cdot 10^{-6}$M; methylamine, 40 mM. Other details as in Fig. 1.

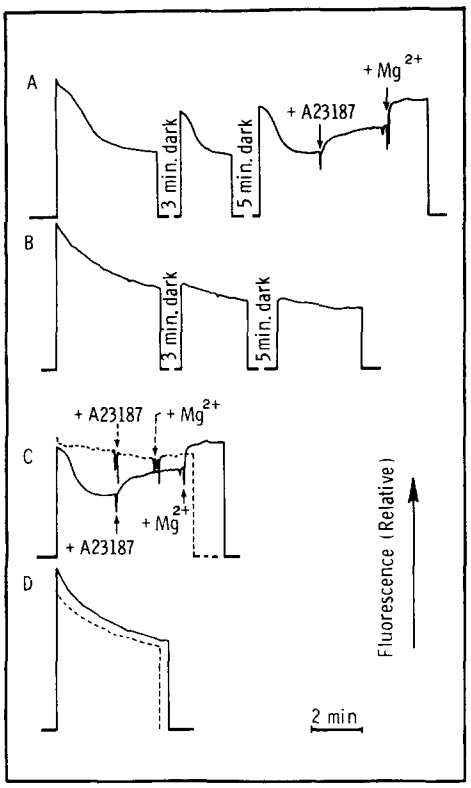

Fig. 5. Dark reversibility of the two components of slow fluorescence quenching. (A) Ionophore-reversible quenching measured at 10.5 % light-intensity. (B) Ionophore-resistant quenching, measured at 100 % light in the presence of A23187 (1.25 μg/ml). DCMU-sensitivity of the two forms of quenching: (C) Ionophore-reversible quenching measured at 10.5 % light in the presence (dotted line) and absence (solid line) of 10^{-5}M DCMU. (D) Ionophore-resistant quenching, measured at 100 % light in the presence of A23187 (solid line) or A23187 and DCMU (10^{-5}M) (dotted line). Other details as in Fig. 1.

DISCUSSION

The data indicate that the phenomenon referred to as slow fluorescence quenching is, in fact, the composite of two processes differing dramatically in their response to light, to uncouplers, and to DCMU, in their sensitivity to pH, and in their dark reversibility.

Ionophore-reversible quenching is readily identified as the slow fluorescence quenching reported by Krause [2, 3] and by Barber and coworkers [5, 13, 14] and attributed to Mg^{2+}-efflux from the thylakoids driven by proton uptake. This phenomenon can clearly best be studied at absorbed light intensities sufficiently low to insure that interference from ionophore-resistant quenching is minimal.

The finding that the extent of ionophore-reversible quenching saturates as light intensity is increased has not previously been reported. (Krause [3] reported half-saturation of fluorescence quenching at 3.5 kerg $\cdot$ cm^{-2} $\cdot$ s^{-1}, but the parameter being discussed was percent quenching, i.e., quenching expressed as a percent of the

maximal fluorescence level. For ionophore-reversible quenching, percent quenching decreases at high light.) Several authors [3, 13] have suggested that Mg^{2+}-mediated quenching in Type A chloroplasts is related to Mg^{2+}-stimulation of fluorescence in Type C (Class II) chloroplasts [15, 16]. The latter is, in turn, thought to represent decreased spillover of excitation from Photosystem II to Photosystem I [17, 18]. The finding of light saturation behavior would then imply some limitation on the transfer of excitation to Photosystem I. Perhaps the limitation is inherent in the transfer mechanism. Possibly the loss of excitation via ionophore-resistant quenching limits ionophore-reversible quenching at high light. At this point, whether ionophore-resistant quenching is the cause or the result of the saturation of ionophore-reversible quenching is unknown.

On the basis of actinic light intensity employed, response to uncouplers and lack of dark-reversibility in the presence of uncouplers, we suggest that the quenching studied by Jennings and coworkers [6] represented primarily the phenomenon defined here as ionophore-resistant quenching. We agree with these authors that this type of quenching is not driven by proton uptake by the thylakoids. The actual mechanism responsible for ionophore-resistant quenching is, however, unclear. We have considered several possibilities.

One possible explanation for ionophore-resistant quenching, namely, chlorophyll photooxidation, has been discarded for a number of reasons: (1) we did not observe any destruction of chlorophyll under the conditions of our experiments and (2) in contrast to chlorophyll photooxidation [19, 20], ionophore-resistant quenching begins without a lag.

A more likely alternative is that ionophore-resistant quenching reflects photoinhibition [21]. Like ionophore-resistant quenching, photoinhibition is irreversible [22] and is a first-order process [22–24]. Photoinhibition in Type C chloroplasts (in air) is correlated with a decrease in the variable fluorescence [23]. The quenching reported by Jennings et al. [6] (equated here with ionophore-resistant quenching) likewise represents a reduction in the variable fluorescence. Finally, like ionophore-resistant quenching, photoinhibition is relatively insensitive to DCMU [24], while the correlated decrease in fluorescence is resistant to reversal by DCMU [23].

Using the formulations of Butler and Kitajima [25], Jennings et al. [6] suggested that a decrease in k_t, the probability constant for energy transfer from the reaction center chlorophyll back to the fluorescent light-harvesting chlorophyll (or the probability constant for a closed reaction center not trapping) was responsible for slow fluorescence quenching. Unfortunately, as the authors state, the mechanism of k_t alteration is unknown. Photoinhibition, which has been attributed to an increase in k_d, the probability of nonradiative decay processes at the reaction center chlorophyll [25] and to traps that remain permanently open such that absorbed quanta are wasted [24], would be consistent with an apparent decrease in k_t.

ACKNOWLEDGEMENTS

The helpful discussions of Dr. P. G. Sokolove and the technical assistance of Ms. Mary Jo Carlo are gratefully acknowledged. This research was supported in part by a grant from the National Science Foundation (GB-38237). A23187 was the gift of Dr. Robert L. Hamill.

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