12-(9-ANTHROYL)-STEARIC ACID AND ATEBRIN AS FLUORESCENCE PROBES FOR ENERGETIC STATES OF CHLOROPLASTS

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1. Introduction

Ever since the introduction of the chemiosmotic hypothesis [1] for the coupling of phosphorylation to electron flow, the role of the two major components (pH gradient, $\triangle$pH, and membrane potential, $\triangle\psi$) of the total electrochemical energy gradient utilized for ATP synthesis has been actively investigated in several systems [2–9]. The importance of both the proton gradient [6,7] and the membrane potential [8,9] aspects of the total proton motive force has been demonstrated in chloroplast systems.

In this report, a comparison of the effects of antibiotics (valinomycin, gramicidin, nigericin) on the light and dark responses of the fluorescence probes 12-(9-anthroyl) stearate (AS) and atebrin illustrates their potential usefulness in the investigation of the relation of the membrane potential to the energy conservation mechanism.

It is believed that these probes may provide valuable information regarding an energetic membrane state involved in the coupling of phosphorylation to photosynthetic electron flow, including any participating membrane conformational changes. The experiments reported in this paper demonstrate a light-induced, phenazine methylsulfate (PMS) catalyzed, biphasic increase in AS fluorescence inhibited by uncouplers of phosphorylation e.g. NH_4Cl and suppressed by phosphorylating conditions (ADP, P_i, Mg^{2+}). Valinomycin (in the presence of K^+) in the dark produced a diffusion-potential-induced stimulation of AS fluorescence which may be correlated to a stimulation of ATP synthesis by a membrane potential [9]. The effect of valinomycin on the light response of the fluorescence probe atebrin, reported here, which monitors some function of the 'energized state' in chloroplasts [10–13], could also be correlated with the effects of valinomycin on AS fluorescence. Thus, the fluorescent probe, AS, used here for the first time with chloroplasts, is suggested to report an 'energy state' of the thylakoid membrane.

2. Materials and methods

Class II chloroplasts were isolated from oats *(Avena sativa)* and resuspended in the homogenizing medium: 50 mM phosphate buffer containing, at pH 7.8, 10 mM NaCl and 400 mM sucrose. Chlorophyll (Chl) concentration was determined by the method of MacKinney [14].

Spectrofluorimetric experiments were performed on an instrument previously described [15]. Probe fluorescence was excited at 366 nm (3.2 nm bandwidth, C.S. 760 filter) and monitored at 460 nm (AS) or 510 nm (Atebrin) (6.4 nm bandwidth) at 90° to the measuring beam. Saturating actinic light was passed through Corning C.S. 3-73 and C.S. 2-59 glass filters plus a heat filter (#7740) and a 5 cm water filter; this light was blocked from the measuring monochromator with a Corning C.S. 4-96 filter.

Photophosphorylation was measured by the method of Nishimura et al. [16], as described by Dilley [17]. Light-induced proton transport was measured with a Sargent glass pH electrode (attached to a Corning model 12 meter), using saturating light passed through a Corning C.S. 3-69 filter plus a heat filter.

All experiments were carried out using 3 ml volume. Valinomycin was purchased from Calbiochem.

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3. Results and discussion

The fluorescence probe AS was originally synthesized and described by Waggoner and Stryer [18], and has recently been used in a study of the lipid bilayer in influenza virus [19]. Based on a consideration of the structure of AS (see fig. 2A) as well as previous work done in other systems [18,19], we felt that AS had a very high probability of locating in a relatively hydrophobic, interior portion of the thylakoid membrane, and so might be useful in monitoring any structural, conformational or other events which are postulated to be involved in the mechanism of coupling in the energy conservation steps of biological systems [20].

In fig. 1, the effect of saturating actinic light on the steady state fluorescence of AS molecules bound to chloroplasts capable of supporting (10 μM PMS-induced) cyclic electron flow is shown. As DCMU does not alter this effect (table 1), it is a true response to 'cyclic' electron flow.

Light stimulates a biphasic (rapid and slower phases) increase in intensity of AS fluorescence; the average extent of the increase under these conditions was nearly 50% (see table 1). (Non cyclic electron flow employing methyl viologen (as electron acceptor) also produced the light response, but at a very much reduced rate and extent (not shown). The above-described effect was quite reversible, although the percentage response becomes somewhat smaller after several successive illuminations with the actinic light. The slow increase seen in the 'dark' (366 nm measuring beam only) may be due to the fact that the beam intensity is not negligible or may be attributed to a slow migration of the probe to its preferred binding site inside the membrane.

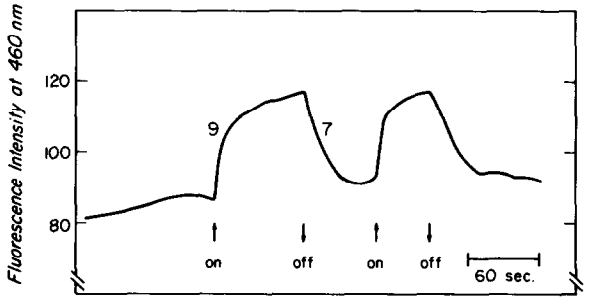

Fig. 1. Conditions as in table 1, except [Chl] = 20 μM.

In table 1, the effects of some ionophorous antibiotics and uncouplers on the dark and light AS fluorescence responses are presented, along with a comparison of the effects of these compounds on photophosphorylation and the extent of light-induced proton transport. The fact that all these treatments alter the light-stimulated enhancement of AS fluorescence in some way suggests that some aspects of bioenergetic significance are being monitored. The data of table 2 support this suggestion, where it is shown the the addition of ADP under conditions permitting substantial cyclic photophosphorylation (see table 1), markedly inhibits the extent of the AS change in light. As phosphorylation dissipates the 'high energy state', AS fluorescence decreases. However, if utilization of the 'high energy state' is prevented by blocking a late step in energy conservation by the energy transfer inhibitor phlorizin, the light response of AS recovers in a manner approximately proportional to the concentration of phlorizin.

The dark responses of AS fluorescence to the ionophores and uncouplers (table 1) are also interesting and may tell us more about the nature of the event(s) that AS fluorescence changes in chloroplasts are reflecting. Valinomycin and nigericin, but not gramicidin and NH_4Cl, produce an increase in 'dark' AS fluorescence. The especially marked valinomycin-induced increase is shown in fig. 2A. The data of Schuldiner et al. [9] are especially relevant here. They found that following 1 min of pre-illumination, valinomycin (in the presence of KCl) added to the *dark* stage resulted in large increases in the yield of ATP formation, due to the formation of a diffusion potential, and especially under conditions made suboptimal with regard to a limited proton concentration gradient. It is reasonable to suggest that since our conditions are similar, the valinomycin-induced diffusion potential is also responsible (in part, at least) for the AS fluorescence change in the dark. In our experiments, however, no ADP is present to collapse an energetic gradient, and the valinomycin is added more than a few seconds after the preillumination step, at which point much of the proton gradient will have collapsed.

It was observed [9] that with valinomycin, ATP synthesis in the dark stage, in a post-illumination experiment, was much *faster;* the time for completion of

Table 1
Effects of various antibiotics, 3-(3,4-dichlorophenyl)-1,1-dimelhyurea (DCMU) and NH_4Cl on fluorescence levels of probes, ATP formation and proton transport

Additions	Per cent changes in AS fluorescence at 460 nm		Per cent changes in atebrin fluorescence at 510 nm		ATP formation μmoles/mg · chlorophyll/hr	Relative extent proton transport nmoles H^+
	Dark	Light	Dark	Light		
None	Slow increase ~ 30 in 3 min	~ + 45	0	−70	500	+16.5 ± 2
10 μM valinomycin	+ 80 to 135 rapid phase	+ 3–10	+25	−70	380	+17.5 ± 2
1 μM nigericin	+ 25	0	+20	− < 3	0	−20 ± 2
5 μM gramicidin D	0	+ 0 to 5	+21	−10	0	−18 ± 2
5 mM NH_4Cl (± 1.5 mM ADP)	0	0	–	–	0	0
1 μM DCMU	0	+41	–	–	–	–

Probe fluorescence was measured utilizing a reaction mixture containing: 3.3 mM NaP_i, 3.3 mM $MgCl_2$, 16.7 mM KCl, 0.01 mM PMS, 10 μM Chl, pH 7.8. Probe concentrations were: AS, 14.5 μM; atebrin, 5 μM. 'Dark' changes in probe fluorescence represent those occurring in the presence of the 366 nm beam only; 'light' changes signify an increase or decrease in fluorescence intensity of the probe upon the onset of the saturating red actinic light. (PMS does fluoresce, but not significantly at 460 nm where AS emission is measured. Controls containing all the chemicals, in same volume, produced negligible changes.)

For ATP determination, the concentration of ADP added was 1.6 nm; see also Materials and methods. The *extent* of proton transport was taken as the difference between the steady state levels in light and dark; positive values indicate uptake, negative values represent an efflux. Unlike the control, since the rate of photophosphorylation in the presence of valinomycin was decreasing with time, the rates presented represent an initial rate for the first 10 sec of illumination. However, the rate of ATP formation became inhibited fairly soon; when measured over the first 60 sec, it was decreased to only 140 μmoles/ mg · chlorophyll/hr. A similar trend was seen with 5 μM valinomycin. This data then is consistent with the inhibition of photophosphorylation seen by Telfer and Barber [22] with concentrations of valinomycin above 0.1 μM. This decay in rate with time may represent the action of valinomycin in dissipating ΔpH (extent of proton transport).

Table 2
Effects of phosphorylating conditions on fluorescence levels of probes

Experiment	Additions	Per cent changes in AS fluorescence at 460 nm		Phlorizin-induced recovery of AS light response
		Dark	Light	Per cent changes
1	None	slow increase	+30	
	1.6 mM ADP	0	+12	18
	0.5 mM phlorizin	−30 (see legend)	+15	
2	None	slow increase	+45	
	1.6 mM ADP	0	+17	35
	1.0 mM phlorizin	−53 (see legend)	+27	

Reaction and measuring conditions as in table 1. Phlorizin was significant absorption at 366 nm, explaining the artifactual dark fluorescence lowering.

synthesis decreasing from 20–30 sec to about 5 sec. Atebrin, known to reflect the energized state of the membrane [10–13; 21], was then used. Valinomycin markedly decreased by a factor of 25 (fig. 2B) the half-time of the light-off recovery of the light induced, PMS catalyzed atebrin fluorescence quenching, in a time range not unlike that of the above ATP experiments. Thus, under these conditions atebrin seems to probe the collapse of the diffusion-potential related energetic state.

In this paper, the ability of the AS probe to monitor the 'energy state' of the thylakoid membrane is established for the first time. The fact that agents that will dissipate the proton gradient (NH_4Cl, nigericin) or create the diffusion potential (valinomycin, with K^+) or dissipate both the proton and membrane po-

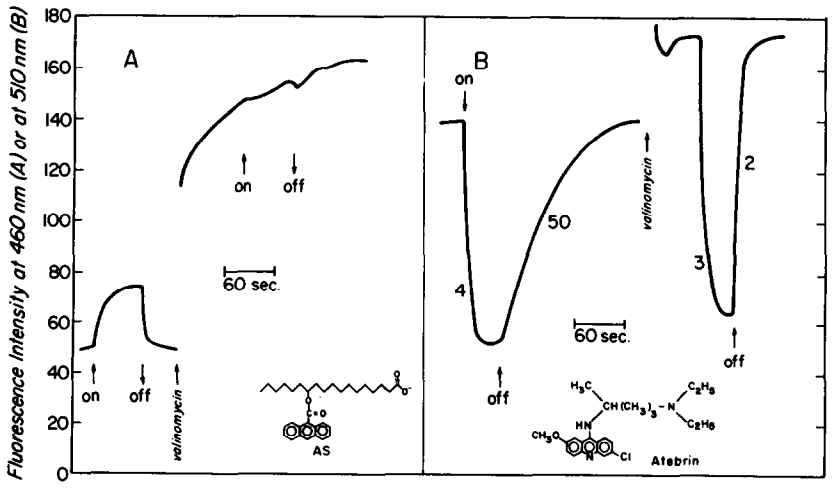

Fig. 2. Conditions as in table 1, A: 14.5 μM AS, B: 5 μM atebrin. [Valinomycin], 10 μM. Overall half-times indicated are in sec.

tential (gramicidin) successfully eliminate the light-induced AS fluorescence increase suggests that AS is simply reflecting some high energy state of the membrane, and that the destruction of one of the two components of the chemiosmotic hypothesis is sufficient to remove light-induced fluorescence changes. The response of this probe is similar but not identical to the well known probe atebrin. For example, 10 μM valinomycin suppresses 80–90% of the light-induced AS fluorescence increase, but does not affect the atebrin fluorescence decrease although it speeds up the recovery of fluorescence change by 25-fold. Why there is a dark increase in AS fluorescence with nigericin, which unlike valinomycin dissipates the proton gradient rather than the membrane potential, cannot be easily explained with our present data. Izawa and Good [23] point out that nigericin has been observed to release accumulated K^+ from membranes as well as acts in abolishing active K^+ uptake. In this sense, nigericin could, perhaps, also create some diffusion potential albeit less efficiently than valinomycin. This could then explain the 'dark' effect of nigericin. In the future, further work with the AS and atebrin could yield valuable information relating to the contribution of the membrane potential and associated 'membrane states' to energy conservation.

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