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ATP Formation Onset Lag and Post-Illumination Phosphorylation Initiated with Single-Turnover Flashes. I. An Assay Using Luciferin-Luciferase Luminescence

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Abstract

The great sensitivity of the luciferin-luciferase ATP detection system allows direct observation of ATP formation derived from single-turnover flashes in a thylakoid reaction mixture. The method can measure the energization threshold—the number of flashes required for the initiation of ATP formation—as well as detect post-illumination ATP formation after the last flash of a flash sequence. We describe the characteristics of this post-illumination phosphorylation which can be observed after a series of phosphorylating flashes (PIP^+) or when the assay for ATP formation was performed in a "traditional" manner where the ADP and P_i were added after the flash-energization period (PIP^-).

Comparing PIP^+ yields and kinetics of the PIP^+ decay under various treatments can give information about membrane energization events only if it is clearly established that different PIP^+ yields and decay rates are not due to limitations of the luciferase-catalyzed reaction. Experiments showing that the PIP^+ ATP yield and kinetics were due to membrane-limited deenergization events (proton efflux) rather than luciferase limitations include: (1) An uncoupler, nigericin, added after the last flash reduced the PIP^+ yield, but had no effect on the luciferase reaction. (2) The kinetics of the luminescence after adding standard ATP were much faster than the PIP^+ kinetics. (3) Valinomycin and K^+ stimulated the PIP^+ yield but had no influence on the luciferase reaction. (4) Lowering the pH from 8 to 7 increased both the PIP^- (an assay independent of luciferase kinetics) and the PIP^+ ATP yields, an expected result owing to the greater endogenous buffering power encountered by the proton gradient when the external pH is 7.

In spite of the last-mentioned point, the threshold flash number for ATP formation onset was the same for pH 7 and 8 (valinomycin, K^+ present) at slow flash frequencies. This is consistent with a membrane-localized rather

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than a delocalized gradient. The accompanying reports (W. A. Beard, G. Chiang and R. A. Dilley, and W. A. Beard and R. A. Dilley, *J. Bioenerg. Biomembr.*) show that different conditions can lead to observing either localized or delocalized proton gradient coupling in the PIP^+ event and the ATP onset threshold flash number.

Key Words: Energization threshold; luciferin-luciferase; post-illumination phosphorylation; single-turnover flash; thylakoid membrane.

Introduction

Chloroplast and mitochondrial ATP formation is believed to be driven by the protonmotive force, ΔP,[3] developed by the proton-translocating electron transport systems embedded in those organelle membranes. Whether the proton gradients are strictly delocalized, localized within the membranes, or developed as some mixture of the two is an unresolved question (Ferguson, 1985). Testing the various hypotheses concerning energy coupling will be aided by having accurate measurements of the initial events of electron and proton transport and the associated ATP formation.

The Berlin group, for example, has used light or electrical field pulses or acid-base jumps with rapid mixing techniques to resolve early events of energy coupling (Gräber *et al.*, 1984). Ort and colleagues (Graan *et al.*, 1981; Graan and Ort, 1981; Flores and Ort, 1984) have combined repetitive single-turnover xenon flash energization to precisely control the turnover of the redox components, and a sensitive [^{32}P]ATP isolation procedure (Smith *et al.*, 1976) to follow the early events of photophosphorylation. Horner and Moudrianakis (1983, 1986) have used rapid mixing and quenching techniques and millisecond illumination to follow the early events of photophosphorylation and the ability of thylakoids to form ATP in the subsequent dark period, i.e., post-illumination phosphorylation.

The methods to extract the [^{32}P]ATP from the unreacted $^{32}P_i$ and the rapid mixing and quenching are time-consuming and laborious. During the last 10 years the luciferin-luciferase ATP detection method has been developed to study ATP formation driven by single-turnover flash energization (Lundin *et al.*, 1977; Galmiche and Girault, 1982; Schreiber and Del Valle-Tascon, 1982; Beard and Dilley, 1986a,b; DeWolf *et al.*, 1985; Dilley and Schreiber, 1984; Lemaire *et al.*, 1985; Theg and Dilley, 1986). The sensitivity and the simplicity of this method allows fast and accurate assays to be

[3]The abbreviations used are: Δp, protonmotive force; PIP, post-illumination phosphorylation; $\Delta\psi$, transmembrane electrical potential difference; ΔG_{ATP}, phosphoryl group transfer potential of ATP formation; Tricine, *N*-tris(hydroxylmethyl)methylglycine; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; CF_0, intrinsic membrane portion of the coupling factor complex; CF_1, extrinsic membrane portion of the coupling factor complex; DTT, dithiothreitol; Chl, chlorophyll.

performed. Used under the proper conditions, this method can measure the energization threshold, i.e., the number of flashes required for initiation of ATP formation, as well as measuring post-illumination ATP formation after thylakoid energization with single-turnover flashes. We describe herein the characteristics of this post-illumination phosphorylation which can be observed after a series of phosphorylating flashes or when the assay for ATP formation was performed in a "traditional" manner where the ADP and P_i were added *after* the light-energization period. In this and the accompanying papers (Beard and Dilley, 1988; Beard *et al.*, 1988), we will show that important information about membrane energization for ATP formation can be obtained from flash-initiated post-illumination phosphorylation.

Materials and Methods

Thylakoid Isolation and Luminescence Detection for ATP Assay

Chloroplast thylakoids were isolated from growth chamber-grown spinach (11-h photoperiod, 19°C day and 16°C night temperatures) as described by Ort and Izawa (1973). Chlorophyll assay was by the technique of Arnon (1949). ATP formation was measured by the luciferin-luciferase luminescence detection method (Dilley and Screiber, 1984; Lundin *et al.*, 1977). Luciferin luminescence (560 nm) was measured with a photomultiplier (EMI 9634QR) protected by a Corning 9782 (blue) filter. The Corning filter allowed most of the luminescence to pass but cut off most of the red exciting light passed through a Schott RG 630 filter. Saturating single-turnover xenon flashes (10 μs at half-height) were provided by a xenon lamp (EG&G-193). The flash light was focused on a flexible light guide connected to a stirred, 1-ml reaction chamber maintained at 10°C. The illumination was over the entire cuvette contents. Luminescence exiting through the glass bottom of the cuvette entered a light guide connected to the photomultiplier tube, the signal from which was amplified and recorded as described in Beard and Dilley (1986a). Addition of reagents could be made during an assay through a syringe needle port.

The basic reaction medium was 50 mM Tricine-KOH (pH 8.0), 10 mM sorbitol, 3 mM $MgCl_2$, and 0.1 mM methylviologen. Specific reaction conditions for the phosphorylation assays are indicated in the figure or table legends. For assays performed at pH 7.0, 50 mM MOPS-KOH replaced Tricine. The pH of all solutions was adjusted at 10°C. The luciferin-luciferase ATP-dependent luminescence was obtained using the LKB ATP monitoring reagent. The contents of one vial were dissolved in 1.5 ml of cold distilled water, and 150-μl aliquots were frozen for later use. Luciferin-luciferase suspension (10 μl) was added to each assay mixture. To keep background

luminescence due to sources other than flash-dependent ATP to a minimum, it was necessary to purify commercial ADP from contaminating ATP by use of Dowex 1 ion-exchange resin (Cl^- form, 200–400 mesh) according to Shavit and Strotmann (1980). To avoid formation of ATP from ADP by thylakoid adenylate kinase, 10 μM diadenosine pentaphosphate (Sigma) was added to the reaction mixtures.

Electron Transport Under Flashing Light

The number of electrons transferred per flash was obtained by measuring the number of protons released accompanying water oxidation (Graan and Ort, 1982). Thylakoids, 20 μg Chl/ml, were suspended in 2.5 ml of reaction mixture consisting of 0.5 mM Tricine-KOH (pH 8.0), 50 mM sorbitol, 15 mM KCl, 3 mM $MgCl_2$, 0.4 mM ferricyanide, 1.6 μM nigericin, and 0.4 mM 2,5-dimethyl-*p*-benzoquinone at 10°C. For reactions carried out at pH 7.0, 0.5 mM MOPS was substituted for Tricine. Each reaction solution was adjusted to the appropriate pH before beginning the assay. Recordings of pH were obtained using a Corning combination pH electrode 476050 connected to a Corning Model 12 pH meter, with a sensitivity of 0.045 pH unit full scale. Proton production was measured from a series of 100 single-turnover saturating flashed given at a rate of 5 Hz.

Membrane Potential by the Electrochromic 515 nm Signal

The electrochromic absorption band shift (515–540 nm) was measured in the same reaction cuvette as the phosphorylation assays under identical conditions (coupled), except for the omission of the luciferin-luciferase reagent. A bifurcated light guide was used to deliver the actinic flashes and the measuring beam to the sample. The amplified signal was improved by averaging eight, 20-flash (5 Hz) sequences (separated by a 1-s dark period) using a Nicolet 1072 signal averager. The electrochromic absorption change assays under basal conditions were performed as above but with the omission of ADP. Additionally, four separate thylakoid samples were flashed (20 flashes at 5 Hz) twice each with a 3-min dark period between flash trains to improve the signal resulting from the electrochromic absorption change observed under basal conditions.

Results

Post-Illumination Phosphorylation Resulting from a Train of Phosphorylating Single-Turnover Flashes

The great sensitivity of the luciferin-luciferase ATP detection system allows direct observation of single-turnover flash-driven ATP formation in a

thylakoid reaction mixture, as seen in Fig. 1 and previously shown (Beard and Dilley, 1986a,b; DeWolf *et al.*, 1985; Dilley and Schreiber, 1984; Galmiche and Girault, 1982; Lemaire *et al.*, 1985; Schreiber and Del Valle-Tascon, 1982; Theg and Dilley, 1986). A new aspect, introduced here, is the fact that this system also detects post-illumination phosphorylation which occurs after a sequence of energizing flashes, wherein ATP formation occurred during the flash train. This post-illumination phosphorylation is referred to as PIP^+ in Figs. 1A and B, the "+" indicating that ADP and P_i were present during the flash sequence (or absent in the case of PIP^-). After a brief explanation of the system, the validity of using it to measure post-illumination ATP formation will be establihsed.

Figures 1A and B show typical ATP formation assays initiated by a train of single-turnover flashes given at a rate of 5 Hz. The transient spikes are a result of residual actinic light passing through the filter set and serve as a useful event marker. The response time of the recorder was not fast enough to permit complete relaxation of the spike before the next flash. Therefore, the initial portion of PIP^+ was estimated by extrapolating the signal to a time corresponding to 200–300 ms after the last flash, the response time of the luciferin-luciferase system (DeLuca and McElroy, 1974; Lemasters and Hackenbrock, 1976). This resulted in less than a 1 nmol ATP/mg Chl correction, and typical PIP^+ yields ranged from about 5 to 15 nmol ATP/mg Chl.

Figure 1A illustrates a typical tracing in the absence of valinomycin, while Fig. 1B had 400 nM valinomycin and enough K^+ to allow effective dissipation of any electrical potential built up by the flash-induced electron and proton transfer reactions (Graan and Ort, 1981, 1982). In the presence of valinomycin, the number of flashes required to initiate ATP formation is increased from 3 to 15 flashes (Fig. 1), indicating that valinomycin allows rapid K^+ movement so as to dissipate the $\Delta\psi$ component of the protonmotive force.

Figures 1A and B show that ATP formation continued after the last flash, giving PIP^+ yields of 2.7 and 5.8 nmol ATP/mg Chl in the absence and the presence of valinomycin, respectively. A semilog plot of the PIP^+ time course observed in Figs. 1A and B is presented in Fig. 2. The PIP^+ event plots as a first-order phenomena with an apparent rate constant (with or without valinomycin) of about $0.77\,s^{-1}$ ($t_{1/2} = 900$ ms). Because the duration of the flashes was very short (10 μs width at half height) and separated by dark periods of 100 ms or longer, nearly all the ATP formed was during the post-illumination period, a point clearly established by Horner and Moudrianakis (1983) using rapid quench techniques.

It is essential to establish whether the observed PIP^+ signal is due to ATP formed as previously accumulated protons efflux in the dark period or

A
B
LUMINESCENCE (ATP FORMED)
2s
12s
PIP+
0.1 nmol ATP
– Valinomycin
+ Valinomycin
2s
$t_{\frac{1}{2}}$ = 530 msec
$t_{\frac{1}{2}}$ = 1.2 sec
PIP+
0.1 nmol ATP
Calibration

Fig. 1. Single-turnover flash-initiated phosphorylation measured by luciferin-luciferase luminescence. Flashes were delivered at a rate of 5 Hz to thylakoids containing 10 μg of Chl suspended at 10°C in 1 ml of reaction mixture containing 50 mM Tricine-KOH (pH 8.0), 10 mM sorbitol, 3 mM $MgCl_2$, 2 mM KH_2PO_4, 0.1 mM ADP, 0.1 methylviologen, and 10 μM diadenosine pentaphosphate. DTT, 5 mM, was included to protect critical luciferase sulfhydryls. The vertical spike was the result of a light leak and served as a useful event marker. The flash lag (actual) for the onset of ATP formation was determined by the first detectable rise in luciferin-luciferase luminescence while the nmol ATP yield per flash was calculated from the linear rise in bioluminescence. The extrapolated lag for the onset of ATP formation was ascertained from where the linear rise in luminescence would intersect a baseline drawn through the initial, nonphosphorylating flashes (see Beard and Dilley 1986a,b; Dilley and Schreiber, 1984). Valinomycin was omitted in A and 400 nM was included in B. The lag for the onset of ATP formation increased from about 3 flashes to 15 flashes after the addition of valinomycin, while the ATP yield per flash was about 0.75 nmol ATP/mg Chl, with or without valinomycin. After 50 flashes, phosphorylation continues to yield 2.7 nmol ATP/mg Chl (PIP^+) in the absence of valinomycin and 5.8 nmol ATP/mg Chl in its presence. PIP^+ was extrapolated to 200–300 ms after the last flash. Inset: Comparison of the kinetics of luminescence due to addition of standard ATP (top trace), to the kinetics of the PIP^+ ATP yield (bottom trace). Just the last five flashes of the flash sequence are shown for the PIP^+ experiment. The data were taken from a different experiment than for Fig. 1A and B, but similar conditions to 1B were used. The indicated time scale was the same for both traces. Three separate experiments were performed, all of which gave very similar data.

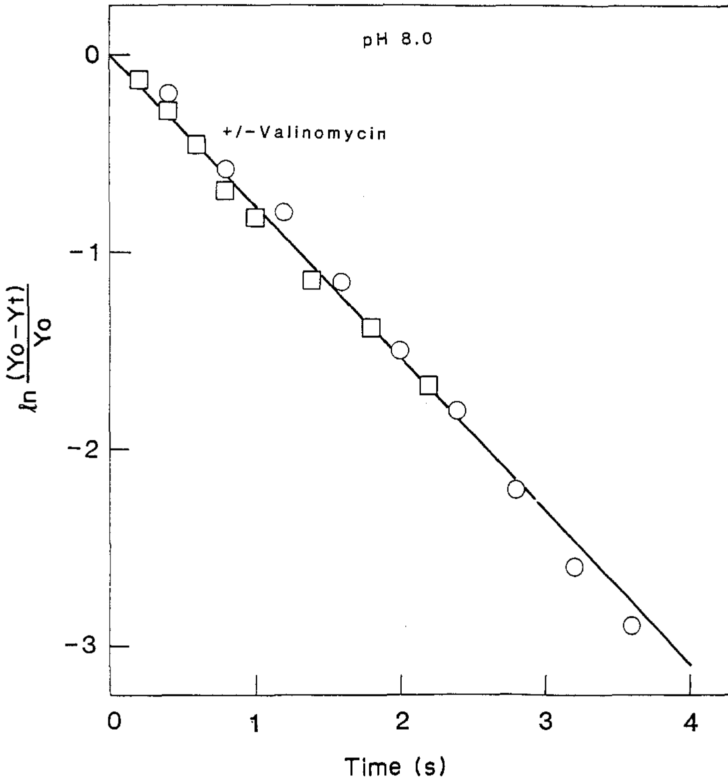

Fig. 2. The effect of valinomycin on the time course of PIP^+ at pH 8. The time course of the PIP^+ ATP yields of Fig. 1 were plotted. Y_o refers to the maximum ATP yield and Y_t refers to the ATP yield at time t after the last flash. The apparent $t_{1/2}$ was 0.9 s without (○), or with (□) 400 nM valinomycin.

whether the slowly appearing ATP has a trivial explanation such as being due to some kinetic limitation of the luciferin-luciferase system or a diffusion limitation of ATP away from the CF_1. We think the former explanation is correct for the following reasons:

(a) The response time of the luciferin-luciferase to added ATP was about $t_{1/2} = 530$ ms (see inset of Fig. 1) in an experiment where the $t_{1/2}$ of the PIP^+ was 1200 ms. Clearly the ATP assay enzyme kinetics were not rate limiting.

(b) An uncoupler (nigericin), added at the last flash of a PIP^+ experiment, inhibited the dark PIP^+ ATP yield, as expected if the PIP^+ ATP formation was due to proton efflux-driven ATP formation rather than some artifact of the assay system. The PIP^+ ATP yield of 10.3 nmol (mg Chl)$^{-1}$ (pH 7.0, 5-Hz flash frequency) was inhibited by over 60%, down to 3.5 nmol ATP (mg Chl)$^{-1}$ when 20 μM nigericin was added during the last flash (the

apparent insensitive PIP^+ was probably due to the manual injection—of necessity rather slow—and mixing of the nigericin). Nigericin should not influence either of the above-suggested artifactual explanations for the PIP^+ signal. That nigericin did inhibit the PIP^+ yield is consistent with there being a storage pool of energetically competent but unused protons built up during the flash train and dissipated in the dark stage.

(c) Related to the point of there being a storage reservoir of protons accumulated in the flash train, the PIP^+ ATP yield is more than can be expected from the energy delivered in the last flash. A PIP^+ ATP yield of 6 nmol ATP/mg Chl requires at least 18 nmol/mg Chl of H^+ to efflux through the CF_0–CF_1 complex, but a single flash only gives about 4.5 nmol H^+/mg Chl (Beard and Dilley, 1988).

(d) A very strong argument is, that in certain cases, the permeable buffer pyridine can influence the kinetics and extent of PIP^+ [see accompanying paper (Beard *et al.*, 1988)]. This excludes an artifactual origin for the PIP^+ ATP yield and kinetics, but is completely consistent with a thylakoid energization origin for the PIP^+ event.

(e) The valinomycin stimulation of PIP^+ ATP yield is consistent with membrane energization being the origin and driving force for the observed PIP^+ signal. On the one hand, valinomycin does not stimulate the luciferin-luciferase response *per se* (see the calibration ATP additions in Figs. 1A and B). On the other, one expects that valinomycin + K^+ would promote a larger ΔpH component in a flash excitation train because $\Delta\psi$ is collapsed by the rapid K^+ efflux and H^+ accumulation replaces that K^+ loss. The greater H^+ accumulation in the valinomycin case and the absence of a $\Delta\psi$ contribution can lead to more PIP^+ yield, primarily because a ΔpH decays more slowly ($t_{1/2} \simeq 1.0$ s) than a $\Delta\psi$ ($t_{1/2} \simeq 0.2$ s, Horner and Moudrianakis, 1985). Also, the valinomycin increase in the PIP^+ ATP yield may be due to the valinomycin-K^+ ionophoric activity keeping the thylakoid membrane system from developing a negative-inside diffusion potential as H^+ ions diffuse out in the ATP-generating efflux. Uribe and Li (1973) and Schuldiner *et al.* (1973) demonstrated this effect with a traditional protocol for post-illumination phosphorylation where the ADP and P_i were added in the dark to thylakoids which had just been pre-illuminated. Under those conditions, the valinomycin-K^+ positive charge delivery to the membrane interior compensates for the H^+ efflux, keeping the membrane $\Delta\psi$ from going negative, thus allowing the dissipating ΔpH component of Δp to express more fully its potential for driving ATP formation. However, in contrast to the Uribe and Li (1973) and Schuldiner *et al.* (1973) protocols, in our case, a K^+ concentration difference was not suddenly imposed across the membrane at the beginning of the dark stage. That is, we did not add KCl at the dark stage as the other workers did.

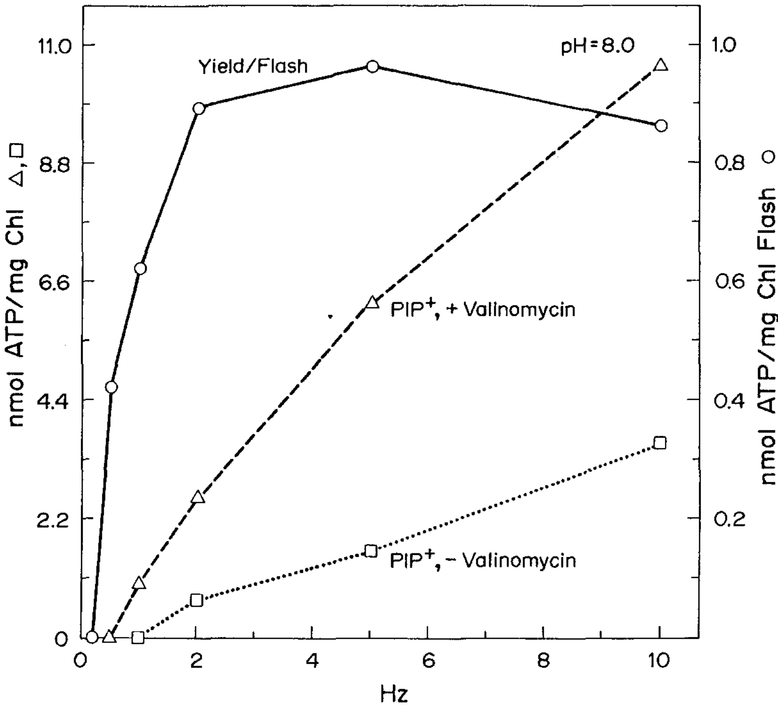

Fig. 3. Flash frequency dependence of the ATP yield per flash and PIP^+ in the absence and presence of valinomycin at pH 8.0. Conditions were identical to those of Fig. 1 except the flash frequency was varied from 0.2 to 10 Hz. (△) PIP^+ in the presence of 100 nM valinomycin; (□) PIP^+ in the absence of valinomycin; (○) ATP yield/flash (−valinomycin).

Figure 3 illustrates that at pH 8 valinomycin stimulated the PIP^+ yield over a range of flash frequencies. PIP^+ was varied by altering the flash frequency of a train of 80 flashes.

A diffusion limitation of ATP (preformed during the flash train) away from the CF_1 is also ruled out as the origin of the increased ATP yield of PIP^+ in the presence of valinomycin, because a diffusion limitation could conceivably show a valinomycin effect of slowing down the ATP appearing in the bulk phase, but a diffusion rate effect should not cause a different net ATP yield.

$\Delta\psi$-Indicating 515 nm Absorption Change

The argument could be offered that the stimulation of PIP^+ by valinomycin may be the result of the depletion of the internal K^+ reservoir during the flash train. If that were the case, then the external K^+ could move

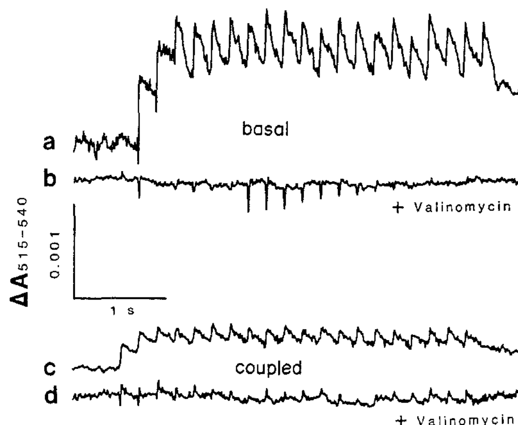

Fig. 4. Effect of valinomycin on the electrochromic absorption change (515–540 nm) under coupled and basal conditions. The reaction conditions are described in Materials and Methods. Traces a and b: thylakoids, four samples each with 15 μg of Chl, were given two 20-flash sequences (5 Hz) separated by a 3-min dark interval in the absence of ADP. Traces c and d: when ADP was included, a single thylakoid sample was given eight 20-flash sequences (5 Hz) separated by about 1 s. Valinomycin, 400 nM, was included in b and d.

back into the lumen during the subsequent dark period, giving rise to a diffusion potential (positive inside), which would supplement the existing protonmotive force and give rise to an extra ATP yield, as has been observed when KCl was added at the end of the light stage (Schuldiner *et al.*, 1973; Uribe and Li, 1973). That possibility can be ruled out in this case because doubling the K^+ in the assay mixture had no effect on the lag for the onset of ATP formation or the PIP^+ ATP yield (Beard and Dilley, 1988).

A depleted internal K^+ reservoir would also allow a flash-dependent $\Delta\psi$ to develop. Figures 4c and d clearly demonstrate that in the presence of valinomycin, and with ADP and P_i present, no appreciable electrochromic absorption change could be detected when 20 flashes were fired at a rate of 5 Hz, eight consecutive times to the same thylakoid sample with 1 s between flash trains. Thus, the thylakoid sample was given 160 flashes in less than 40 s, and there is no evidence that a build-up of $\Delta\psi$ occurred. Figures 4a and b also demonstrate that in the absence of ADP (basal, noncoupled condition), which suppresses the phosphorylating H^+ flux, the usual valinomycin-K^+ concentrations were still adequate to suppress $\Delta\psi$ at and beyond the onset of ATP formation—which would have occurred at about 15 flashes in the plus valinomycin case. Therefore, it is untenable to propose that a diffusion potential (positive inside) was responsible for the observed twofold increase of the PIP^+ ATP yield when valinomycin was present.

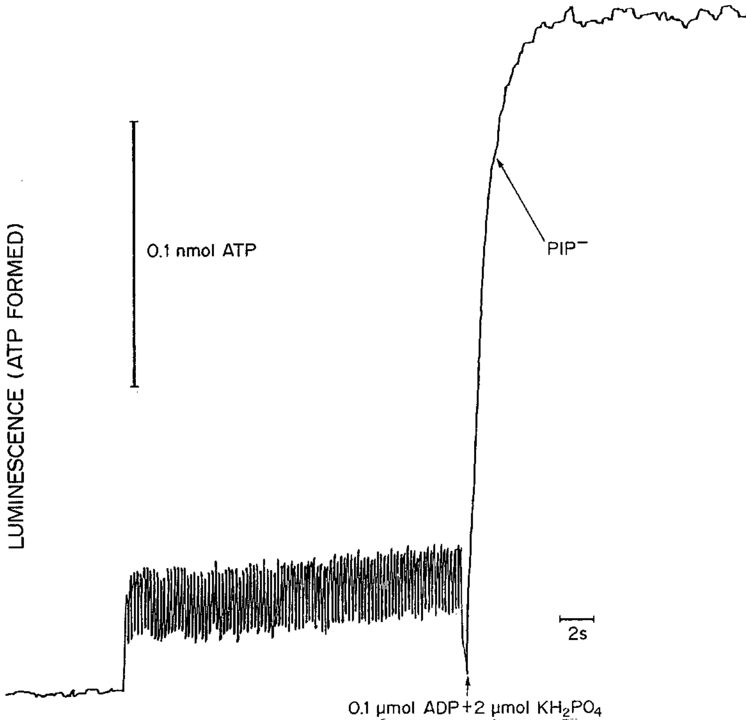

Fig. 5. PIP^- as a result of 100 single-turnover flashes at pH 8.0. Conditions were the same as Fig. 1B except that ADP and P_i were added during the last flash. The Chl content was 13 μg and the final ATP yield was 17.4 nmol ATP/mg Chl. Due to residual ATP present in the ADP, 1.8 nmol ATP/mg Chl was subtracted from the observed PIP^- yield.

Post-Illumination Phosphorylation Resulting from a Train of Nonphosphorylating Single-Turnover Flashes

In the early work, PIP experiments were performed with one or more of the phosphorylating substrates being added after the illumination period and ATP formation was followed in the subsequent dark period (PIP^-). Figure 5 shows a tracing of an analogous experiment with our protocol, performed when the illumination period consisted of a train of single-turnover flashes. The upward spike on the first flash was due to actinic light leaking through the cut-off filter pair (cf. Beard and Dilley, 1986). The spike did not reverse until the last flash because the recorder was not fast enough to return to the base line before the next flash fired. A syringe needle port on the cuvette allowed addition of ADP and P_i immediately after the last flash. The post-illumination ATP yield was 2–3 times greater in such a PIP^- assay as compared to PIP^+ (Fig. 1B). This is the protocol to be used for the experiments reported in the next paper of this series (Beard *et al.*, 1988).

pH Effect on Flash-Initiated Phosphorylation

The PIP^- protocol, as described by Hind and Jagendorf (1963), employed a pH jump accompanying the dark ADP addition, thus permitting a greater ATP yield at the optimum alkaline pH for phosphorylation (dark phase), while the illumination phase was performed at a lower pH to allow for a greater proton accumulation. The ATP yield and the proton accumulation are consistent with the large buffering capacity of endogenous membrane groups near pH 5 (Walz *et al.*, 1974) serving as a reservoir of energetically "competent" protons which can efflux in the high-pH dark stage. Our PIP^- protocol does not use a change of pH between the light and dark phases. We emphasize that the pH dependence of both the PIP^- and PIP^+ ATP yields, particularly in conjunction with permeable buffer (pyridine) effects, reveal important information about the location (bulk phase or membrane-localized) of the accumulated protons that constitute the energy source(s) for driving ATP formation. We also derive information about the relative concentrations of the internal endogenous buffering groups in the pH ranges attained by the energetically competent proton gradient.

Flash Frequency Dependence of PIP^+ and $ATP/2e^-$ Ratios

The flash frequency dependence of PIP^+ at an external pH of 7 in the presence of 100 nM valinomycin is presented in Fig. 6. The PIP^+ ATP yield at pH 7 is 2–3 times greater than at pH 8 for 5-Hz flash frequency (compare with Fig. 3) even though no pH jump accompanied the transition to the post-illumination phase of the assay. The efficient PIP^+ at pH 7 is consistent with the unexpected high ATP yield per flash (Fig. 6). The $P/2e^-$ ratios in those experiments were similar for pH 7 and 8 (Table I). With continuous illumination, ATP formation at pH 7 is less than 20% the rate at pH 8, and the $P/2e^-$ ratio at pH 7 is less than 0.7 while at pH 8 values near 1.1 are common (Gould and Izawa, 1973). The similar $P/2e^-$ ratios at pH 7 and 8 observed with flash excitation allow the ATP yields of the PIP^+ experiments

Table I. Flash-Initiated Electron Transport and ATP Formation[a]

pH	Electron transport (nequiv./mg Chl flash)	ATP formation (nmol ATP/mg Chl flash)	$P/2e^-$
7.0	1.74 ± 0.05	0.75	0.86
8.0	2.10 ± 0.11	0.95	0.90

[a]The reaction conditions for the electron transport assay are described under Materials and Methods and for ATP formation in Fig. 1. For electron transport, the pH of the reaction mixture was adjusted to the desired pH with dilute HCl prior to illumination. Results are the means ±S.E. of five observations. The nmol ATP/mg Chl flash are for the 5-Hz flash frequencies taken from Figs. 3 and 6 for pH 8.0 and 7.0, respectively. Because the ATP yield per flash is constant from 1 Hz to 10 Hz flash frequency, the calculated $P/2e^-$ values will also be constant over the flash frequency range.

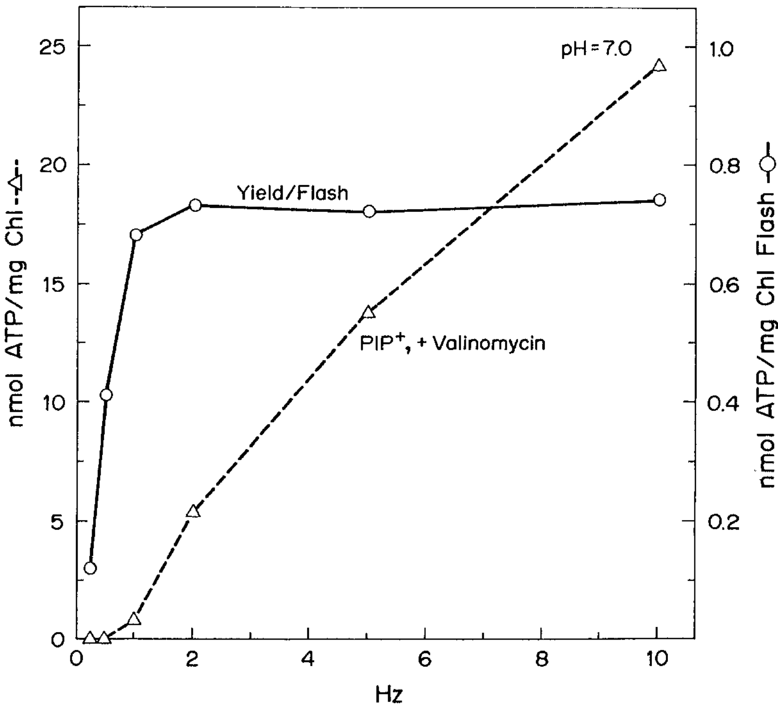

Fig. 6. Flash frequency dependence of the ATP yield per flash and PIP^+ in the presence of valinomycin at pH 7.0. Conditions were as in Fig. 1 except that 50 mM MOPS-KOH (pH 7.0) replaced Tricine and the flash sequence consisted of 100 flashes delivered at a rate that was varied from 0.25 to 10 Hz. (○) ATP yield/flash; (△) PIP^+ in the presence of 100 nM valinomycin. Although the ATP yield/flash was nearly identical to those at pH 8, PIP^+ was stimulated significantly (compared with Fig. 2).

to be compared directly. The ATP yield per flash in Fig. 3 reached a maximum at a flash frequency of 2 Hz as reported earlier using a [^{32}P]ATP to follow ATP formation (Graan and Ort, 1982). Similar results were found at pH 7, as illustrated in Fig. 6.

Flash Number Dependence of PIP^+ and PIP^- Yields at pH 7 and 8

Figure 7 depicts, and allows easier comparison of, the flash titrations of PIP^+ at pH 7 and 8, at 5 Hz frequency. PIP^+ reaches a maximum at around 100 flashes at pH 7 and about 75 flashes at pH 8. The higher yield at pH 7 is consistent with the large, low pK (around 5) buffering pool of thylakoid membranes (Walz *et al.*, 1974) and the efficient ATP formation (i.e., high $P/2e^-$ ratio) which can occur at low pH (Table I). Figure 7 also indicates that detectable PIP^+ ATP formation begins near 20 flashes at pH 8 and 30 flashes at pH 7.

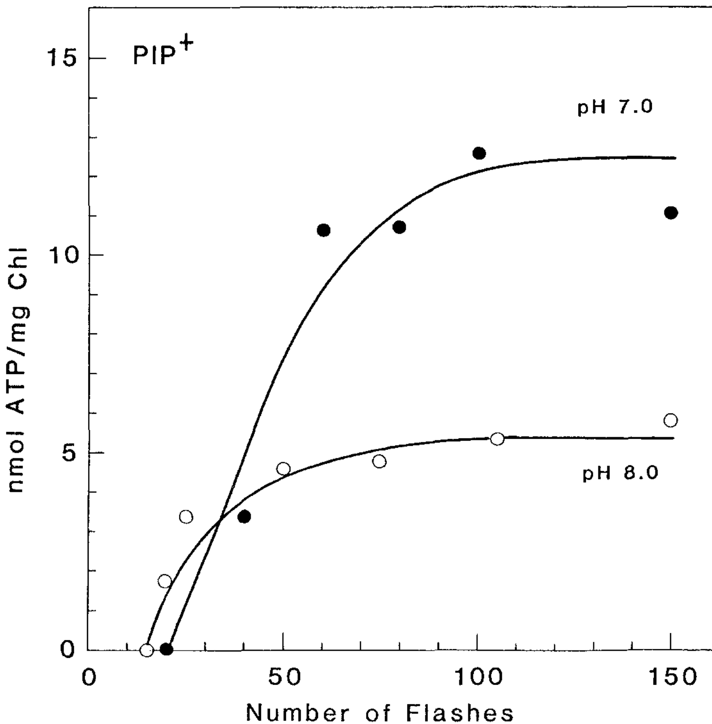

Fig. 7. Flash titrations of PIP^+ at pH 7 and 8 at 5 Hz. Conditions were as in Fig. 1B except that MOPS-KOH replaced Tricine at pH 7 and the number of flashes was varied up to 150 flashes. PIP^+ saturates at around 75 flashes at pH 8 and at 100 flashes at pH 7. The actual ATP onset lags were 20 flashes at pH 8 and 35 flashes at pH 7. Therefore, a significant PIP^+ was observed in the "vicinity" of the onset of ATP formation. (○) pH 8; (●) pH 7.

Figure 8 indicates that the flash titrations of PIP^- (experiments such as that shown in Fig. 5) were similar to those of PIP^+ except that the ATP yields were increased. The greater PIP^- ATP yields are due to a greater H^+ accumulation under basal, noncoupled conditions [the accompanying paper (Beard *et al.*, 1988) provides detailed measurements of this] because the phosphorylating H^+ flux does not occur during the flash train. Again, it was observed that a greater PIP^- ATP yield was expressed at the lower pH (i.e., pH 7).

Flash Frequency Dependence of the Onset Lag for ATP Formation

The onset lag for ATP formation can also be easily measured using the luciferin-luciferase technique, as has been previously demonstrated (Beard and Dilley, 1986a, b; Dilley and Schreiber, 1984; Theg and Dilley, 1986). In the presence of valinomycin-K^+ to collapse $\Delta\psi$, ATP formation is driven by

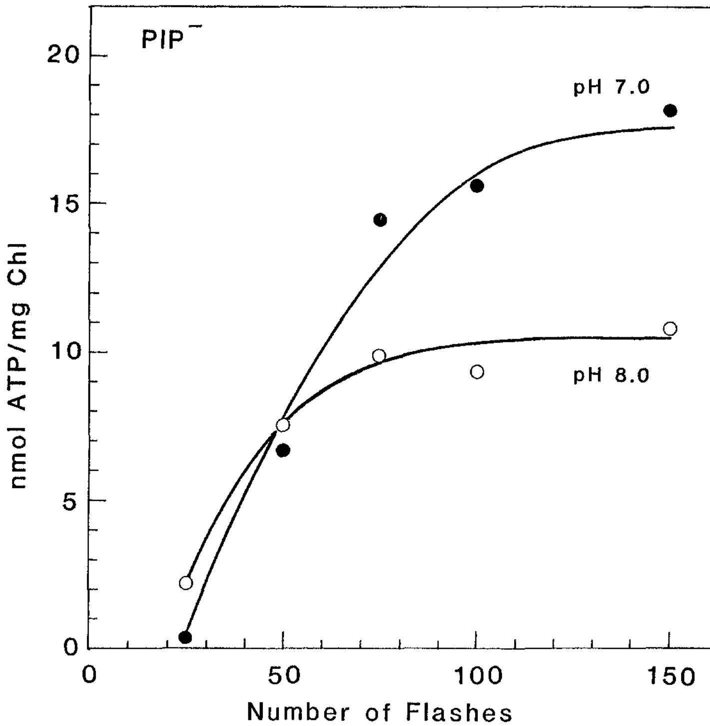

Fig. 8. Flash titrations of PIP^- at pH 7 and 8. Conditions were as in Fig. 5 except that MOPS-KOH replaced Tricine at pH 7 and the number of flashes was varied up to 150 flashes. The PIP^- titrations are similar to those in Fig. 7 except that the ATP yields are higher. (○) pH 8; (●) pH 7.

a protonmotive force consisting solely of ΔpH. Figure 9 illustrates that in the presence of valinomycin, the onset of ATP formation was relatively insensitive to the flash frequency at pH 8. Thylakoids required about 17 flashes before the onset of ATP formation was detected. Surprisingly, at pH 7 the onset lag for ATP formation was very sensitive to the flash frequency. From the slope of the pH 7 plot (slope = 2 s) it is suggested that a slow (2 s), dark event was limiting the onset of ATP formation after the thermodynamic energy requirement had been established. A limitation at the level of the luciferin-luciferase system is unlikely since there is only a 25-ms lag for light emission upon the addition of ATP at room temperature (DeLuca and McElroy, 1974). Although the lag for light emission using the luciferin-luciferase system has been found to be very temperature sensitive (Deluca and McElroy, 1974), a limitation of the ATP detection system can be ruled out as a cause of the pH 7 flash frequency effect, because the lag for the onset of ATP formation was *not* sensitive to flash frequency when $\Delta\psi$ was the

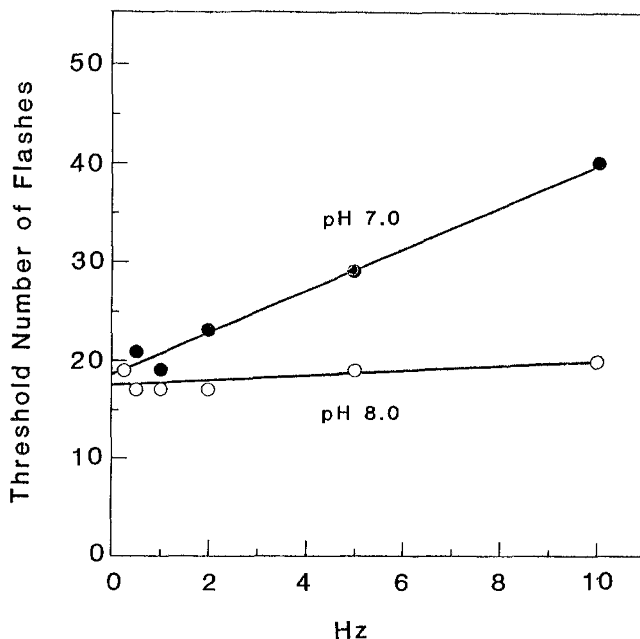

Fig. 9. Flash frequency titration of the actual onset lag of ATP formation in the presence of valinomycin at pH 7 and 8. Conditions were as in Fig. 1B except that MOPS-KOH replaced Tricine at pH 7. The onset of ATP formation was not sensitive to flash frequency at pH 8 but demonstrated a strong sensitivity at pH 7. The sensitivity of the actual onset lag for ATP formation at pH 7 indicates that a 2-s dark event must occur after the thylakoids have attained the energetic requirement to phosphorylate ADP. The lines in the figure were drawn from a least-squares analysis and intersected the y-axis at 17 flashes at pH 8 and at 19 flashes at pH 7. The slopes were 0.3 s (pH 8) and 2.1 s (pH 7). The correlation coefficients were 0.74 and 0.99 for pH 8 and 7, respectively. (○) pH 8; (●) pH 7.

Table II. The Effect of Flash Frequency and Valinomycin on the Onset Lag for ATP Formation at pH 7.0[a]

Flash frequency	Valinomycin	Onset (actual/extrapolated)
1	−	7/19
5		7/19
1	+	22/30
5		30/42

[a]The reaction conditions were as in Fig. 1 except that 50 mM MOPS-KOH (pH 7.0) replaced the Tricine. Valinomycin was present at a concentration of 400 nM where indicated. The actual and extrapolated onsets for ATP formation were derived as indicated in Fig. 1. The results are the means of three determinations and the S.E. was 1 flash or less.

major component of the protonmotive force (i.e., in the absence of valinomycin) at pH 7 (Table II). The extrapolated lag for the onset of ATP formation at pH 7 indicates that a lag of about 19 flashes was required to overcome the thermodynamic threshold for ATP formation.

Discussion

Single-Turnover Flash-Initiated Post-Illumination Phosphorylation Assayed by the Luciferin-Luciferase ATP Detection Method

The luciferin-luciferase ATP detection system has been a useful analytic tool in studying ATP formation initiated with single-turnover flashes in thylakoids (Beard and Dilley, 1986a,b; DeWolf *et al.*, 1985; Dilley and Schreiber, 1984; Galmiche and Girault, 1982; Lemaire *et al.*, 1985; Schreiber and Del Valle-Tascon, 1982; Theg and Dilley, 1986). However, a number of considerations should be given particular attention when applying this assay for continuous monitoring of ATP. The ATP concentration should remain far enough below the K_m so that the bioluminescence is related linearly to the ATP concentration. The luminescence intensity is proportional to ATP concentration up to about 1 μM ATP (Lundin *et al.*, 1976). The luciferase concentration should be low enough so as not to change the ATP concentration or give rise to interference as a result of product inhibition, but must be high enough to give adequate sensitivity. The reaction conditions will also affect the sensitivity of the system. Luciferase is sensitive to sulfhydryl reagents (Alter and DeLuca, 1986), certain anions (Denburg and McElroy, 1971), temperature (DeLuca and McElroy, 1974), and pH. For example, DTT stabilizes the enzyme and Cl^-, a commonly used anion in reaction buffers, is quite inhibitory, and cannot be used above about 10 mM.

Kinetic analysis of the luciferase reaction indicated that there was a lag of 25 ms at 25°C (pH 7.8) before light emission was observed and this lag was very temperature sensitive (DeLuca and McElroy, 1974). After the onset of light emission, it takes about 250–300 ms to reach peak intensity (Deluca and McElroy, 1974; Lemasters and Hackenbrock, 1976). The lag and the time required to reach 50% peak intensity are independent of ATP concentration and luciferase concentration, indicating that the onset of light emission is controlled by something other than ATP binding (DeLuca and McElroy, 1974). The kinetic properties of luciferase will be altered when the ATP assay is performed under different reaction conditions (i.e., pH). Therefore, caution should be used in drawing conclusions from experiments performed under different conditions unless it can be demonstrated that there is not a limitation at the level of the luciferin-luciferase system. Figure 1 (inset) shows

that for our conditions the response time of luciferase to ATP concentration changes was not rate limiting in regard to detecting the post-illumination (PIP) ATP formation by thylakoids.

The measurement of PIP is the new aspect of the luciferin-luciferase ATP detection system introduced here. The sensitivity of the PIP^+ ATP yield to permeable buffers (Beard and Dilley, 1986a,b, 1988; Beard *et al.*, 1988), nigericin, valinomycin (Figs. 1 and 3), and flash frequency (Figs. 3 and 6) convincingly demonstrates further than the PIP^+ signal is not the result of the kinetic limitation of the assay system, or an ATP diffusion limitation, but must represent membrane deenergization as accumulated protons diffuse out through the CF_0–CF_1 complex.

The increased yield of PIP^+ at pH 7 as compared to pH 8 cannot be accounted for by a possible change in the rate constant of the luciferase reaction by lowering the reaction pH. PIP^- showed a similar stimulation upon dropping the external pH from 8 to 7 (Fig. 8), and since for PIP^- only the absolute ATP yields were plotted (i.e., there is no ATP formed during the flash train), this indicates that the stimulation of PIP^+ at pH 7 (Fig. 7) was real. It is most likely due to the larger endogenous buffering capacity (Walz *et al.*, 1974) reached by the accumulated protons (therefore more protons stored prior to the PIP^- or PIP^+ stages) when the external pH was 7 compared to 8.

P/2e⁻ Ratios

The $P/2e^-$ ratios observed under our conditions were about 1 at both pH 7 and 8, an unexpected finding given that under continuous illumination the $P/2e^-$ ratio is much lower at pH 7 compared to pH 8 (Gould and Izawa, 1973). Ort and colleagues (Flores and Ort, 1984; Graan and Ort, 1982; Hangarter and Ort, 1985, 1986a) typically found the $P/2e^-$ values to be around 1.2 for single-turnover flash-initiated phosphorylation at pH 8.4 and 4°C. The high $P/2e^-$ ratio observed at pH 7 is consistent with the high flash yields that Galmiche and Girault have observed with multiple flashes, but not with single flashes (Galmiche and Girault, 1982; Lemaire *et al.*, 1985). Likewise, Schlodder *et al.* (1982) have shown the yield of ATP, from a series of externally applied voltage pulses or 20-ms light pulses, to be independent of the external pH between 6.5 and 8.6. Thus, when thylakoids are energized with short pulses separated with "dark" periods, events can occur which are not limited by dark, coupling factor enzymatic turnover time (after the coupling factor has been activated, see above). Under steady-state conditions (i.e., continuous illumination), these dark events may express themselves as a slower rate of photophosphorylation and hence a depressed $P/2e^-$ ratio. Alternatively, the high $P/2e^-$ ratio at pH 7 may represent the operation of a

Q-cycle or redox-linked proton pump operating more efficiently than at pH 8 (Cramer *et al.*, 1987).

Flash Frequency Dependence of the Onset Lag for ATP Formation

Figure 9 indicates that the lag for the onset of ATP was insensitive to the flash frequency (up to 10 Hz) in the presence of valinomycin at pH 8. At pH 7, however, the onset lag for ATP formation required a dark step of about 2 s after thylakoids had developed the ability to phosphorylate ADP. This may involve a slow activation step at the CF_1 complex at the low pH (deprotonation of a critical carboxyl?). The activation state of the CF_1, as influenced by light and dithiothreitol treatment, has been shown to cause changes in the threshold flash number for ATP formation onset, when the ΔG_{ATP} is low (Hangarter *et al.*, 1986). Since the $P/2e^-$ ratio was similar at pH 7 and pH 8 (Table I) in a flash train of 5 Hz, the slow step does not appear to express itself once ATP formation has been initiated—further implicating activation phenomena as a cause of the change in the ATP onset lag, rather than steps in the CF_1 catalytic reaction cycle. The sensitivity of the ATP onset lag to flash frequency at pH 7 was not due to a limitation of the luciferin-luciferase system since in the absence of valinomycin, the onset lag was *not* sensitive to flash frequency (Table II). There is no reason to believe that valinomycin may change the kinetics of the luciferin-luciferase system, but as a check for this, we found that the presence of valinomycin did not change the time required to reach 50% peak intensity of a calibration at pH 7 or 8 (data not shown). Figure 2 also indicates that valinomycin did not alter significantly the formation kinetics of PIP^+. The insensitivity of the ATP onset lag at pH 7 to flash frequency in the absence of valinomycin may indicate that ΔpH and $\Delta\psi$ are not equivalent in the activation of the coupling factor.

The ATP onset lags extrapolated from the y-axis in Fig. 9 indicate that ATP formation begins after 17 flashes at pH 8 and 19 flashes at pH 7. If, in the presence of valinomycin, a ΔpH of 2.3 units is required to overcome the thermodynamic requirement to form ATP (Gräber *et al.*, 1984), then the pH of the lumen probably will not decrease more than 0.14 (2.3/17) pH units per flash. Junge *et al.* (1979) have estimated the internal pH to drop only 0.06 pH units/flash starting at a pH near 8. The drop in lumen pH, however, *would not* be expected to be a linear function of flash number since the buffering capacity of the lumen increases with decreasing pH (Walz *et al.*, 1974). Decreasing the external pH 1 unit (to pH 7) should, therefore, have *increased* the number of flashes required to titrate the threshold buffering pool because the lumen pH should drop to around 4.7 before an energetically competent proton gradient is established, thus encountering the known, large pool of

endogenous carboxyls with pK values near 5–5.2 (Walz *et al.*, 1974). A number of reports have indicated that the threshold buffering pool for ATP formation onset was insensitive to added internally accumulated buffers (Beard and Dilley, 1986a,b; Graan *et al.*, 1981; Hangarter and Ort, 1985; Horner and Moudrianakis, 1983, 1986; Ort *et al.*, 1976). The relative insensitivity of the onset lag length for ATP formation to external pH under the conditions used here also suggests that the lumen was not involved in protonation events leading to energization of ATP formation. Rather, we can better explain the data by postulating localized proton accumulation domains having a lower buffer capacity and perhaps a different buffering power profile than the lumen.

Concluding Remarks

The techniques described here for detecting either PIP^- or PIP^+ ATP formation will be valuable tools for experiments designed to probe events associated with proton–membrane interactions during energy coupling. In the past, PIP assays were performed in a manner similar to the protocol employed to measure the PIP^- ATP yield (Hind and Jagendorf, 1963). The stimulation of the ATP yield by low pK_a permeable buffers in such PIP^- experiments demonstrated that bulk-phase proton gradients can drive phosphorylation under those conditions (Avron, 1972; Nelson *et al.*, 1971; Vinkler *et al.*, 1980). In an accompanying paper (Beard *et al.*, 1988), we demonstrate that under the conditions we normally use, the PIP^+ ATP yields *were not* sensitive to the permeable buffer pyridine, indicative of a localized mode of energy coupling. But, most interestingly, PIP^- ATP yields *were* sensitive to pyridine, consistent with a component of bulk-phase protonic energy coupling under the PIP^- energization protocol. Approaches such as these should help identify the location (membrane-associated or lumen-associated) and size of the thylakoid energization threshold proton buffering pools, along with the p*K*s which may contribute to those pools.

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