PROTON GRADIENTS AS POSSIBLE INTERMEDIARY ENERGY TRANSDUCERS DURING ATP-DRIVEN REVERSE ELECTRON FLOW IN CHLOROPLASTS

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1. Introduction

After light activation of the latent adenosine triphosphatase (ATPase), addition of ATP in the dark drives reverse electron flow in isolated chloroplasts as evidenced by the oxidation of cytochrome *f* and the reduction of *Q* [1,2]. According to the chemiosmotic hypothesis [3] proton gradients should be obligatory energy transducing intermediates in this process. Indeed, transmembrane proton gradients were shown to be created during the action of the ATPase [4,5] and, when artificially induced, drive reverse electron flow by themselves [6,7].

As obligatory intermediates, the buildup of proton gradients would be expected to be kinetically at least as fast as the induction of reverse electron flow under all experimental conditions. Thus, treatments which slow down the development of the transmembrane proton gradient would be expected to induce a corresponding slow-down in the development of the ATP-driven reverse electron flow.

In this report we shall describe the construction of an apparatus which enabled us to simultaneously monitor the ATP-driven development of ΔpH and reduction of *Q* under a variety of conditions, and thus test the above prediction.

Abbreviations: Q, The primary electron acceptor of photosystem II; ΔpH, Proton concentration gradient across the chloroplast thylakoid membrane; SF-6847, 3,5,di-tertbutyl-4-hydroxybenzylidene-malononitrile

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2. Materials and methods

Spinach chloroplasts were prepared [8] and chlorophyll determined [9] essentially as previously described. The chloroplasts were finally suspended in a small volume of 0.4 M sucrose, 0.05 M NaCl at a concentration of about 2 mg chlorophyll/ml. The standard reaction mixture contained: Tricine, pH 8.0, 15 mM; NaCl, 20 mM; $MgCl_2$, 5 mM; phenazine methosulphate, 1 μM; dithiothreitol, 5 mM; 9-aminoacridine, 2 μM; and chloroplasts containing about 20 μg chlorophyll/ml. To activate the ATPase the mixture was illuminated for three minutes with intense heat-filtered white-light from a slide-projector (about 10^5 ergs × cm^{-2} × s^{-1}). Monitoring was initiated within 5–10 s after the light was turned off. Twenty seconds after the light was turned off 3 μl 10 mM ATP were introduced with a microsyringe to initiate the reaction. The reaction mixture (about 0.5 ml) was stirred throughout by a magnetic stirrer and maintained, unless stated otherwise, at a constant temperature of 20°C by a continuous flow of water from a thermostated bath. Rapid cooling was effected by the valve-controlled flow system described earlier [10].

The measuring apparatus is illustrated in fig.1. It employed three bundles of optical fibers, one of which introduced a very weak blue (390–440 nm; about 50 ergs × cm^{-2} × s^{-1}) measuring light to the sample; the second permitted a photomultiplier to monitor the blue–green fluorescence (through a filter combination transmitting 505–535 nm) of 9-aminoacridine, indicating ΔpH [11], and the third allowed a second photomultiplier to monitor the red

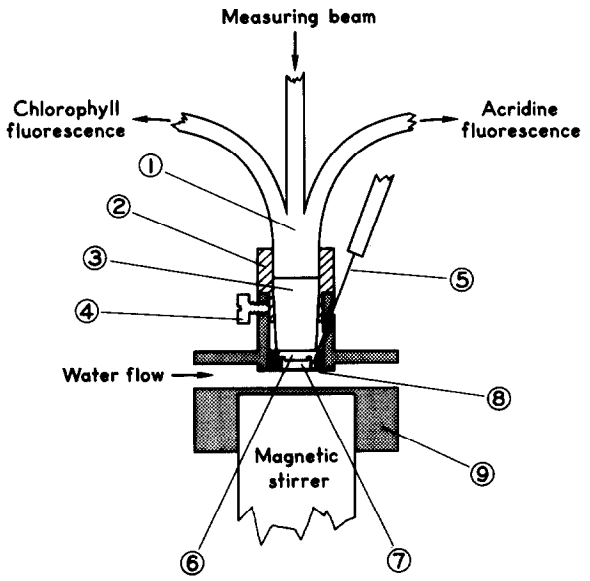

Fig.1. Measuring device for simultaneously observing ATP-driven changes in chlorophyll and 9-aminoacridine fluorescence. (1) Trifurcated fiber optics. (2) Cover of reaction chamber constructed of an external polyvinyl-chloride ring and an internal transparent lucite rod (3), held in place by a nylon screw (4). (5) Removable syringe for injection of additions. (6) Chamber for reaction mixture. (7) Rotating magnetic stirring disc (Radiometer #D 4030), (8) Aluminum container which serves also as a heat conductor from the thermostated flowing water. (9) Polyvinyl-chloride body.

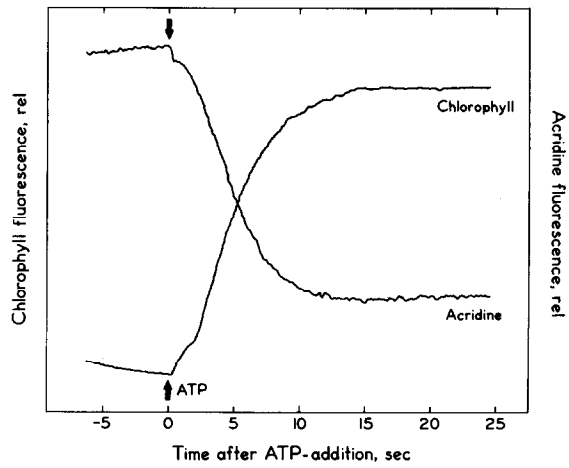

Fig.2. Simultaneous recording of ATP-driven buildup of ΔpH and reduction of Q. Reaction conditions as described under Materials and methods.

fluorescence (through a filter transmitting light longer than 655 nm) of chlorophyll *a* indicating the redox state of Q [12]. The measuring light was filtered through Corning filters 4-96 and 5-58; the 9-amino-acridine fluorescence measuring photomultiplier was screened by a Corning 4-96 filter and Wratten filters 58 and 64 and the chlorophyll fluorescence measuring photomultiplier by a Corning 2-64 filter. For ATP-ase-activation the fiber-bundle was temporarily removed and the cuvette was illuminated directly from above.

3. Results

3.1. *Simultaneous measurement of acridine and chlorophyll fluorescence*

Figure 2 illustrates the simultaneous recording of 9-aminoacridine and chlorophyll fluorescence under the standard conditions described. After the ATPase-activating light was turned off both the acridine and chlorophyll fluorescences were approaching a steady-state level dictated by the intensity of the weak measuring light; the acridine fluorescence increasing, indicating the decay of the ΔpH formed during the illumination with the activating light [11] and the chlorophyll fluorescence decreasing, indicating the reoxidation of the reduced Q formed by the same illumination [12]. At room temperature (20–25°C) both signals reached steady-state levels by about 15 s. These levels were within 10% of the 'dark' steady-state levels (F_o for chlorophyll and F_{max} for 9-aminoacridine) with the standard intensity of measuring light employed. On adding 50 μM ATP to the continuously stirred suspension, a marked increase in chlorophyll fluorescence occurred indicating the reduction of Q by reversed electron flow [1,2]. Simultaneously a marked decrease in 9-aminoacridine fluorescence was observed indicating the formation of ΔpH [4,5], The overall kinetics of both responses were comparable. The new steady-state achieved was maintained for long periods depending on the concentration of ATP added. The magnitude of the change in chlorophyll fluorescence was normally about 100% of that of the F_o-level or 20% of the variable fluorescence. The magnitude

of the quenching of 9-aminoacridine fluorescence was about 50% corresponding to a ΔpH of 3–4.

The response time of the setup was most probably limited by the mixing time and was estimated at about 0.1 s. ATP, at concentrations exceeding 50 μM, caused a perceptible quenching of the 9-aminoacridine fluorescence which was evident as a very rapid decrease in the fluorescence level, much faster than the slow ATP-dependent quenching (see fig.2). It had no perceptible quenching effect on the chlorophyll fluorescence. The kinetics of the chlorophyll fluorescence change often included an initial small rapid change followed by a lag period. A similar, but generally less pronounced lag was observed in the acridine fluorescence change. The lags were accentuated in the presence of inorganic phosphate (see below). These phenomena have not been further studied in the present investigation.

Several experiments were performed to optimize conditions for observing the phenomena. Among the parameters tested it was found that around 1 μM phenazine methosulphate was optimal for a maximal chlorophyll fluorescence response and that a minimum of about 20 μM ATP was necessary for a maximal response, with higher concentrations eliciting a similar response but with progressively increased initial quenching of acridine fluorescence, as described above.

3.2. *Uncoupler effects*

Uncouplers are commonly suggested to act by dissipating proton and other ionic gradients. It was, therefore, of interest to check whether the rate of the ATP-dependent reduction of Q would decrease in correspondence with the decrease in the buildup of ΔpH. Figure 3 shows the effects of adding increasing concentrations of the excellent uncoupler SF-6487 [13,14]. It is evident that both the reverse electron flow and the ΔpH were affected and to a similar degree.

Other uncouplers tested, like gramicidin and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), behaved similarly.

3.3. *Buffer effects*

Several compounds were previously shown to act as internal buffers in chloroplasts [15–17]. Such compounds should slow down the formation of ΔpH driven by the ATPase, and thus may be expected to also slow down the reduction of Q. Figure 4 shows the effect of adding increasing concentrations of three

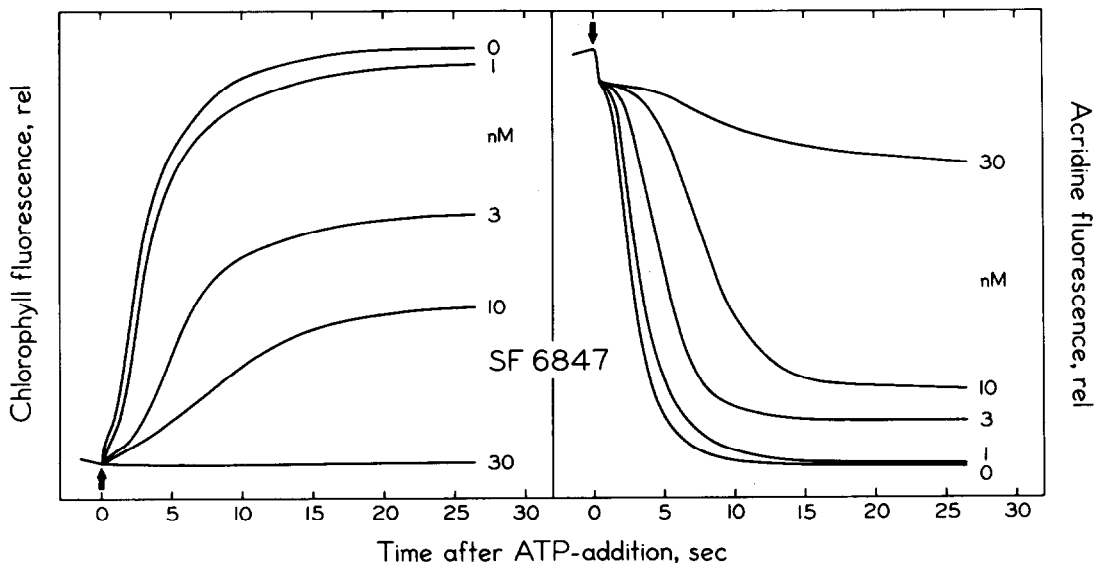

Fig.3. The effect of the uncoupler SF-6847 on the kinetics of ATP-driven buildup of ΔpH and reduction of Q. Reaction conditions as described under Materials and methods. SF-6847 was dissolved in methanol and added before activation to the indicated final concentrations in volumes not exceeding one percent.

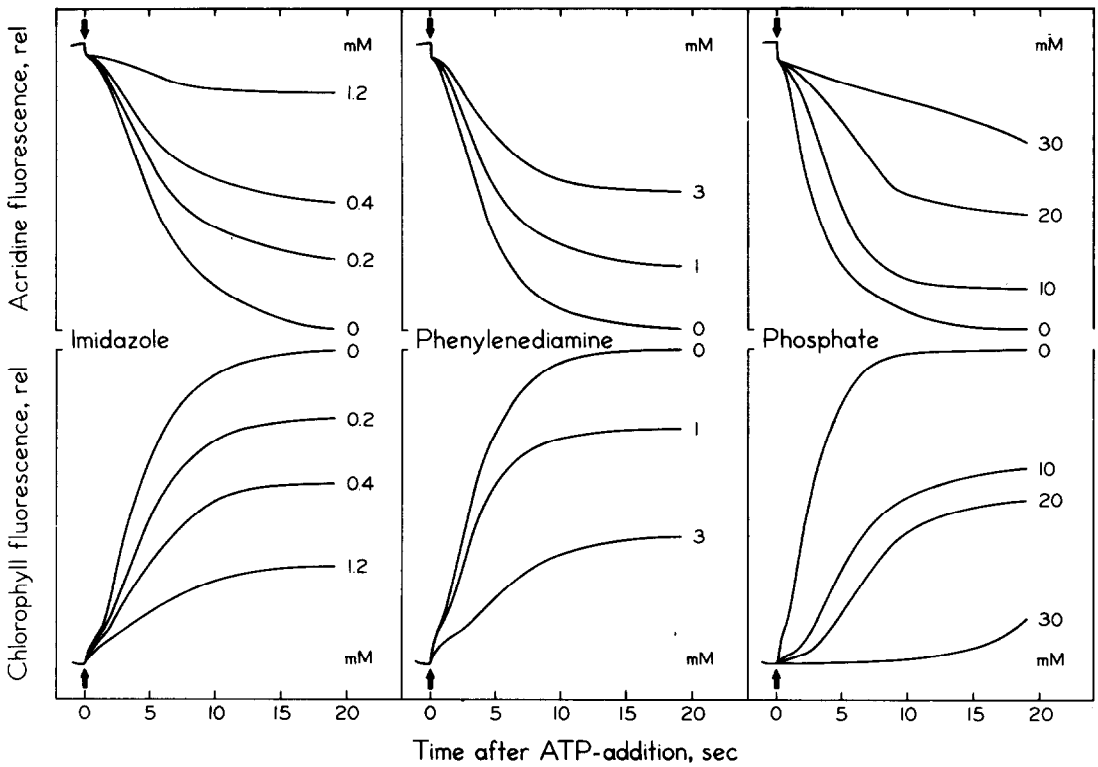

Fig.4. The effect of internal buffers on the kinetics of ATP-driven buildup of ΔpH and reduction of Q. Reaction conditions as described under Materials and methods. Buffers pre-adjusted to pH 8.0 were added to the indicated final concentration before activation.

such buffers on the ATP – induced ΔpH buildup and reverse electron flow. All three caused a decrease in rate and extent of the ATP induced ΔpH, as expected, and a similar change in the ATP – induced reverse electron flow.

It is of interest to note that imidazole and *p*-phenylenediamine behave as expected also in slowing down the dark decay of the ΔpH induced during activation (not shown in the figures), but orthophosphate did not. Orthophosphate was also unique in inducing rather marked lag phases particularly in the chlorophyll fluorescence increase (see fig.4).

Compounds like *N*-Tris(hydroxymethyl)-methylglycine (tricine), *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) or 3-(*N*-morpholino) propanesulfonic acid(MOPS), which do not enter into the inner thylakoid space [18] and thus do not serve as good internal buffers, did not elicit any significant response in concentrations up to 30 mM. Interestingly, bicarbonate at concentrations up to 30 mM also did not have any effect (see ref. [17]).

3.4. *Temperature effects*

Temperature was previously shown to have a relatively small effect on the light induced quenching of acridine fluorescence [19]. Figure 5 shows the effect of lowering the temperature from 20–3°C on the ATP-induced acridine and chlorophyll fluorescence changes. A decrease in rate and increase in extent was observed in both responses on lowering the temperature.

It is of interest to note that the activation of the ATPase seems to occur almost as well at 3°C as at 20°C (not shown).

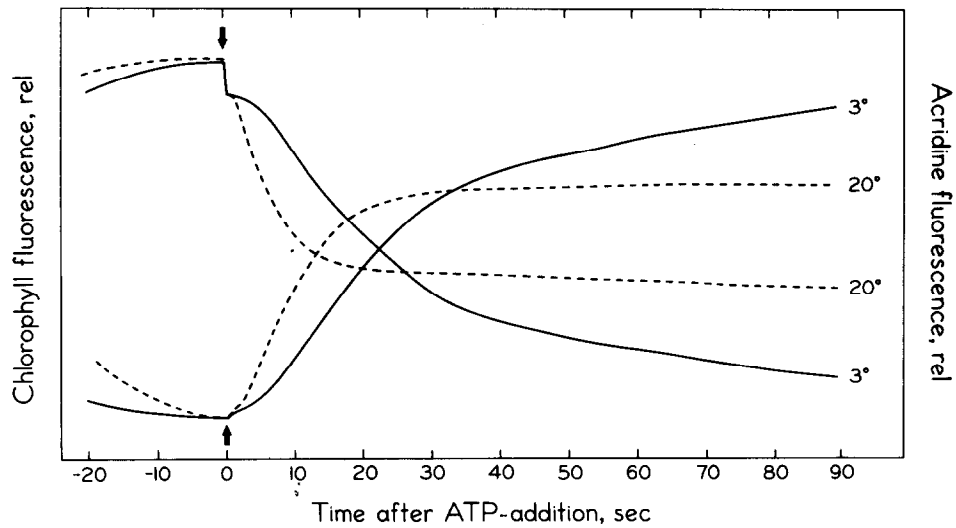

Fig.5. The effect of temperature on the kinetics of ATP-driven buildup of ΔpH and reduction of Q. Reaction conditions as described under Materials and methods for the 20°C sample. For the 3°C sample, activation was at 20°C for 165 s, at which point the sample was rapidly cooled to 3°C and illumination continued for a further 15 s. Decay in the dark before addition of ATP was for 60 s in the 3°C sample (instead of 20 s), because of the slow decay of the light-induced signals at this temperature (see [9]).

4. Discussion

The technique developed allows simultaneous monitoring of changes in pH-gradients and electron flow, and therefore makes it possible to correlate the two parameters under a variety of conditions. The technique may find application in a variety of systems other than the one investigated in this communication. The major limitation with regard to rapid kinetic measurements is the relatively slow distribution of 9-aminoacridine across the membrane in response to a transmembrane pH-gradient with a time constant of about 1 s. Accurate quantitative comparisons are difficult, since the chlorophyll fluorescence yield is not linearly related to the redox state of Q [20] and the 9-aminoacridine quenching is not linearly related to the ΔpH [10].

We have tried to correlate the ATP-driven buildup of ΔpH and reverse electron flow under a variety of conditions. The chemiosmotic hypothesis [3] predicts that ATP-driven reverse electron flow operates through the intermediary creation of a high-energy state in the form of ΔpH (in chloroplasts). Thus, it would be expected that under all conditions the buildup of ΔpH should be at least as fast as the rate of reverse electron flow. Any treatment which decreases the rate of buildup of ΔpH should have a similar effect on the rate of the observed reverse electron flow. This is not a requirement of other theories of energy coupling such as the chemical or conformational hypotheses.

The results presented are essentially in agreement with the above predictions of the chemiosmotic hypothesis. Thus, slowing the buildup of ΔpH by a variety of uncouplers, internal buffers, and temperature lowering brought about a corresponding change in the rate and extent of the observed reduction of Q by reverse electron flow. This in itself does not, of course, prove the validity of any one theory, since all the observed ATP-driven reactions are relatively slow (half-time in the order of a few seconds) and may permit complete equilibration between a hypothetical high-energy intermediate and the observed proton gradients (see [21]).

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