INITIAL FORMATION OF ATP IN PHOTOPHOSPHORYLATION DOES NOT REQUIRE A PROTON GRADIENT

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1. Introduction

The role of a transmembrane proton gradient and/or membrane potential[+] as the initial driving force for ATP synthesis in photophosphorylation is investigated in this report by application of sensitive measurements of ATP formation using millisecond light exposure. Evidence is provided that, within a few milliseconds after illumination, ATP synthesis by chloroplast thylakoids can occur in the absence of a significant proton gradient. This initial synthesis of ATP is later replaced by a more rapid synthesis occurring in the presence of an appreciable transmembrane proton gradient.

Previous experimentation has established that steady-state phosphorylation occurs with presence of little membrane potential but an appreciable transmembrane proton gradient [1–4]. Such data and demonstrations that illuminated chloroplasts take up protons that can be used to drive ATP synthesis in a following dark period (post-illumination phosphorylation) [5,6], are consistent with the capacity of the proton gradient to drive ATP formation. Membrane potential created by valinomycin-K^+ has also been shown to aid ATP formation [7,8].

Upon illumination of chloroplasts in presence or absence of ADP and P_i, a transmembrane potential develops very rapidly [1,4,9,10], and this potential subsequently disappears. Experiments have been reported [11] in which ATP was synthesized in response to external voltage pulses. This indicates that ATP synthesis can take place under conditions where only an electric field exists across the membrane. It thus seemed reasonable to us that measurements of the ATP formed in single millisecond flashes of increasing duration with use of valinomycin-K^+ to abolish membrane potential and of nigericin to abolish transmembrane proton gradient would allow evaluation of the possibility that ATP formation in the light can be driven by membrane potential or other energized membrane state in the absence of sufficient proton gradient.

2. Materials and methods

Chloroplast membranes from spinach leaves were prepared as in [12] and chlorophyll was determined as in [13].

For short term single flash photophosphorylation experiments, 1.0 ml reaction mix as described in the legends was exposed to light in a narrow test tube (1 cm diam.) by use of a Uniblitz model 225 electronic shutter controlled by a Uniblitz model 310B shutter timing unit (Vincent Associates, Rochester, NY 14607). A 300 W slide projector provided 1.6×10^6 ergs $\times$ $cm^{-2} \times s^{-1}$ when the shutter was open. Solutions containing 2.0 ml 1.0 M acid or 1.0 ml ADP and $^{32}P_i$ were added rapidly (< 0.1 s)

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[+] The designation 'transmembrane proton gradient' refers to the difference in proton activity on each side of the membrane and the designation 'membrane potential' refers to the transmembrane electric potential

from a 3.0 ml syringe fitted with no. 18 needles.

Three variations of the sequence of light exposure and ADP + P_i addition were used. In one chloroplast, membranes were illuminated for 15 s followed by ADP + P_i addition and continued illumination (prior-illumination photophosphorylation); in a second ADP and P_i were added in the dark immediately following cessation of illumination (post-illumination phosphorylation) and in a third ADP and P_i were present in the dark prior to illumination (photophosphorylation).

In prior-illumination photophosphorylation experiments, chloroplast membranes were exposed to light for 15 s, the shutter closed, 1.0 ml containing ADP and $^{32}P_i$ injected, and the shutter triggered to immediately open automatically for the desired amount of time. When the shutter closed 2.0 ml 1.0 M perchloric acid were immediately injected.

In measurements of post-illumination phosphorylation the shutter was not reopened after ADP and P_i addition and the sample was quenched with perchloric acid after 1 min incubation in the dark. Contrary to most previous post-illumination phosphorylation experiments [5] the pH of the light and dark phases were always the same to facilitate comparison with photophosphorylation.

For photophosphorylation experiments a 1.0 ml solution containing ADP and $^{32}P_i$ was added to 1.0 ml solution containing chloroplast membranes in the dark followed by shutter opening for the desired amount of time and quenched immediately.

Immediately after quenching, 1 μmol ATP and 10 μmol P_i were added to the acidified reaction mixtures and separations made as in [14]. Methyl viologen was used as the electron acceptor to avoid possible lags due to photoconversions that occur with phenazine methosulphate [15].

Valinomycin was from Sigma Chem. Co. and nigericin was kindly supplied from Dr R. J. Hosley of the Lilly Res. Labs.

3. Results

3.1. *pH dependency of illumination time required to initiate post-illumination phosphorylation*

Post-illumination ATP synthesis depends on the formation of a sufficient transmembrane proton gradient in the light [5]. Also, the net rate of proton influx shows little change with pH [16]. This leads to the prediction that if external pH is lowered to approach the pH where maximum internal buffering capacity exists (external pH 6.5), the time of exposure to light needed to allow ATP synthesis in the dark phase will be longer. Experiments to test this are shown in fig.1. No ATP synthesis is observed in the dark when incubation in the light was shorter than about 50 ms, at pH 8.3, 200 ms at pH 7.5, and 500 ms at pH 6.8. Significantly, the presence of

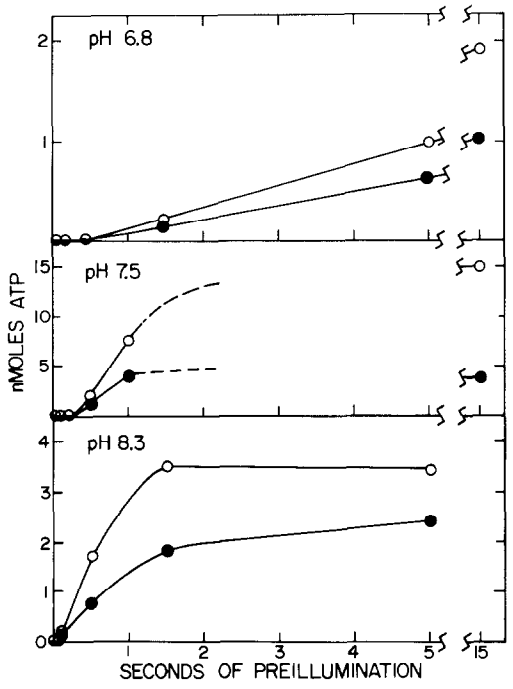

Fig.1. Post-illumination phosphorylation at different pH values. Post-illumination experiments were done as in section 2. A 1.0 ml reaction mix at the pH values indicated containing 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 1 mM methyl viologen, 33 mM Na-Hepes, chloroplast membranes (0.1 mg chl./ml), and when indicated 5 μl 0.4 mM ethanolic solution of valinomycin, was placed in a test tube in front of the electronic shutter. A 1.0 ml reaction mix containing the same constituents except for the addition of 2 mM ADP, 4 mM $^{32}P_i$ (2×10^6 cpm/μmol) and the omission of the chloroplasts, was placed in a syringe. A second syringe contained 2.0 ml 1.0 M $HClO_4$. The first reaction mix was illuminated for the indicated length of time, followed by the immediate addition of the second reaction mix. The $HClO_4$ was added 1 min after the shutter turned itself off. (○) Without 1 μM valinomycin; (●) with 1 μM valinomycin.

valinomycin and KCl did not affect the length of illumination time required for onset of ATP formation in the dark, although a lower extent of ATP synthesis was observed.

3.2. *Early time course of photophosphorylation with and without prior illumination*

Data of fig.1 show that a measurable time period is essential to establish a sufficient proton gradient for subsequent formation of ATP in the dark. If a similar proton gradient is required for initiation of ATP formation in the light, then a similar lag in onset of ATP formation would be expected when thylakoids with ADP and $^{32}P_i$ added in the dark are first illuminated. Experiments showed that this was not the case (see also [17,18]). Representative results are given in fig.2; the main portion of the figure shows the time course up to 1 s and the insert the continuation of the same samples up to 20 s. Without prior illumination, ATP formation commences as soon as the light is turned on, within experimental error, and definitely without a lag approaching the 50 ms as seen in fig.1 at the same pH 8.3. The initial ATP formation is thus driven by something other than a transmembrane proton gradient. At pH 8.3, this alternative energization is not able to achieve the maximal rate of ATP formation. Similar results are demonstrated at pH 6.8 where a lag of 500 ms is expected but an immediate onset of ATP synthesis is observed without prior illumination (fig.3,4).

Also shown in fig.2 is the time course for ATP formation with the chloroplasts illuminated before ADP and $^{32}P_i$ addition. Within error of the experimental procedure the rate of ATP formation is linear from the onset. The sample without prior illumination achieves the maximal phosphorylation rate within 1 s.

It was shown that at lower light intensities there is a lag in the onset of ATP synthesis following initial illumination, and that this lag disappears at high light intensities [17,19]. In [19] the experimental procedure was not sufficiently sensitive to detect an immediate onset of a lower rate of ATP synthesis at high light intensities and in [17] the repetitive technique employed results in a situation closely resembling a preilluminated sample. If the subsequent linear synthesis shown in fig.2 was extrapolated to the time axis, this would indicate a lag of about 100 ms. The important point is that ATP formation is occurring in this first 100 ms even thought at a lower rate.

3.3. *The effect of valinomycin–KCl on initiation of ATP synthesis*

An attractive possibility appeared to be that the

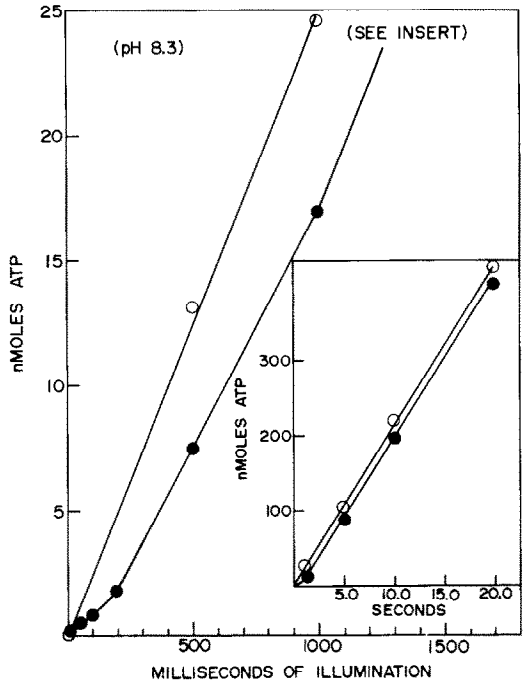

Fig.2. Time course of ATP synthesis by thylakoids with and without prior illumination. A 1.0 ml reaction mix containing 5 mM $MgCl_2$, 20 mM NaCl, 0.1 mM EDTA, 1 mM methyl viologen, 33 mM Na-Hepes and chloroplast membranes (0.073 mg chl./ml) at pH 8.3, was placed in a test tube in front of the electronic shutter. A 1.0 ml reaction mix containing the same constituents but with 2 mM ADP, 4 mM $^{32}P_i$ (2×10^6 cpm/μmol) and no chloroplast membranes was injected rapidly into the test tube then exposed to light for the indicated amount of time. For experiments with illumination prior to addition of ADP and P_i, 15 s illumination of the first reaction mix took place and the reaction mix containing ADP and $^{32}P_i$ was added in short dark intervals before exposure to light for the indicated amount of time. Quenching was done by injecting 2.0 ml 1 M $HClO_4$ as in section 2. Minor corrections were made from separate measurements for the amount of [^{32}P]ATP synthesized in the dark in the short period elapsing between the time the light was turned off and $HClO_4$ added. (○) Chloroplasts exposed to light prior to the addition of ADP and $^{32}P_i$; (●) chloroplasts kept in the dark prior to the addition of ADP and $^{32}P_i$.

initial formation of ATP upon illumination is driven by a membrane potential. Ample evidence exists for the transient formation of a membrane potential following illumination [1,9], although during steady-state photophosphorylation little or no membrane potential is present [1]. If the initial ATP formation is driven by membrane potential, the addition of a valinomycin plus K^+, which annihilates the potential, should block ATP synthesis until a sufficient proton gradient is established.

Tests of the effects of valinomycin were more readily made at pH 6.8 where there is a longer and more readily measured illumination time required to initiate post-illumination phosphorylation (fig.1), while photophosphorylation still proceeds at reasonable rates. Results of parallel experiments on photophosphorylation and post-illumination phosphorylation are shown in fig.3. As noted in the left panel of the figure, without prior illumination ATP formation started without delay in absence of valinomycin but ~500 ms are required for the onset of ATP synthesis in the presence of valinomycin-K^+. The right panel of the figure shows that the same time period of light phase is required for onset of ATP synthesis in a post-illumination experiment under the same conditions, without or with valinomycin.

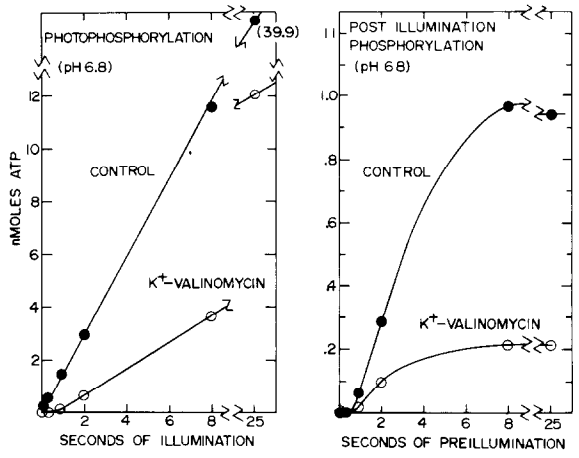

Fig.3. The effect of KCl and valinomycin on photophosphorylation and on post-illumination phosphorylation. Photophosphorylation and post-illumination phosphorylation were carried out as in section 2. A reaction mix contained 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 1 mM methyl viologen, chloroplast membranes (0.073 mg chl /ml) and 33 mM Na-Hepes, at pH 6.8. Where indicated, 4 μl 0.5 mM ethanolic solution of valinomycin was added to the solution containing the chloroplasts. A reaction mix containing the same constituents but with 2 mM ADP and 4 mM $^{32}P_i$ (6.1×10^6 cpm/μmol) and with no chloroplasts, or valinomycin was rapidly injected prior to turning on the light or immediately following light exposure for the indicated amount of time, as in section 2.

3.4. *The effect of nigericin on initiation of ATP synthesis*

Nigericin is known to induce a rapid $K^+ \rightleftharpoons H^+$ exchange in the thylakoid membrane and by so doing inhibits establishment of a proton gradient. It is a complete inhibitor of steady-state photophosphorylation [20]. Nigericin, however would not be expected to rapidly dissipate a membrane potential. If the initial ATP synthesis upon illumination (fig.2) is driven by a rapidly induced membrane potential, nigericin should allow an initial ATP synthesis when the light is turned on under conditions where valinomycin and KCl inhibit ATP formation. Results in fig.4 show that this is indeed the case.

The left panel of fig.4 shows the linear photophosphorylation at pH 6.8 in absence of nigericin and the

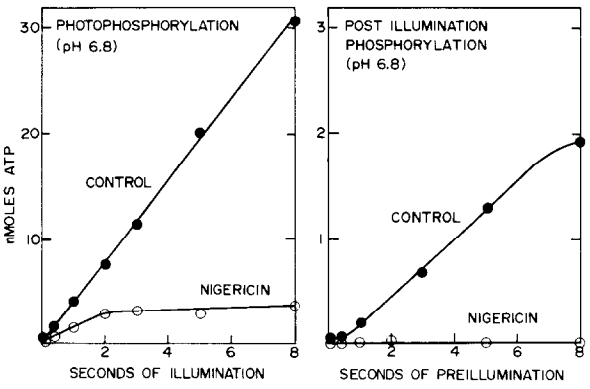

Fig 4. The effect of nigericin on photophosphorylation and post-illumination phosphorylation. A 1.0 ml solution containing 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 1 mM methyl viologen, 33 mM Na-Hepes, at pH 6.8, and chloroplast membranes (0.178 mg chl./ml) were mixed with 1.0 ml of the same solution containing also 2 mM ADP and 4 mM $^{32}P_i$ (5.9×10^6 cpm/μmol) but no chloroplasts. Where indicated 20 μl 10^{-5} M nigericin in an ethanolic solution was added to the solution which contained the chloroplast membranes. Exposure to light, quenching and analyses were performed as in section 2.

important demonstration that in presence of nigericin there is an initial small synthesis of ATP followed by complete inhibition. The right panel shows that, as anticipated, nigericin inhibits completely post-illumination phosphorylation and that, as already shown (fig.1,3), in the absence of nigericin about 500 ms illumination are required for the onset of subsequent ATP formation in the dark.

4. Discussion

The following results give evidence that the initial formation of ATP following illumination is not driven by a transmembrane proton gradient:

1. At pH 8.3 with saturating light, ATP formation commences within a few milliseconds whereas post-illumination phosphorylation experiments show that about 50 ms illumination are required to establish sufficient pH gradient to drive ATP synthesis. Still longer lags are observed in post-illumination phosphorylation at lower pH values, with no lag in photophosphorylation.
2. Valinomycin plus K^+, which abolish membrane potential, stop the initial but not the subsequent ATP synthesis.
3. Nigericin, which blocks formation of transmembrane pH gradient, does not stop an initial small ATP formation but inhibits subsequent ATP synthesis.

From these findings we conclude that upon illumination of chloroplast thylakoids an initial ATP synthesis can occur that is not driven by a transmembrane proton gradient.

A likely energy source for this initial ATP formation is a transient membrane potential. The very rapid initial establishment of such a potential upon illumination has been demonstrated [9]. The same basic mechanism, namely ATP synthesis driven by protonmotive force [21], could prevail in the initial and subsequent steady-state phosphorylation. Our experiments do not conclusively establish the nature of the initial energization of ATP synthesis, but the abolishment by valinomycin-K^+ means that the energized state is dissipated by transmembrane K^+ movement and thus favor a membrane potential as the initial driving force. This possibility has been suggested [17] from data with permeant ions.

It should also be considered that some mode of energization other than a membrane potential might be responsible for the initial phosphorylation. It was suggested [22] that an intramembrane proton movement might be involved. In presence of valinomycin and K^+ the onset of ATP formation was observed [22] prior to the time calculated necessary for a sufficient proton gradient to be established. In our experiments, however, both a transmembrane potential and a proton gradient coexist. This is based on the observation that ATP synthesis in presence of nigericin takes place for 2 s (fig.4) whereas the onset of ATP synthesis in presence of valinomycin and K^+ starts with a delay of about 500 ms only (fig.3). In view of these results an extra driving force for ATP synthesis does not seem to be necessary.

Brief comments may be helpful about some secondary effects. The maximal rate of ATP synthesis in presence of valinomycin is inhibited (fig.3). This is in harmony with data showing some inhibition of photophosphorylation by valinomycin [23,24]. The extent of post-illumination ATP synthesis is lowered by valinomycin (fig.3). This is anticipated in accord with observations showing that valinomycin-K^+ accelerate dissipation of a transmembrane pH gradient [25]. The initial rate of ATP synthesis is less in the presence of nigericin possibly because, in addition to catalysing a $H^+ \rightleftharpoons K^+$ exchange, a faster dissipation of the membrane potential occurs in its presence. The biphasic kinetics seen at pH 8.3 in photophosphorylation is not apparent when the same experiment is run at pH 6.8 (fig.3,4). This may be due to the much higher turnover of the ATP synthase at pH 8.3 versus pH 6.8 [26]. Hence, at pH 8.3 the available energy supply may be rate limiting initially, allowing a slower synthesis rate to take place until energy input has reached its high steady-state level. At pH 6.8 the turnover rate of ATP synthase may be rate limiting and ATP synthesis starts at its maximal rate allowed by the energy supply.

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