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THE ONSET OF PHOTOPHOSPHORYLATION CORRELATES WITH THE RISE IN TRANSMEMBRANE ELECTROCHEMICAL PROTON GRADIENTS

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Summary

The onset of photophosphorylation was determined by exposing chloroplast thylakoids to either single or multiple light flashes of varying duration. In agreement with the results of Ort et al. (Ort, D.R., Dilley, R.A. and Good, N.E. (1976) Biochim. Biophys. Acta 449, 108—124), the permeant buffer imidazole in the presence of valinomycin and K^+ did not greatly delay the onset of phosphorylation driven by multiple flash activation. In single flashes, however, the lag in the development of phosphorylation was much longer and imidazole caused a further delay. A significant ΔpH was generated by the multiple flash regime. The onset of photophosphorylation is, therefore, consistent with the rise in transmembrane ΔpH.

Introduction

A large number of experiments support Mitchell's [1, 2] concept that transmembrane gradients in proton electrochemical activity couple ATP synthesis to electron transport in chloroplast thylakoids (see Ref. 3 for a review). Recently, however, a role for intramembrane pH gradients in photophosphorylation was suggested [4, 5]. In the initial stages of illumination of thylakoids the transmembrane electrical potential difference ($\Delta\psi$) should be large and the transmembrane proton activity gradient (ΔpH), small [6]. As co- and or counter ion fluxes occur, $\Delta\psi$ is collapsed and ΔpH increases. In the steady state of illumination, ΔpH predominates [7]. In agreement with the notion that both $\Delta\psi$ and ΔpH can drive ATP synthesis, Ort and Dilley

[4] and Vinkler et al. [8] found that permeant ions delayed the onset of photophosphorylation. However, no further delay was noted by Ort et al. [5] when permeant buffers (e.g. imidazole) were also present even though the internal buffering by these reagents should increase the time required to generate a significant ΔpH. This observation led to the suggestion that protons in a space within the membrane could leave the thylakoids through the proton-linked ATPase complex before they equilibrated with the internal space. That is, intramembrane pH gradients were proposed to drive phosphorylation. Multiple flashes separated by a dark period were used. In this communication, we show that imidazole delays the onset of ATP synthesis induced by single flashes and that multiple flashes result in the build up of ΔpH.

Chloroplast thylakoids were isolated from market spinach [9]. Photophosphorylation was assayed at 15—16° C in reaction mixtures (250 μl) that contained: 20 mM Tricine-NaOH (pH 8.0), 0.5 mM methyl viologen, 50 mM KCl or 100 mM mannitol, 5 mM $MgCl_2$, 2 mM potassium phosphate (pH 8.0) containing 370 kBq of $^{32}P_i$ per μmol, 0.04 mM EDTA, thylakoids equivalent to 25 μg of chlorophyll and other additions as indicated. Illumination ($1.75 \cdot 10^6$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} of white light) times were varied by Uniblitz model 225L2AOX5 electronic shutter and model 310 shutter controller. After illumination, 250 μl of cold 4% trichloroacetic acid were added and 400 μl aliquots of the deproteinized reaction mixtures assayed for esterified P_i [10]. Non-illuminated samples served as controls. A slow increase (14 pmol $\cdot$ mg^{-1} chlorophyll $\cdot$ s^{-1} in the amount of nonextractable ^{32}P was noted with time. The amount of nonextractable ^{32}P in zero time control samples ranged from 3.4 to 4.5 nmol/mg chlorophyll. The control values were subtracted from the experimental ones. For multiple flash experiments, the maximum correction for the controls was less than 10% even at the shortest time of illumination. ΔpH was calculated from hexylamine uptake determined by silicone fluid centrifugation [7, 11] using a light intensity of $5 \cdot 10^5$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}.

In the absence of valinomycin and K^+, 2 mM imidazole had little if any effect on the onset of photophosphorylation induced by multiple (Fig. 1a) or single (Fig. 1b) flashes. The amount of ATP formed per flash was, however, somewhat inhibited by imidazole (Fig. 1a and 1b). These results are not unexpected since $\Delta\psi$ is probably the major driving force for ATP synthesis in short periods of illumination [4, 8, 12]. With single flashes a significant delay in the onset of phosphorylation, similar to that observed by Vinkler et al. [8], was detected. This lag may reflect dissipation of $\Delta\psi$ by relatively rapid counter ion movements and/or the need to convert the coupling factor part of the ATPase complex to an active form [13].

The negative time intercepts seen in multiple flash experiments (Figs. 1a and 3a) are artifacts. Several hours were usually required to complete the illuminations when multiple flashes were used and some loss of phosphorylation activity occurred during this period. Intercepts of 0—10 ms were obtained when the illuminations were performed over a shorter period or when the illuminations were carried out in order of decreasing flash duration (not shown).

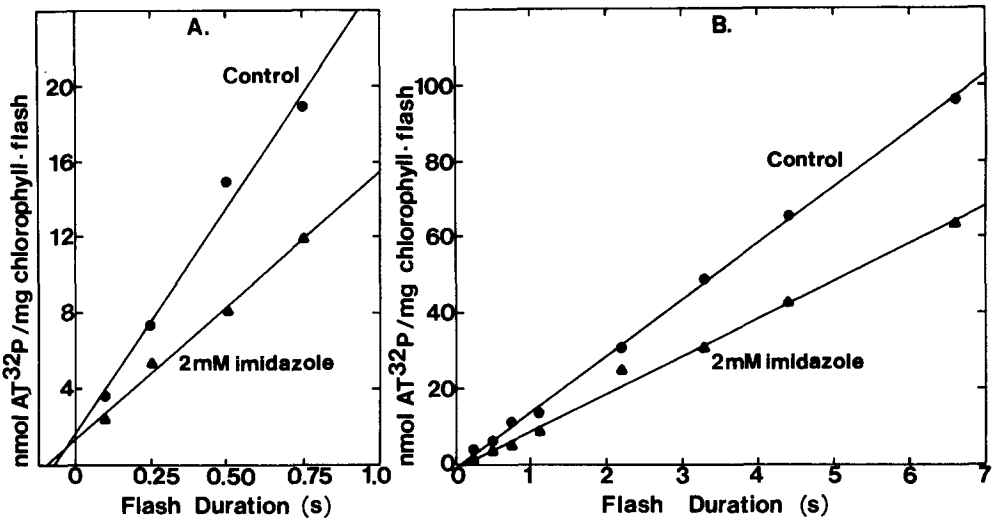

Fig. 1. Effect of 2 mM imidazole on the time course of ATP synthesis. Mannitol (100 mM) was used in place of KCl. In A, 24 flashes, separated by 15 s dark periods, were given. The samples were illuminated in order of increasing flash duration. In B, single flashes were given. Intercepts of about 0.1 s were calculated. Least squares regression analysis was used to calculate the intercepts for all experiments. The linear correlation coefficients ranged from 0.99 to 1.00. The experiments were performed using the same thylakoid preparation with the experiment shown in Fig. 1A being run first.

In the presence of 0.5 μM valinomycin and 50 mM KCl to facilitate the breakdown of $\Delta\psi$, the onset of steady state phosphorylation was delayed for 30 ms in multiple flash experiments (Fig. 2a and Ref. 4) and for 250—500 ms in single flash experiments (Figs. 2b, 3b, 3c). This lag presumably is the time required to generate a ΔpH of sufficient magnitude to drive phosphorylation. Variations in internal buffering power and in proton permeability of thylakoid preparations may explain the differences in the lag times.

In the presence of valinomycin and KCl, imidazole did not delay the onset of phosphorylation induced by multiple flashes (Fig. 3a and Ref. 5).

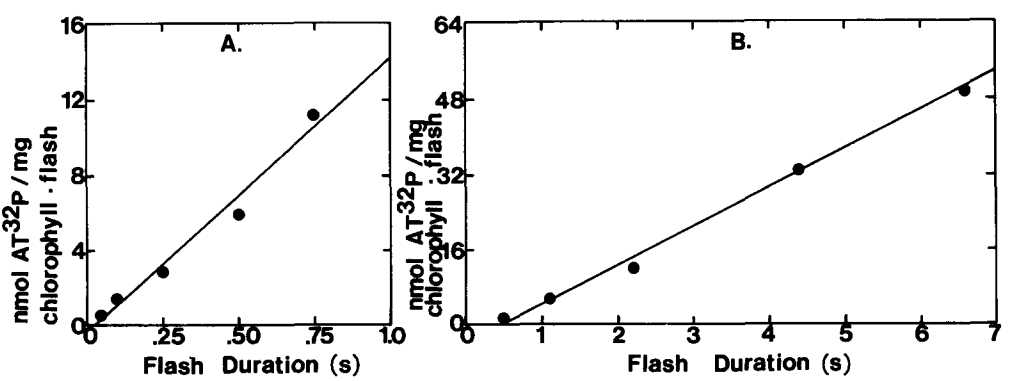

Fig. 2. Single and multiple flash time courses for ATP synthesis in the presence of 0.5 μM valinomycin and 50 mM KCl. In A, 24 flashes separated by 15 s dark periods were given. The intercept is about 30 ms. The samples were illuminated in order of decreasing flash duration. In B, single flashes were given. The intercept is 500 ms. These experiments were performed with the same thylakoid preparation with the experiment shown in panel A being run first.

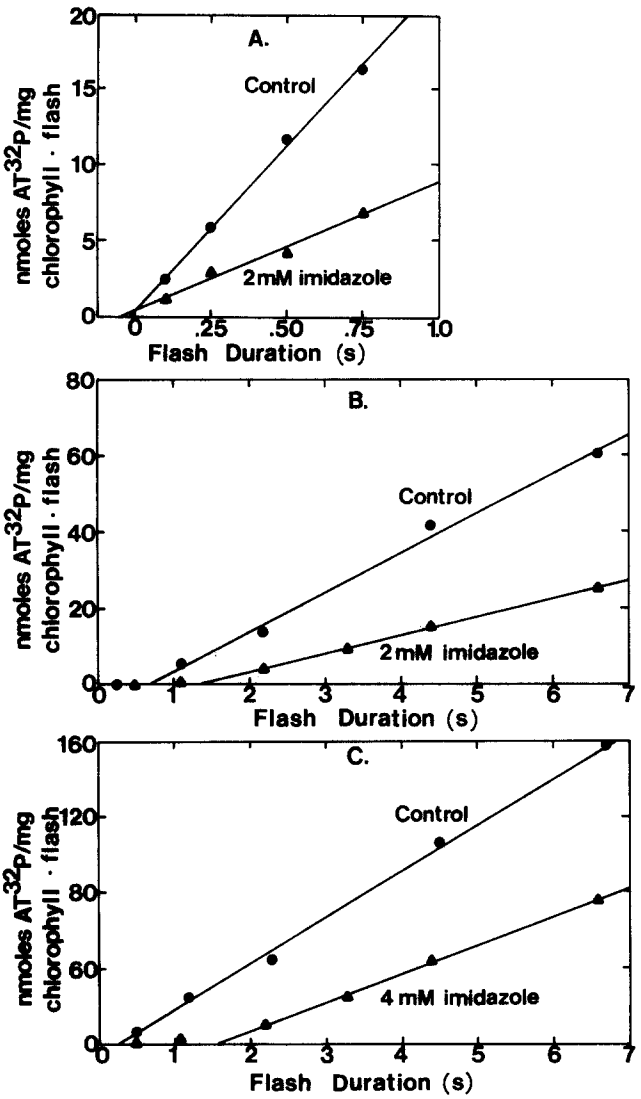

Fig. 3. Effect of imidazole on the time course of ATP synthesis in the presence of 0.5 μM valinomycin and 50 mM KCl. In A, multiple flashes were given as described in the legend to Fig. 1. Intercepts are −23 and −67 ms in the presence and absence of imidazole, respectively. In B and C, single flashes were used. The intercepts are: B, 630 ms in the absence of imidazole and 1310 ms in the presence of 2 mM imidazole; C, 230 ms in the absence of imidazole and 1580 in the presence of 4 mM imidazole. Experiments A and B were performed with the same thylakoid preparation. The experiment shown in C was run with a different preparation.

With single flashes, however, delays of 0.7 s and 1.3 s were observed in separate experiments at 2 and 4 mM imidazole, respectively (Figs. 3b and 3c). These delays are within the range of those predicted by Ort et al. [5]. As pointed out previously [5], however, an exact prediction of the lag is difficult to achieve since the thylakoids probably accumulate imidazole during the illumination, thus increasing the internal buffering capacity and the internal volume.

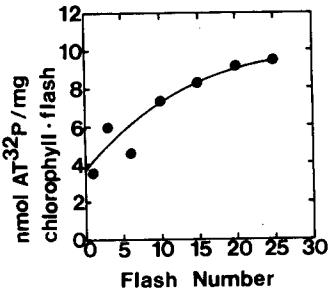

Fig. 4. Effect of flash number on ATP yield per flash. Thylakoids were given 250 ms flashes interspersed with 15 s dark periods. KCl (50 mM) and 0.5 μM valinomycin were present. The precision of the data from samples given a low number of flashes is relatively poor since the light-dependent incorporation is less than 50% of the total non-extractable $^{32}P_i$.

Despite the 15 s delay between flashes, a significant ΔpH may still be generated during the multiple flash regime. This notion is consistent with the observation that the ATP yield per flash is higher with multiple flashes than with single flashes. The ATP yield per flash in the presence of valinomycin and K^+ increases with the number of flashes, as shown in Fig. 4. The ATP yield per flash also increased with increasing flash number when imidazole was present and also in the absence of valinomycin. Moreover, after a train of sixteen, 200 ms flashes ΔpH values of 1.86 and 1.31 were measured in the absence and presence of 4 mM imidazole, respectively. The ΔpH values determined in this manner are undoubtedly underestimated since the amine responds rather slowly to pH differentials. During illumination periods, ΔpH is likely to be considerably higher.

These data show that the kinetics for the onset of phosphorylation are consistent with those of the development of transmembrane gradients in electrochemical proton activity. Recently, Junge et al. [14] presented evidence that neutral red reports pH changes in the thylakoid inner aqueous compartment, even though the dye is bound to the thylakoid membrane. The rate of proton release into the inside is very fast. Thus, there is no need to invoke intramembrane pH gradients which only slowly equilibrate with transmembrane gradients.

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