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APPLICATIONS OF FLUORESCENCE QUENCHING TO BIOLOGICAL PROBLEMS: "HIGH-ENERGY STATE" QUENCHING OF CHLOROPLAST FLUORESCENCE

Since the appearance of the first commercial fluorescence meters, nearly twenty years ago, fluorescence techniques are playing an ever increasing role in the biochemical, the biophysical, and the biomedical laboratories [3, 8, 20, 25]. Fluorescence theory and methods, such as those pertaining to fluorescence quenching, have been applied extensively to the study of biological molecules, membranes, and membrane-bounded organelles and cells. One particular system, about which much has been learned from the application of fluorometry, are the photosynthetic membranes of higher plant chloroplasts and algae. These membranes are unique because they contain chlorophyll (Chl), the lipid-like pigment which plays a central role in trapping solar photons and transducing their energy into electrochemical and redox potentials. Chl amounts to about 10% of the membrane mass and exists as two main variants, slightly differing in their molecular structure, Chl *a* and Chl *b*. Of the two, only Chl *a* is fluorescent *in vivo*.

Since the pioneering observations of Kautsky, in 1931 [12] it is known that the fluorescence yield of Chl *a in vivo* varies in response to the functional state of the photosynthetic membranes. The variation affects part of the emitted fluorescence (identified as "variable fluorescence") and it is now ascribed to the following causes [7, 19].

1. The oxidation state of the reaction centers of photosystem II, which utilizes photons in order to liberate molecular oxygen from H_2O and produce weak reductants at a midpoint potential of about 0V. Centers with electron deficient components trap and dissipate the excitation of Chl *a* without fluorescence emission.

2. The presence of metal cations, especially Mg^{2+}, in the thylakoid interior. The state is characterized by increased Chl *a* fluorescence.

3. The state of "high energy", namely the existence of chemical and electrical potentials across the thylakoid membrane. This state has been proposed to exert a quenching effect on the fluorescence of Chl *a in vivo*.

In this review, I shall endeavour to summarize the current status of ideas pertaining to the quenching effect of the high energy state, assuming some knowledge of the presently accepted theory on the mechanism of photosynthesis and the associated terminology. The problem is of considerable contemporary interest since a number of recent publications from various laboratories seriously question tenets held firmly only 3 years ago. In what follows, I shall use the term fluorescence to identify the ns (prompt) emission by Chl *a*. The effect of the high energy state on Chl *a* luminescence (delayed light) was recently reviewed by Lavorel [13].

In order to resolve the effect of the high energy state on the fluorescence of Chl *a in vivo* it is necessary to block electron transport through the reaction centers of photosystem II with metabolic poisons such as dichloro-dimethylophenylurea (DCMU) and at the same time to

employ a catalyst to accelerate the cyclic electron transport through the reaction centers of photosystem I. This process utilizes photons to produce electrochemical potentials across the thylakoid membrane with no net output of oxidants and reductants (for a review, see ref. [2]). In this way, the reaction centers of photosystem II are permanently closed, and therefore whatever fluorescence changes are observed have their origin in the operation of the cyclic electron transport alone. Cyclic electron transport is catalyzed by several compounds such as dichlorophenol indophenol (DCIP) diaminodurene (DAD), menadione (vitamin K) and phenazine methosulfate (PMS). PMS differs from the other catalysts because it is not a quinone; it is an organic cation becomes an electroneutral free radical by adding one electron to its molecular structure [27].

The first to show quenching of chloroplast fluorescence in the presence of PMS and menadione was Arnon and his coworkes [1]. They interpreted this result as indicating excitation energy "spillover" from the fluorescing photosystem II Chl *a* to the nonfluorescing Chl *a* of photosystem I, in order to provide the energy required for the fast running cyclic electron transport. Since, however, DCMU was not included in the reaction mixture, the quenching could have resulted also from a direct oxidation of the reaction centers of photosystem II by the added compounds.

Quenching of chloroplast fluorescence in the presence of both DCMU and PMS was first reported in a review article by Govindjee et al. (ref. [6]; experiments of L. Yang and Govindjee) but the first through study of the phenomenon was by Murata and Sugahara [16]. These authors established that maximal quenching required intense excitation, in excess of 30 kergs·cm^{-2}s^{-1}, and that the effect is equally distributed among the three fluorescence bands of Chl *a* *in vivo* at low temperature (77°K). In addition, they observed that the quenching effect of PMS was even more pronounced when excess ascorbate was included in the reaction mixture, and that uncoupless of the photophosphorylation, such as atabrin and carbonyl cyanide chlorophenylhydrazone (CCCP) inhibited the fluorescence quenching. Since these compounds are known to dissipate the light-induced electrochemical potentials of chloroplasts, it was suggested that these potentials (i.e. the high energy state) are the generic cause of the light-induced quenching in the presence of PMS. It must be pointed out, however, that the required concentrations for the inhibition of the quenching were well above those required to uncouple photophosphorylation.

Similar results were obtained with algae and isolated chloroplasts by Mohanty and Govindjee [14] and Mohanty et al. [15], who also mentioned that the quenching is associated with a decline in the fluorescence lifetime of Chl *a*; hence it is a true decrease of the quantum yield of Chl *a* fluorescence *in vivo*.

Other groups of researches studied light-induced fluorescence quenching in chloroplasts and bacteria, employing quinoid compounds such as DAD as catalysts of the electron transport. With this system, Wraight and Crofts [26] observed that compounds which make the chloroplast thylakoids freely permeable to protons (eg. NH^+Cl and the antibiotic dianemycin) could reverse the light-induced quenching, whereas compounds which dissipate the transmembrane electric potential (such as K^+-valinomycin) were less effective. This led them to the postulate that the observed quenching is primarily related to the proton concentration difference in and out of the thylakoid, that is established as a result of the cyclic electron transport. Similar ideas were promoted by Cohen and Sherman [5] and Sherman and Cohen [23] who studied light-induced fluorescence quenching with isolated chloroplasts and *Rhodopseudomonas spheroides* chromatophores. In particular, they have shown that a) subchloroplast particles show less light-induced quenching in the presence of

DAD; b) NH_4Cl reverses both the light induced quenching and the light-induced proton uptake in chloroplasts; and c) removal of the coupling factor protein, by means of EDTA washing, abolishes both the fluorescence quenching and the proton uptake. Cohen and Sherman used quite high concentrations of DAD (330 μM), and it is possible that part of the effect they were observing was simply a direct quenching interaction between excited Chl *a* and the added cofactor [17].

We may summarize the evidence in favor of a quenching effect on the fluorescence of Chl *a* *in vivo*, that is due to the existence of a high energy state in the thylakoids, as follows:

1. Fluorescence quenching is observed at conditions which are required for fast cyclic electron transport, namely strong excitation, presence of suitable catalysts, and presence of the photosystem II inhibitor DCMU.

2. Quenching requires the thylakoids and the thylakoid membrane to be intact. This is also a prerequisite for the formation of transmembrane electrochemical potentials.

3. In some cases, uncouplers of photophosphorylation abolished or reversed the quenching. Proton transporting uncoupless are very effective with DAD-induced quenching, but they are ineffective with PMS (Table I).

Table I

Percent inhibition by photophosphorylation uncouplers of the light-induced quenching of chloroplast fluorescence in the presence of DCMU, and PMS or DAD*

Uncoupler	% inhibition	
	PMS	DAD
NH_4Cl, 5 mM	0	100
$CH_3NH_2 \cdot HCl$, 15 mM	0	90
Valinomycin, 25 μM	30	100
CCCP, 5 μM	25	100
Nigericin, 1 μM	–1.3	–
Dianemycin, C 1 ng/ml	–	100

*Data compiled from Wraight and Crafts [26], Schmidt and Rurainski [22], and Homann [9].

In the last few years, new evidence obtained by several laboratories independently, suggests the possibility that other mechanisms, quite distinct from the proposed high energy quenching of chloroplast fluorescence, may also operate in the case of PM. I have shown [17, 18], for example, that oxidized and ascorbate-reduced PMS (i.e. the PMS free radical) are very effective quenchers for excited Chl *a* in solution. Taking the mean lifetime of Chl *a* fluorescence in methanol to be $\tau = 6.9$ ns [4], the calculated collision rate constant was estimated to be $k = 4.06 \times 10 M^{-1} s^{-1}$. Since this magnitude is in the range of the diffusion-imposed limit for bimolecular reactions, it follows that every single collision between Chl *a* and PMS is a

quenching event. This is in accordance with the delocalization of the positive charge over the entire PMS molecule, and the delocalization of the excitation of Chl *a* over the π-electron system of the chlorin ring.

In vivo, part of the observed quenching of chloroplast fluorescence is also due to the direct interaction between excited Chl *a* and PMS, while the rest is due to an indirect, cyclic electron transport mediated effect. The latter is reversed during an ensuing dark rest of the chloroplasts; the first is not. This property allowed us to contract PMS concentration curves for the direct and the indirect quenching. Directly quenched chloroplast fluorescence shows no saturation with increasing PMS. On the other hand, the indirectly quenched fluorescence (dark reversible part) saturates at about 2–6 μM PMS [17, 18].

Further doubts on the role of the high energy state in the light-induced fluorescence quenching by PMS were raised by Slovacek and Bannister [24]. These authors observed that a) the light-induced proton uptake by unpoisoned chloroplasts is not attended by fluorescence quenching; b) the light-induced fluorescence quenching in the presence of PMS and DCMU is not attended by synchronous proton uptake; and c) in the presence of ascorbate, quenching is accompanied by proton uptake, but the resulting pH gradient can be dissipated with FCCP and NH_4Cl without restoration of the fluorescence to the original level.

In independent experiments, Schmidt and Rurainski [22] and Homann [9] discovered that simultaneously with the light-induced fluorescence quenching, there is an absorption decrease at 388 nm (ΔA388), the principal absorption band of oxidized PMS. The surprising feature is that as much as 1 PMS/2 Chl appear to have been transformed, a figure much too high to be accounted for by a photosynthetic chain reduction of the added PMS. In a subsequent publication, Homann [10] reported that the bleaching of the 388 nm absorption is not attended by an increase at 448 nm, that could signal the appearance of reduced PMS free radical. Homann, further, observed that with suitable symport anions, such as Cl^-, up to 6 PMS/Chl is accumulated in the membrane, perhaps on nucleophilic sites of the membrane that have been exposed as a result of the cyclic electron transport. No adequate explanation is provided, however, why this accumulation is associated with ΔA388.

It is quite possible that the light-induced accumulation of PMS in the membrane phase and the light-induced quenching in the presence of PMS are two distinct phenomena occuring simultaneously. For example, from the data in ref. [17] it can be calculated that at maximal photochemical quenching of chloroplast fluorescence, the ratio of PMS/Chl is only 0.1–0.2, far less than the binding capacity of 6 PMS/Chl. Also, ascorbate-reduced [16–18, 24] and glucose-oxidase reduced [21] PMS, is a more effective quencher than the oxidized PMS for Chl *a in vivo*. Since the reduced species is the electroneutral free radical of PMS, it is hard to visualize how this will accumulate on exposed nuclophilic sites of the membrane.

In conclusion, it appears that PMS quenches the fluorescence of DCMU-poisoned chloroplasts under strong exciting light by means of one or more of the following, mutually nonexclussive mechanisms: (1) direct quenching of excited Chl *a* [17, 18]; (2) excitation energy spillover from fluorescent photosystem II Chl *a* to the nonfluorescent photosystem I Chl *a* [1, 24]; (3) quenching by PMS after reduction (poising) by photosystem I and penetration into the membrane phase, [22]; and (4) quenching due to transmembrane electrochemical gradients, as evidenced by the inhibition effect of valinomycin (Table I).

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ZASTOSOWANIE GASZENIA FLUORESCENCJI DO ZAGADNIEŃ BIOLOGICZNYCH GASZENIE „WYSOKOENERGETYCZNE" FLUORESCENCJI CHLOROPLASTÓW

Streszczenie

Kofaktory cyklicznego transportu elektronow takie, jak DAD i PMS gaszą fluorescencję chloroplastów zatrutych przez DCMU na drodze kilku procesow, obejmujących: gaszenie bezpośrednie, gaszenie poprzez

transbłonowe gradienty elektrochemiczne oraz pobudzeniowy „spillover" z fluoryzujących chlorofili fotosystemu II do niefluoryzujących chlorofili fotosystemu I. Równoczesny, indukowany przez światło zanik pasma absorpcji PMS przy 398 nm może nie być bezpośrednio związany z procesami gaszenia fluorescencji.

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