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ANTHROYL STEARATE: A FLUORESCENT PROBE FOR CHLOROPLASTS

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Summary

Experiments with chloroplast membranes suggest that the fluorescent probe anthroyl stearate (AS) is an indicator of an energetic membrane state involved in photophosphorylation. The light-induced increase in AS fluorescence as catalyzed by phenazine methosulfate is reversible, and sensitive to ADP + P_i and the energy transfer inhibitor phlorizin. An increase in AS fluorescence was also observed when either a gradient of H^+ (using injection of acid) or cations (using high concentrations of the ionophores valinomycin or nigericin) was established. The possible relationship of these data to the chemiosmotic hypothesis or a conformational state of the energy transduction process in photosynthesis is discussed.

1. Introduction

The chemiosmotic hypothesis[1] for the coupling of phosphorylation of ADP to electron flow has gained wide support in recent photosynthesis research, and so the role of the two major components (pH gradient, ΔpH, and membrane potential, $\Delta\psi$)of the total protonmotive force (pmf) used for ATP synthesis has been under intensive investigation in several systems[2-9]. Both the proton gradient[6,7] and the membrane potential[8,9] have been demonstrated to be of significance in chloroplasts.

In this paper, experiments are reported in which the fluorescent probe AS is used in chloroplasts for the first time; AS was originally synthesized and described by Waggoner and Stryer[10] and has been used in other systems.[11,12] Based on analysis of X-ray diffraction data[13] and spectroscopic studies,[14] the AS molecule is believed to bind to a relatively hydrophobic site in a membrane. It has, however, been pointed out that the anthroyl group is sufficiently large that

"precise localization within the hydrophobic region of the bilayer is not possible"[11]; we will adopt a similar position in this report. Insofar as AS fluorescence is responsive to its environment, we judged that it 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.

2. Materials and Methods

Class II chloroplasts were isolated from oats (Avena sativa, var. Cleland), peas (Pisum sativa), or lettuce (Lactuca sativa) and resuspended in the homogenizing medium: 50 mM phosphate buffer containing, pH 7.8, 10 mM NaCl and 400 mM sucrose. Chlorophyll (Chl) concentration was determined by the method of MacKinney[15], with a Cary 14 spectrophotometer.

The reaction mixture for the experiments described here contained 3.3 mM $MgCl_2$, 3.3 mM NaP_i, 16.7 mM KCl, 15 μM AS and chloroplasts equivalent to 10-15 μg chlorophyll/ml in a 3 ml volume at pH 8. Electron flow was catalyzed by PMS (5-10 μM) or ferricyanide (0.33 mM). Spectrofluorimetric measurements of probe fluorescence were performed as described previously[16]; excitation was at 365 nm, and emission was monitored at 460 nm. Saturating actinic light was passed through Corning C.S. 2-59 and C.S. 3-73 glass filters plus a heat filter (#7440) and 5 cm water filter; this light was blocked from the measuring monochromator with a Corning C.S. 4-96 filter.

Thorough mixing of added chemicals was insured by use of cell stirrer, explaining the loss of some information observed in some figures. High speed centrifugation was accomplished with a Beckman model L3-50 ultracentrifuge. Photophosphorylation was measured by the method of Nishimura et al.[17], as described by Dilley.[18]

3. Results

In fig. 1, the effect of saturating actinic light on the steady state fluorescence of AS in chloroplasts supporting cyclic electron flow is shown to be reversible. The extent of the light-induced increase varied between about 25-50%, depending at least in part on the state of the membrane preparation and its capacity to demonstrate electron transport and formation of the high energy state. The data also reveals a slow increase in the "dark" AS fluorescence. This could conceivably reflect some slow movement

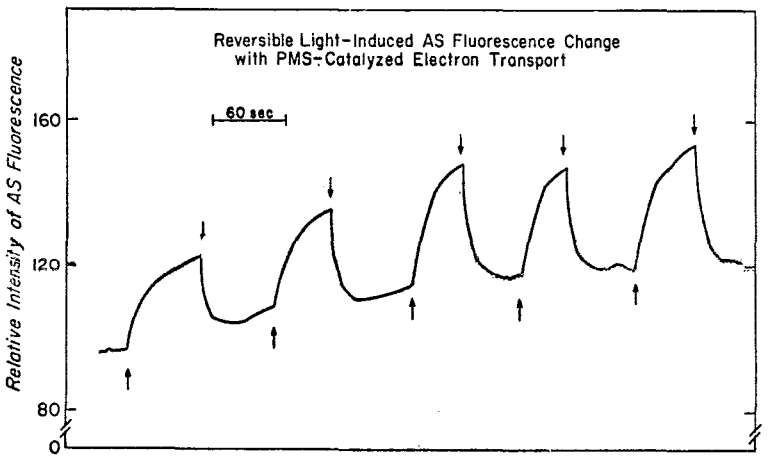

Fig. 1. Reversible light-induced AS fluorescence change with PMS-catalyzed electron transfer in lettuce chloroplasts. Upward arrows: actinic light on; downward arrows: light off.

of the probe deeper into a hydrophobic region of the thylakoid membrane. However, the fact that the light-induced response remains intact suggests that no extensive migration occurs to place the AS molecule in a significantly different "site".

In looking at the AS fluorescence response to light as a function of probe concentration, we found that the percentage increase did not vary in the range 5-30 μ M. This suggests that most of the AS in this concentration range is bound. Other independent binding data supports this; when 35 μ M AS was incubated with chloroplasts equivalent to 20 μ M Chl and the membrane material was cleared by high speed centrifugation, only a small percentage of the ultraviolet absorption of this probe was found as unbound AS in the supernatant.

The data of table 1 suggests that a state of bioenergetic significance is being monitored by the AS probe. The addition of ADP under conditions permitting substantial cyclic photophosphorylation ($\sim$ 500 μ moles ATP/mg Chl/hr), markedly inhibits the extent of the AS fluorescence change in light. As phosphorylation dissipates the "high energy state" (HES), AS fluorescence decreases. However, if utilization of the HES is prevented by blocking

Table 1

Effects of Phosphorylating Conditions on the Light-Induced Fluorescence of AS

Expt.	Additions	Percent increase in AS fluorescence induced by light	Phlorizin-induced recovery of the AS response (% changes)
1	none	30	
	1.6 mM ADP	12	18
	0.5 mM phlorizin	15	
2	none	45	
	1.6 mM ADP	17	35
	1.0 mM phlorizin	27	

a late step in the energy conservation by the inhibitor phlorizen, the light response of AS "recovers" in a manner approximately proportional to the phlorizin concentration. (Phlorizin has significant absorption at the excitation wavelength for AS, 366 nm, producing an artifactual dark fluorescence lowering which has been accounted for in data table 1.)

The well-known acid-base phosphorylation experiments of Jagendorf and Uribe[6] suggest a further means of securing information on the relationship of AS fluorescence to an energized state in chloroplasts; they found that imposition of ΔpH across the thylakoid membrane in the dark promoted phosphorylation. When transitions of 2 and 3 pH units are produced by the rapid addition of small volumes of concentrated acid and base, rapid changes are observed in the response of AS fluorescence (fig. 2a, 2b). However, a similar change is observed in the same system but without chloroplasts (fig. 2c). (The larger change observed in fig. 2c is probably due to the absence of reabsorption of the blue fluorescence with no chlorophyll present.)

If the pH of the buffer-only medium is gradually reduced, the large increase in fluorescence of fig. 2c is, however, not seen. Apparently, AS responds to a gradient of positive charge under these conditions. We suggest a likely explanation for these results to be that high local concentrations of positive charge become bound to the negatively charged moiety of the AS molecule, thereby encouraging micelle formation, local hydrophobicity, and an increase in

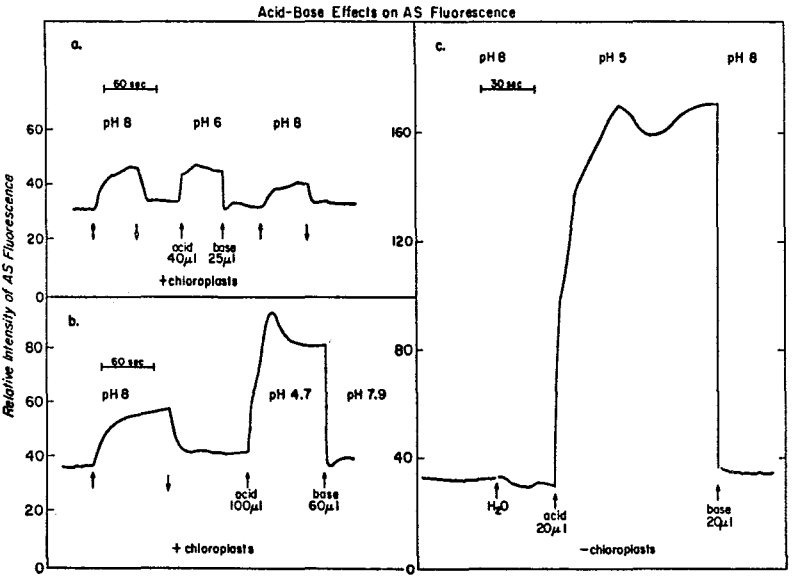

Fig. 2. A comparison of the PMS-catalyzed, light-induced change in AS fluorescence with ΔpH induced changes in oat chloroplasts. The pH of the medium was rapidly shifted by injection of acid (0.2 M succinic) or base (1 M Tris) in the specified volumes. The final pH after each injection is indicated. Assay mixture for (c) is without chloroplasts, using 1 ml sample. Unlabelled upward arrows: actinic light on; downward arrows: light off.

quantum yield. These results do not insure that this is the same mechanism responsible for changes in AS fluorescence observed in the membrane-mediated case. Thus, while this fact complicates the interpretation of the acid-base AS fluorescence changes in terms of an energized state, it may implicate the formation of ΔpH or other "charge gradient" in the observed response of AS fluorescence to actinic light. Perhaps AS responds to a sudden local change of H^+ concentration in the membrane.

Valinomycin, in carrying K^+, can act in establishing a diffusion potential if Cl^- is the anion, since Cl^- is less permeable in the chloroplast membrane. In this manner, Schuldiner et al.[9] utilized a relatively high concentration (10 μM) of valinomycin in the presence of KCl to stimulate ATP synthesis by

means of a membrane potential. They found that following one minute of pre-illumination, valinomycin added to the dark stage resulted in large increase in the yield of ATP formation, due to the formation of a diffusion potential. We have reported[16] that under similar conditions, and in the dark, valinomycin induced a large (up to 135%) increase in AS fluorescence. In fig. 3 it is demonstrated that a similar increase occurs in the light as well, and is dependent on the concentration of valinomycin. The raw data of fig. 3

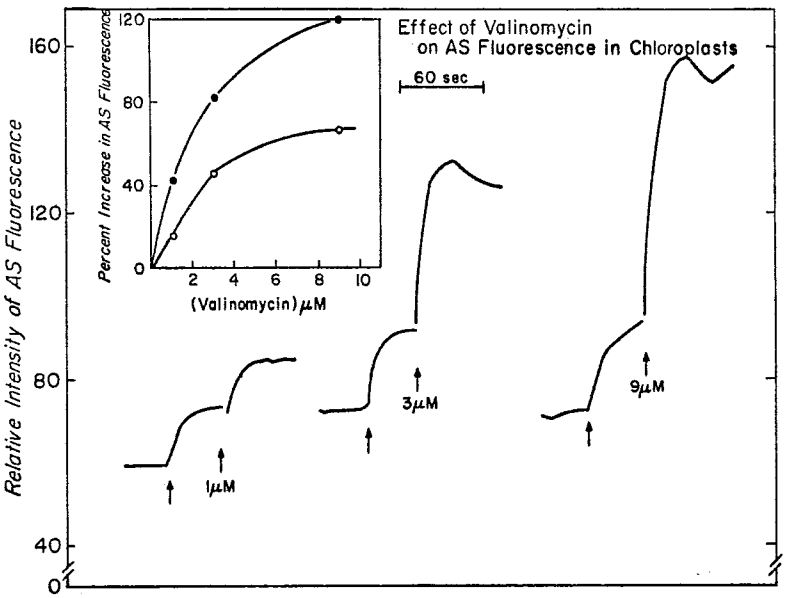

Fig. 3. The effect of valinomycin on light-induced AS fluorescence in oat chloroplasts. Unlabelled upward arrows: actinic light on; valinomycin added at the indicated concentrations. Explanation for the inset in the text.

is shown in the inset in terms of a percentage increase of AS fluorescence above the dark (closed circles) and light-increased (open circles) levels. At 10 μM valinomycin, the effect saturates and the maximum valinomycin-induced increase in the light (∿65%) is still well below the figure for the valinomycin-induced increase in the dark (see above). However, using the observation that the light- and valinomycin-stimulation of AS fluorescence are additive, the total maximum percentage increase (∿120%) compares favorably with the figures for dark only addition.

If valinomycin is transporting the positive charges of K^+ ions across the thylakoid membrane, then one can consider that the AS fluorescence response is due to: (i) the charge gradient itself, (ii) an effect caused by (i), e.g., a $\Delta\psi$ induced membrane conformational change that in turn enables or assists in ATP synthesis (not to be confused with the "conformational change hypothesis"[19] as such; also see Witt[20]); or (iii) some combination of (i) and (ii). As a first attempt in investigating the possibility of (i), especially in view of the acid-base experiments in buffer alone (fig. 2c), we imposed a localized "charge gradient" on free AS by rapidly injecting large amounts of salt. In fig. 4 it is shown that the injection of NaCl (0.45 mmole, producing a final concentration of 150 mM) does induce an increase in AS fluorescence, which then declines as equilibrium of charge occurs. As stated earlier in reference

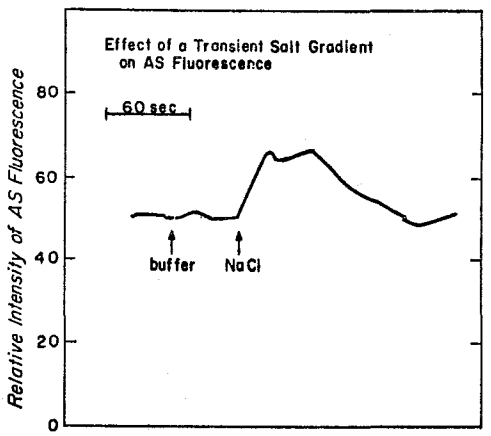

Fig. 4. The effect of a transient salt gradient on AS fluorescence. Assay mixture is the same as for fig. 3, without chloroplasts. Volumes of addition were 90 µl; concentration of stock NaCl was 5 M.

to the acid-induced effects, it cannot be asserted that this same mechanism is involved in the membrane situation, only that AS is capable of responding to such perturbations. In addition, the AS response is not likely to be a direct reflection of the primary light-produced $\Delta\psi$ itself, inasmuch as it is known to be formed with a half-time of 20 nsec (at least in the one turnover situation, see Witt[20]).

Nigericin is another cyclic antibiotic compound which acts as an ionophore

in a number of biological membranes, including chloroplasts. We have found that the addition of 0.5 μM nigericin eliminates the light-induced increase of AS fluorescence[16] (also fig. 5a); nigericin at this concentration is known to uncouple phosphorylation, presumably by accelerating the decay of ΔpH, as H^+ are allowed to efflux down the concentration gradient and K^+ are exchanged in an electrically neutral manner. Nigericin, especially at concentrations higher than normally used for uncoupling conditions (e.g., 2 μM), when added in the dark (not shown here) produces an AS fluorescence increase not unlike the response observed for valinomycin. If it is assumed that nigericin acts only in a 1:1 K^+:H^+ exchange, this result is perplexing. However, work of Ferguson et al.[21] and Shavit et al.[22] indicates that nigericin at high concentrations can catalyze a one-way movement of charge, i.e., charge transfer across the chloroplast membrane. Also, Henderson[23] notes that in bilayer experiments, high concentrations of nigericin decrease the membrane resistance in promoting electrogenic (electrical potential is generated) cation transport. On this basis, it appears that nigericin at higher concentrations is acting to create some diffusion potential, although less efficiently than valinomycin.

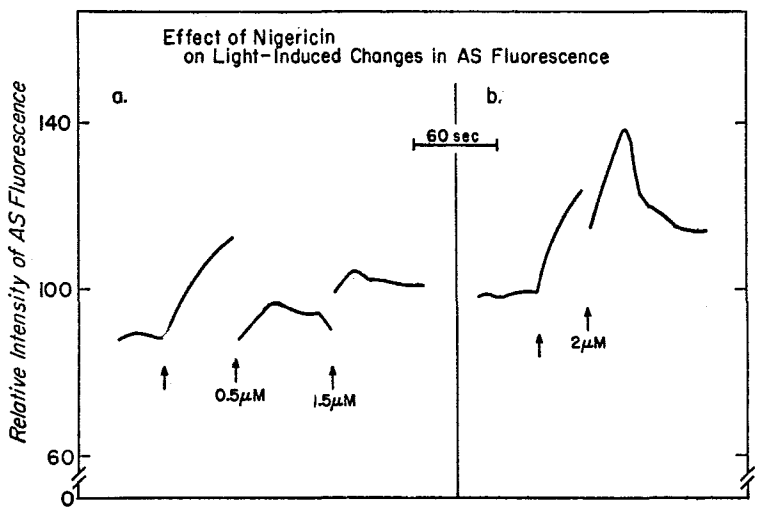

Fig. 5. The effect of nigericin on light-induced changes in AS fluorescence in pea chloroplasts. Unlabelled upward arrows: actinic light on; nigericin added as indicated.

The alternate modes of action of nigericin at different concentrations become manifest when the ionophore is added in the "light stage". In fig. 5a, 0.5 μM nigericin is shown to inhibit the light-induced increase in AS fluorescence and maintain the intensity close to the dark baseline. But the addition of more nigericin to achieve a final concentration of 2 μM results in an increase, partially "restoring" the light-augmented fluorescence intensity. Approximately the same final effect results when 2 μM nigericin is added at once (fig. 5b). The transient fluctuation observed as 0.5 μM nigericin is added may reflect the fact that this concentration is near the "threshold" between the high and low concentration responses.

4. Discussion

In analyzing the results of this report, we assume (allowing modification) the viewpoint of the chemiosmotic hypothesis[1], in which $\Delta\psi$ and ΔpH are components of the total protonmotive force (pmf) that gives rise to ATP synthesis. Extensive investigation in Witt's laboratory (reviewed in ref. 20) indicates that with "one-turnover" light flashes ATP can be synthesized with essentially only the $\Delta\psi$. In the general steady-state case, however, it appears that photophosphorylation is coupled to field-driven ion flux, specifically a proton efflux; the electrical potential gradient $\Delta\psi$ and chemical potential gradient ΔpH are suggested to cooperate in driving out protons and establishing any further steps or intermediates necessary for ATP synthesis. Evidence has been presented here to indicate that the fluorescence probe AS is an indicator of some aspect of the HES in photosynthetic energy conversion in chloroplasts.

The data of figs. 2-5 suggest that AS is responsive to a gradient of positive charge and/or any subsequently created membrane state as a result. Such a state could be thought of in terms of a change in membrane conformational state developing from charge redistributions within or across the thylakoid, placing the membrane components in a state affording most efficient utilization and channeling of the energy stored in the pmf. As Witt[20] has suggested, such a conformational change could be functional in the support of the steady-state synthesis of ATP, but is formed as a consequence of a "charging step" and is therefore distinct from the "conformational change hypothesis"[19] as such.

It was mentioned above in reference to the results of fig. 2 that AS may

monitor a localized alteration of H^+ concentration in the thylakoid membrane. In this regard we direct the reader's attention to the modified chemiosmotic theory of Williams.[24] In this proposal, he has designated a key role to the localized H^+ concentration formed within the hydrophobic membrane as hydrogen carriers become oxidized, as contrasted to the H^+ gradients subsequently established across the membrane. Recent work of Izawa and Ort[25] could be viewed by them to be most consistent with Williams' hypothesis.

In explaining the membrane potential stimulation of ATP synthesis, Schuldiner et al.[9] suggest that $\Delta\psi$ assists in driving out those protons which are coupled to phosphorylation, as in Witt's concept and the chemiosmotic hypothesis. The valinomycin data of fig. 3 are consistent with an AS-monitored step in HES being established through cooperative action of $\Delta\psi$ and ΔpH,as well as through a "charge gradient" alone. Further investigations with the fluorescence probe AS, especially in conjunction with other techniques, should prove useful in the study of photosynthetic membranes to uncover new information with regard to the mechanism of energy transduction and the role of $\Delta\psi$, ΔpH and membrane high energy states. (A paper describing comparison of AS with atebrin has been recently submitted for publication.[26])

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