QUENCHING OF UNCOUPLER FLUORESCENCE IN RELATION ON THE "ENERGIZED STATE" IN CHLOROPLASTS

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1. Introduction

Proposed mechanisms [1–3] by which uncoupling compounds dissipate energy in mitochondria and chloroplasts are essentially based on observations that uncouplers interact with these organelles at the level of the osmotically active membranes. The energy conserving systems generate an "energized state" (high-energy compound [4], electrochemical membrane potential [5] or high-energy conformation of the membrane proteins [6]) which can be used to drive ATP synthesis, ion transport and other energy-requiring processes. The "energized state" can be detected by measuring these processes. In the presence of an uncoupler the energy cannot be recognized beyond the site of action of the uncoupler. However, the uncoupler itself should "see" the "energized state" during the uncoupling activity. A possible interaction between the uncoupler and the "energized state" was investigated by making use of the fluorescent properties of some uncouplers. Fluorochromes have been used in studies on isolated proteins [7] for the determination of molecular weights, ligand orientations [8], conformational changes [9] and relaxation phenomena [10] and as "polarity probes" and "spectroscopic rulers" [11]. In mitochondria and submitochondrial particles energy-dependent uncoupler-inhibited fluorescence changes have been observed, using different fluorescent probes [12–16]. However, if a fluorescent probe is used for quantative determination of the "energized state" ("titration of ∿") it must have a sufficiently high affinity for the system to dissipate the energy and to inhibit all other energy-utilizing reactions. In other words: the fluorochrome itself must be an uncoupler.

The experiments reported in this paper show that there is a stoichiometric relationship between the energy generation in spinach chloroplasts and the fluorescence quenching of the uncoupler atabrine. The required energy may be provided by electron transport, ATP hydrolysis or a pH gradient [17].

Abbrevations
DCMU: 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea;
diquat: *N,N'*-ethylene-2, 2'-dipyridilium dibromide;
DTE : dithioerythritol;
FCCP : carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone;
S13 : 5-chloro,butan-3-ol,2'-chloro,4'-nitro-salicylanilide;
TTFB : 4, 5, 6, 7-tetrachloro-2-trifluoromethyl-benzimidazole.

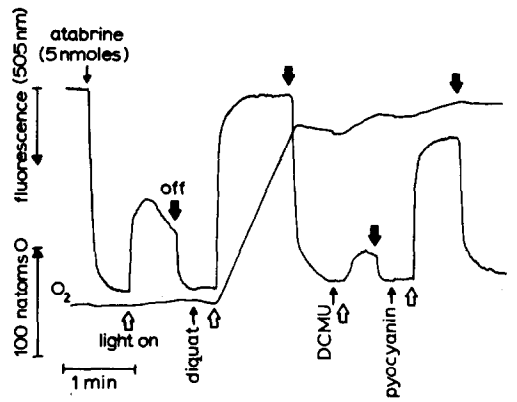

Fig. 1. Effect of electron transport on the fluorescence of atabrine. For the incubation conditions, see Materials and methods. 3 mM DTE was added. The concentration of diquat was 5 μM, that of DCMU 1 μM and that of pyocyanin 5 μM. The chlorophyll concentration was 74 μg/ml.

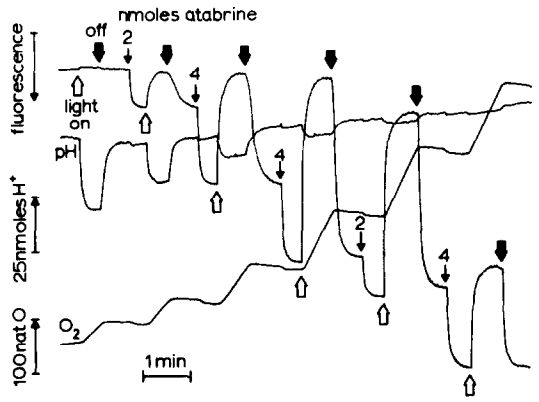

Fig. 2. Effect of increasing atabrine concentrations on the light-induced fluorescence quenching, electron transport and the pH rise. Atabrine concentrations as indicated. The buffer concentration was 4 mM. The chlorophyll concentration was 71 μg/ml.

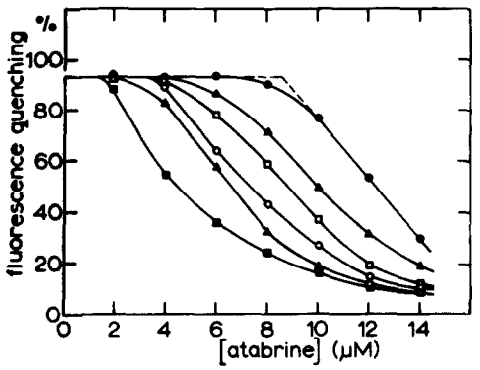

Fig. 4. Dependency of the fluorescence quenching on the atabrine concentration in the presence of different uncouplers. Conditions as fig. 3. The chlorophyll concentration was 65 μg/ml. —●— no uncoupler added; —○— 0.22 μM S13; —■— 0.34 μM S13; —□— 1.3 μM FCCP; —△— 1600 μM methylamine; —▲— 22 μM chlorpromazine.

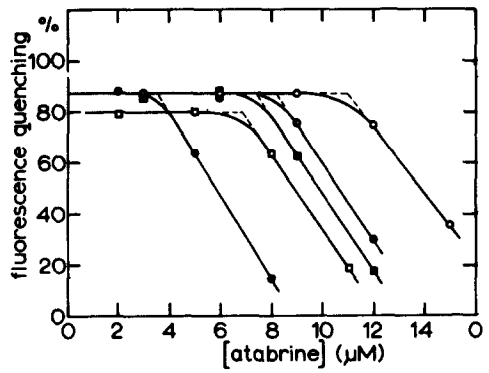

Fig. 3. Dependency of the fluorescence quenching on the atabrine concentrations at different light intensities and in the presence of inhibitors. The incubation medium contained, in addition, 5 μM diquat, 3 mM DTE and 78 μg chlorophyll/ml. —○— 21.8 mW/cm^2 (saturating light-energy); —●— 7.52 mW/cm^2; —●— 2.05 mW/cm^2; —■— 0.25 μM DCMU, 21.8 mW/cm^2; —□— 4 μM antimycin, 21.8 mW/cm^2.

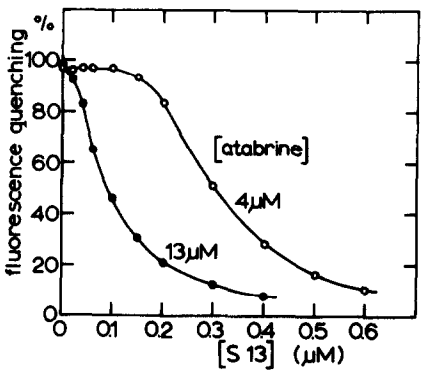

Fig. 5. Effect of S13 on the fluorescence quenching at different atabrine concentrations. Conditions as in fig. 3. Chlorophyll concentration: 78 μg/ml. Atabrine and S13 concentrations were as indicated.

2. Materials and methods

The preparation of spinach chloroplasts and the apparatus for simultaneous measurement of fluorescence, oxygen concentration and pH were as described previously [18]. The excitation light was filtered by a 405–436 nm filter (Eppendorf). Emission was measured "front-face" through a 505 nm interference filter (Vitatron) with a 1P21 (RCA) photomultiplier. The cuvette could be illuminated by a 24 V, 150 W quartz-iodide lamp, of which the light was passed through a 6-cm water layer and a red filter (Schott RG1). The signals were recorded on a multi-channel recorder (Rikadenki Kogyo Ltd.).

Unless otherwise stated, the chloroplasts were incubated in the following reaction medium (final concentrations): 160 mM sucrose, 40 mM NaCl, 20 mM

tris (hydroxymethyl)methylaminoethanesulfonic acid buffer (pH 7.8) and 3 mM $MgCl_2$. All reactions were carried out in a volume of 1.0 ml at 25°C.

Electron transport was measured as oxygen uptake mediated by diquat. The peroxide formed was removed with DTE.

Total chlorophyl was determined according to Wathley and Arnon [19].

3. Results and discussion

When atabrine is added to a chloroplast suspension in the dark the fluorescence of this compound increases linearly with increasing concentration up to at least 0.1 mM. If atabrine is present in uncoupling concentrations (2–10 μM) the fluorescence is quenched when the chloroplasts are illuminated. Fig. 1 shows that without added electron acceptor the maximal quenching is about 40% of the fluorescence signal; furthermore there is a rapid decay. Under conditions of non-cyclic electron transport mediated by diquat *plus* DTE this quenching amounts to nearly 100% and there is no decay in the light. Addition of the electron transport inhibitor DCMU gives severe inhibition of the fluorescence quenching in the light while DCMU-independent cyclic electron transport, mediated by pyocyanin, partly restores the effect. In further experiments it was found that the magnitude of the fluorescence quenching is not only dependent on electron transport, but also on the concentration of atabrine and on the chloroplast concentration. In the experiment shown in fig. 2 consecutive additions of atabrine to a chloroplast suspension in the dark and the light-induced fluorescence quenching were recorded simultaneously with oxygen uptake and pH [20] changes. As can be seen, inhibition of the fluorescence quenching, maximal stimulation of electron transport and complete inhibition of H^+ uptake were obtained in the same concentration range of atabrine. It seems likely therefore that the decrease in fluorescence quenching reflects the saturation of the energy-dissipating system. The saturating atabrine concentration is proportional to the chlorophyll concentration as long as other conditions are optimal and amounts to about 65 mmoles atabrine per mole of chlorophyll. In fig. 3 the light-induced quenching is plotted against the uncoupler concentration. Decreasing the velocity of electron transport by using non-saturating light intensities or electron-transport inhibitors like DCMU or antimycin results in a lowering of the saturating concentration of atabrine. This observation underlines an earlier suggestion [21] that, at least in the mitochondrial system, uncouplers have a fixed maximal *rate* of energy dissipation and not a fixed stoichiometry to the number of energy-conserving *sites* [22, 23]. The same experiment was carried out in the presence of other uncouplers. As fig. 4 shows, the effect of uncouplers is different from that of inhibitors of electron transport. Due to the fact that atabrine competes with the added uncouplers, the curves tend to approach the control curve. The shift of the saturation point is about proportional to the concentration of the added uncoupler; this is shown for S13, but all the other uncouplers tested behave in the same way. Fig. 5 shows that at fixed atabrine concentrations the uncoupler S13 inhibits the fluorescence quenching when the combination of both uncouplers saturates the system. From these experiments it follows that it is possible to determine the affinity of different uncouplers relative to that of atabrine. In table 1 the affinity of some uncouplers for the chloroplast is expressed as moles of uncoupler having the same effect as 1 mole of atabrine. This sequence fits very well with the effectiveness of these uncouplers in inhibiting energy-requiring reactions like ATP synthesis and the light induced pH rise [20]

Table 1
Affinity of different uncouplers for the "energized state" in chloroplasts. The affinity is expressed as moles of uncoupler having the same effect as 1 mole of atabrine in saturating the "energized state".

Uncoupler	moles/mole Atabrine
S13	0.044
TTFB	0.088
FCCP	0.53
Atabrine	1.00
Chlorpromazine	4.49
Laurylamine	6.00
2,4-Dinitrophenol	26.3
Methylamine	296

From these experiments it could be expected that not only electron transport but also other reactions

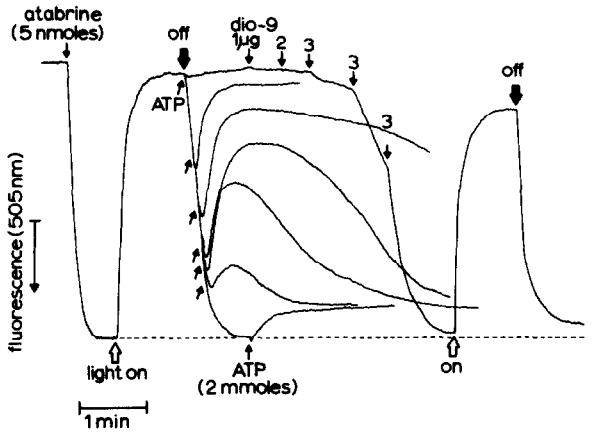

Fig. 6. Fluorescence quenching induced by the light-triggered ATPase. The incubation medium contained, in addition to the standard components, 5 mM DTE. The times of addition of ATP (2 mmoles) were as indicated. Chlorophyll concentration, 78 µg/ml.

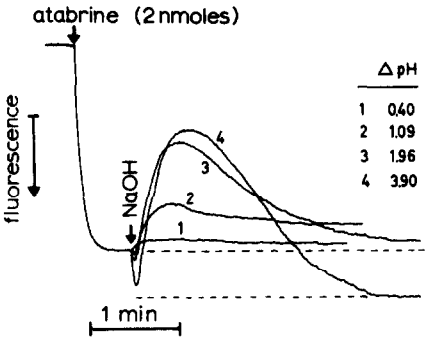

Fig. 7. Fluorescence quenching induced by acid-base transition. The chloroplasts (56 µg chlorophyll/ml) were incubated in the standard incubation medium *plus* 45 µl 0.2 M succinic acid, which results in a pH of the medium of 5.35. Additions of NaOH (2 M) were 2 µl (curve 1), 5 µl (2), 10 µl (3) and 18 µl (4), which resulted in the pH differences as indicated.

leading to an "energized state" would have the same effect on the atabrine fluorescence. In fig. 6 an experiment is pictured in which the fluorescence quenching was maintained in the dark by addition of ATP. As is known, chloroplasts can be induced to hydrolyse ATP at appreciable rates when they are preilluminated in the presence of Mg^{2+} and SH-compounds [24]. When the light is turned off, this "energized state" triggering an ATPase, decays fairly quickly [25]. Form the different recordings in fig. 6 it can be seen that the fluorescence quenching could be restored by ATP to an extent which is dependent on the time of ATP addition. When the decay was completed, ATP had the same influence as when it was added without preillumination. This ATP effect in the dark is very much dependent on the freshness and integrity of the chloroplasts, which is consistent with earlier observations [26] that intact or "Class I" chloroplasts have a much higher dark ATPase than damaged ("Class II") chloroplasts. The ATPase-induced fluorescence quenching is maintained as long as the ATP/ADP ratio is high enough. The antibiotic Dio-9, which inhibits photophosphorylation and the ATPase, inhibits the quenching effect (fig. 6).

Another way of inducing a "high-energy state" is described by Jagendorf and coworkers [17, 27], who found phosphorylation and ATPase induced by a transition of the chloroplasts from a low to a high pH. In fig. 7 it is shown that under similar conditions, where chloroplasts were preincubated with succinic acid at pH 5.35, an acid-base-induced quenching of atabrine fluorescence was produced. In complete darkness addition of NaOH gave rise to a considerable but temporary quenching, which was higher at larger pH differences. At higher pH the fluorescence signal also reached a higher level, due to increased atabrine fluorescence at higher pH.

From the experiments presented it is clear that all three methods of producing an "energized state" in chloroplasts result in a quenching of atabrine fluorescence. Presumably this quenching reflects a binding of the uncoupler to certain structures related to the energy-conservation mechanism, or a change of the environment in which the uncoupler is situated.

Because of the fact that the uncoupler action is so closely linked to the "energized state", fluorescence changes of these compounds can be useful tools in determining such a state.

Similar results have been obtained in rat liver mitochondria and submitochondrial particles from beef heart with atabrine and also with other fluorescent uncouplers like the salicylanilides. These will be described in more detail elsewhere.

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Spectrofluorimetric determination revealed that the quenching of atabrine fluorescence was due to disappearance of the emission spectrum and not to a shift of the wavelength of maximal emission.