A shift in chloroplast energy coupling by KCl from localized to bulk phase delocalized proton gradients

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Spinach thylakoids were resuspended in a buffer including either 200 mM sucrose or 100 mM KCl. Single-turnover flash excitation and the luciferin-luciferase system were used to energize and follow ATP formation. The effect of the permeable buffer pyridine was measured on the ATP formation onset lag and on the post-illumination ATP yield. Consistent with a bulk phase delocalized proton gradient coupling model, thylakoids stored in 100 mM KCl exhibited an increase in these two parameters in the presence of pyridine. Thylakoids isolated in the absence of KCl showed no effect of pyridine on the two parameters indicating a localized energy coupling mode.

ATP synthesis *Protonmotive force* *Salt effect* *Single-turnover flash* *Thylakoid membrane*

1. INTRODUCTION

Energy coupling in chloroplast thylakoids is believed to utilize a protonmotive force to drive ATP formation [1]. Controversy surrounds such mechanistic questions as how protons are processed from the proton-generating events of electron transport into the CF_0-CF_1 complex. It is clear from the early work of Jagendorf and colleagues that protons originating in the inner aqueous phase of chloroplast thylakoids can drive ATP formation in a bulk phase delocalized mode as illustrated from an acid-base shift in the dark [2] or from electron transfer-driven proton accumulation, as shown in the post-illumination ATP formation experiments [3]. However, evidence has accumulated which is difficult to reconcile with a bulk phase delocalized protonmotive force as the primary driving force during phosphorylation (review [4]). Thus, a localized protonmotive force has been postulated [4]. This controversy has been puzzling and disconcerting, and progress in the understanding of energy coupling will be abated until this issue is resolved. If, depending on presently unknown conditions, either localized or delocalized bulk phase proton gradients can drive phosphorylation, it should be possible to find the conditions whereby either one or the other coupling mode is expressed with the same thylakoid preparation. Sigalat et al. [5] have suggested that KCl may influence the mode of coupling, and the work reported here utilized that idea. The results indicated that incubation of isolated thylakoids in the presence of high KCl concentrations induced a bulk phase delocalized coupling which was not observed in its absence.

Our assay utilized single-turnover flashes and the luciferin-luciferase ATP detection method [6,7]. The flash excitation allows control over the magnitude of energization and permits easy detection of the lag for the onset of ATP formation. The sensitive luciferin-luciferase system permits direct observation of post-illumination phosphorylation (PIP) after the last flash in a flash sequence. The effect of permeable buffers on ex-

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; CF_0-CF_1, integral membrane and peripheral portions of the coupling factor complex, respectively; DTT, dithiothreitol; Chl, chlorophyll

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tending the flash lag for the onset of ATP formation in the presence of valinomycin-K^+ and on changing the PIP yield are used as indicators of bulk phase delocalized proton gradient energization [8,9]. The absence of an effect by a permeable buffer on the ATP onset lag [10–12] and PIP ATP yield are taken as indicative of localized proton gradient coupling.

2. MATERIALS AND METHODS

Chloroplast thylakoids were isolated from growth chamber-grown spinach essentially as described by Ort and Izawa [13]. The thylakoids were washed once and resuspended in 200 mM sucrose, 5 mM Hepes (pH 7.5), 2 mM $MgCl_2$, and 0.5 mg/ml BSA or in a resuspension buffer where the sucrose was replaced with 100 mM KCl. The chlorophyll concentration was determined by the technique of Arnon [14].

ATP formation was measured by the luciferin-luciferase luminescence detection method [6,7]. Luciferin-luciferase luminescence was measured with a photomultiplier tube (EMI 9558Q) protected by the filter combination Corning 9782 and Balzers DT-Gruen. Saturating single-turnover flashes with a half-width of 10 μs were delivered by a xenon lamp (EG&G FX-193) at a frequency of 5 Hz. Flash-light was filtered through a Schott RG 630 filter and focused on a flexible light guide connected to a 1 ml reaction chamber which was stirred and maintained at 10°C. The illumination was over the entire cuvette surface. Luminescence exiting through the glass bottom of the cuvette entered a light guide connected to the filter-protected photomultiplier tube. The signal was amplified with a Tektronix 5A22N differential amplifier and then simultaneously recorded on a strip chart recorder and stored in a Tracor Northern signal averager. Addition of reagents could be made during an assay through a syringe needle port.

Unless specified, thylakoids (with or without KCl in the resuspension buffer) were incubated for 3 min before a flash sequence in a reaction medium consisting of 50 mM Tricine-KOH (pH 8.0), 10 mM sorbitol, 3 mM $MgCl_2$, 1 mM KH_2PO_4, 5 mM DTT, 400 nM valinomycin, 0.1 mM ADP, 5 μM diadenosine pentaphosphate (Sigma), and 0.1 mM methyl viologen. In the case of the high-KCl-resuspended thylakoids, the KCl carried over to the assay reaction buffer did not exceed a final concentration of 0.5 mM. The pH of all solutions was adjusted at 10°C. The luciferin-luciferase ATP-dependent luminescence was elicited using the LKB ATP monitoring reagent. The contents of one vial were dissolved in 1.5 ml cold distilled water, and 150-μl aliquots were frozen for later use. To each assay mixture was added 10 μl of the luciferin-luciferase suspension. At the end of each assay, 100 pmol ATP was added to calibrate the signal. To keep background luminescence due to sources other than flash-induced phosphorylation to a minimum, it was necessary to purify commercial ADP from contaminating ATP with a Dowex 1 resin (Cl^- form, 200–400 mesh) according to Shavit and Strotmann [15]. To avoid formation of ATP from ADP by thylakoid adenylate kinase, 5 μM diadenosine pentaphosphate was included in the phosphorylation assay mixture. Further details of reaction conditions are given in the table and figure legends.

3. RESULTS

The sensitivity of luciferin-luciferase toward high Cl^- concentrations [16] did not permit us to have high concentrations of KCl present during the assays. Therefore, to test the suggestion of Sigalat et al. [5] that high concentrations of KCl favor bulk phase delocalized proton gradient coupling, we washed and resuspended our thylakoids, to 2–4 mg Chl $\cdot$ ml^{-1}, in a resuspension buffer which did not include KCl [13] or replaced the 200 mM sucrose in the resuspension buffer with 100 mM KCl (see section 2). The stock thylakoid suspensions were incubated for at least 30 min before the first assay and the observed effects did not change with longer incubation times (3 h).

The effect of the high-KCl storage treatment on the onset of ATP formation was quite dramatic (fig.1). Compared to the usual resuspension buffer, the high-KCl treatment caused the lag for the onset of ATP formation to increase from 13/21 to 30/42 flashes (see legend to fig.1 for explanation of these flash parameters). Table 1 gives a compilation from several different experiments for each treatment documenting the point illustrated in fig.1 (a, − KCl; c, + KCl).

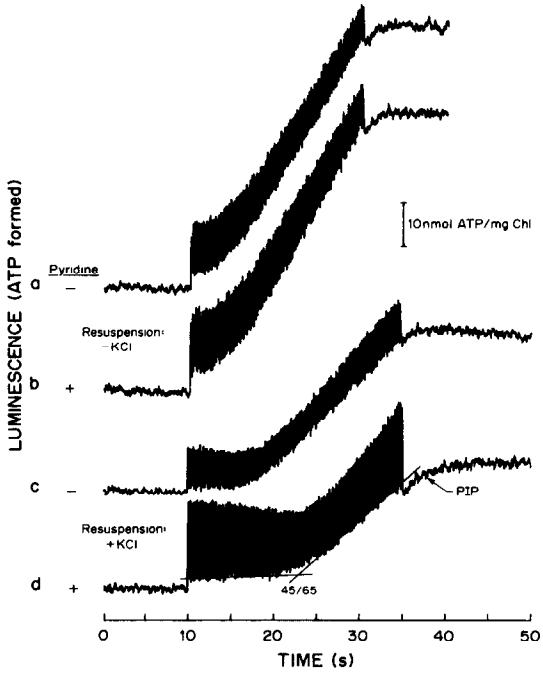

Fig.1. The effect of pyridine on single-turnover flash initiated phosphorylation with thylakoids resuspended in the absence or presence of 100 mM KCl. Thylakoids were washed and resuspended in 5 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 0.5 mg/ml BSA, and 200 mM sucrose (a,b: – KCl) or 100 mM KCl in place of the sucrose (c,d: + KCl). Flashes (100 in a,b; 125 in c,d) were delivered at a rate of 5 Hz to thylakoids containing 14 μM chlorophyll suspended at 10°C in 1 ml reaction mixture containing 50 mM Tricine-KOH (pH 8.0), 10 mM sorbitol, 3 mM $MgCl_2$, 1 mM KH_2PO_4, 5 mM DTT, 0.1 mM ADP, 0.1 mM methyl viologen, 400 nM valinomycin, and 5 μM diadenosine pentaphosphate. The reaction mixture was pH adjusted at 10°C with (b,d) or without (a,c) 5 mM pyridine. The vertical spike is the result of a light leak and served as a useful event marker. The different flash-spike heights are an artifactual result of the sampling time of the signal averager. The flash lag for the onset of ATP formation was determined with the aid of two criteria: (i) the first detectable rise in luminescence (45 in d) and (ii) the back extrapolation of the steady rise in the flash-induced luminescence increase to the x-axis (65 in d). The increase in luminescence after the last flash was due to post-illumination phosphorylation.

As demonstrated by previous experiments using PIP [9,10], pyridine present during the light stage initially delayed the attainment of the energetic threshold and at longer illumination times gave an enhanced ATP yield. This is an expected effect for bulk phase delocalized coupling. The pK_a of pyridine, 5.44 at 10°C [17], is alkaline enough to increase the internal buffering capacity of the lumen so as to cause a delay in the onset for ATP formation and it is also acidic enough so as to serve as a reservoir for protons after the energetic threshold to phosphorylate ADP is reached. The controversy, in the past, has been that some results showed permeable buffer-dependent delays in the onset of light stage ATP formation [8,9], but other data showed no significant delay of the onset of the energetic threshold [10,11]. The significant result illustrated in fig.1 and documented in table 1 is the finding that the thylakoids washed and resuspended in a buffer lacking KCl exhibited little or no effect of pyridine on the length of the ATP onset lag, while in the high-KCl-stored sample, 5 mM pyridine caused the lag parameters to increase by 10/23 flashes. Although the ATP yield/flash was not significantly affected by the KCl or pyridine treatments, it took many more flashes to reach the steady-state ATP yield/flash in the presence of pyridine with thylakoids stored in high KCl. This is again consistent with pyridine 'slowing' the acidification of the lumen by increasing its buffer capacity. In the control sample (– KCl), on the other hand, the lack of a pyridine effect is consistent with localized coupling.

Table 1

Effect of KCl resuspension and pyridine on the onset of ATP formation

Conditions	Number of flashes to the onset of ATP formation	ATP yield per flash [nmol ATP · (mg Chl flash)$^{-1}$]
– KCl storage		
– pyridine	13 ± 1/21 ± 2	0.61 ± 0.02
+ pyridine	14 ± 1/25 ± 1	0.64 ± 0.08
+ KCl storage		
– pyridine	30 ± 3/42 ± 3	0.56 ± 0.05
+ pyridine	40 ± 3/65 ± 5	0.53 ± 0.05

Conditions are as in fig.1. The lags for the onset of ATP formation were determined as described in fig.1 and represent the actual/extrapolated lags. The ATP yield per flash was determined from the linear rise in luminescence (see fig.1). The results are the means of 4 observations ± SE

The luciferin-luciferase technique used in these assays permitted us to detect directly PIP, as shown in fig.1 as the increase in luminescence after the last flash. Thus, the effects of permeable buffers on PIP allow an additional test for distinguishing localized from bulk phase delocalized energy coupling. In the original work on PIP, wherein permeable buffers increased the yield of PIP [18,19], ADP and P_i were not present during the proton pumping (light) stage, but were added in the dark stage. Thus, the proton accumulation in the light was under basal, non-coupling conditions. In the assay used here however, all phosphorylation substrates were present during the flash sequence and ATP formation, as a result of the flash train, was detected. A residual energization occurred over and above that utilized in the 5 Hz flash train and was expressed as PIP. Obviously the PIP yield in these experiments represents energy storage in excess of the energy (protons) delivered just by the last flash because the 4–10 nmol ATP·(mg Chl)$^{-1}$ represents 12–30 nmol H^+·(mg Chl)$^{-1}$ dissipated, and the H^+ accumulation per flash is no more than about 3.5 nmol H^+·(mg Chl flash)$^{-1}$ [20]. In these experiments, pyridine had no effect on PIP yields in the control thylakoids (– KCl), but significantly increased (about 2-fold) the PIP yield in the high-KCl-stored sample (fig.2 and table 2). Since there

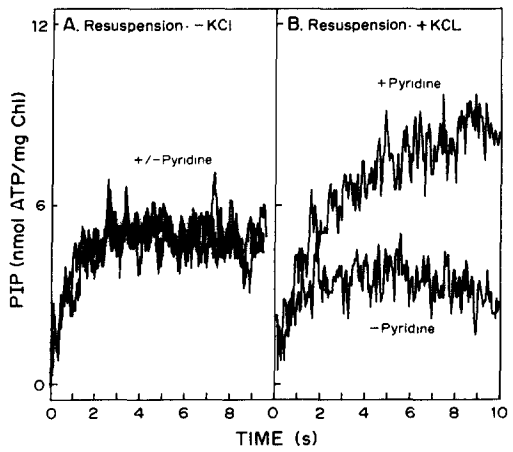

Fig.2. The effect of pyridine on the luminescence resulting from post-illumination phosphorylation with thylakoids resuspended in the absence (A) or presence (B) of 100 mM KCl. The PIP portions of the luminescent signals in fig.1 are presented.

Table 2

Effect of KCl resuspension and pyridine on post-illumination ATP formation

Conditions	Post-illumination ATP yield [nmol ATP·(mg Chl)$^{-1}$]	Total ATP yield during flash train [nmol ATP·(mg Chl)$^{-1}$]
– KCl storage		
– pyridine	4.3 ± 0.4	50 ± 2
+ pyridine	4.3 ± 0.4	51 ± 6
+ KCl storage		
– pyridine	5.4 ± 0.5	48 ± 4
+ pyridine	9.5 ± 0.4	33 ± 5

Conditions are as in fig.1. PIP ATP yield was determined from the increase in signal after the last flash in a flash sequence, while the remaining rise in signal from the onset of ATP formation to the beginning of PIP represents the total ATP yield from the flash train. The results are the means of 4 observations ± SE

was less ATP made in a flash sequence with high-KCl-resuspended thylakoids due to the longer lag and the pronounced curvature in the attainment of the steady state, the effect of pyridine on PIP in fig.2 illustrates the PIP resulting from 125 flashes (+ KCl) as compared to that resulting from 100 flashes (– KCl). Those flash trains allowed similar total ATP yields during the flash sequence for the minus pyridine cases (table 2). This assured that the phosphorylation potential (ΔG_P) will be similar at the onset of PIP for the two thylakoid samples (± KCl). It should also be noted that pyridine had no effect on the PIP yield if control thylakoids (– KCl) were energized with 125 flashes (not shown). The increase in PIP ATP yield observed with high-KCl-resuspended thylakoids in the presence of pyridine indicates that the longer lags for the onset of ATP formation generally observed with this preparation were not due to an uncoupling or an inhibition of electron transport. Uncoupling and electron transport inhibition have been shown to increase the lag for the onset of ATP formation [21,22]. If those situations had occurred, a decreased PIP yield would have been expected; however, the PIP yield was stimulated (table 2). Moreover, measurement of flash-induced electron transfer indicated that the same number of electrons were transferred during a 100 flash se-

quence in both types of thylakoid preparations (not shown).

4. DISCUSSION

The substitution of 100 mM KCl for 200 mM sucrose in the thylakoid resuspension buffer induced a sensitivity, of both the lag for the onset of ATP formation and the PIP ATP yields, toward pyridine in a manner consistent with bulk phase delocalized energy coupling. In previous work which indicated that permeable buffers could increase the lag for the onset of ATP formation [8,9], the conditions for the phosphorylation assays included high concentrations of KCl, whereas the experimental conditions for the first demonstration of a lack of an effect of such buffers on the lag for the onset of ATP formation [10] had only enough KCl present to collapse the membrane potential with the aid of valinomycin. Low KCl was also used in later work using a rapid quench technique to follow ATP formation during, as well as after, the illumination period [12] and that work also indicated an insensitivity of the onset lag of ATP formation toward permeable buffers. With our protocol, thylakoids resuspended and assayed in the absence of KCl did not exhibit pyridine effects on either the onset of ATP formation or the PIP ATP yields, consistent with a localized coupling mode. It should be emphasized that all our assays were performed in the same phosphorylation medium which lacked KCl but had enough Tricine-KOH present to collapse the membrane potential via valinomycin-dependent K^+ flux.

The increase in the lag for the onset of ATP formation induced by pyridine in the high KCl-stored thylakoids is about that calculated for the increase in buffering capacity of the lumen by pyridine under the assumption that a bulk phase delocalized proton gradient could protonate pyridine. If the energetic threshold ΔpH for ATP formation is 2.3 pH units [23], then the calculated extension of the onset of ATP formation by pyridine is 9 flashes compared to the measured 10 ± 3 flash extension (table 1). Because additional pyridine is expected to be taken up as internally located pyridine becomes protonated [18], the calculated extension of the lag is a minimum estimate.

The KCl effect reported here agrees with the suggestion of Sigalat et al. [5]. It appears that the earlier contradictory results concerning localized or bulk phase delocalized proton gradients [8–11] may now be resolved. However, studies to determine the mechanism behind the high KCl concentration inducing a shift from localized to bulk phase delocalized energy coupling remain to be done. One obvious possibility is that the high KCl perturbs critical membrane protein conformations allowing the protons released upon H_2O and plastohydroquinone oxidation to equilibrate with the inner bulk phase. It has been previously hypothesized that thylakoids have localized proton-binding domains [24–26] which permit the translocation of protons from the redox reactions into the CF_0-CF_1 complex without entering the internal bulk phase [7,10,24,27]. The approaches reported here may lead to more definitive experiments concerning the nature and functioning of the putative localized proton processing domains.

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