The Effect of Permeant Buffers on Initial ATP Synthesis by Chloroplasts Using Rapid Mix-Quench Techniques*

Robert D. Horner and Evangelos N. Moudrianakis

From the Department of Biology and McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

The chemiosmotic hypothesis predicts that buffers which permeate chloroplast membranes should delay the formation of the proton gradient at the onset of illumination. If valinomycin and KCl are present to collapse the electrical potential as well, this delay should result in a lag in initial ATP synthesis. Using rapid-mix, acid-quench techniques, we have found that in light-driven ATP synthesis the permeant buffer imidazole does not increase the initial lag caused by the valinomycin-KCl pair. Similar results are obtained under methyl viologen or phenazine methosulfate/ascorbate-mediated photophosphorylation and are independent of the internal volume of the chloroplasts. Furthermore, we have observed that chloroplasts can synthesize significant amounts of ATP in darkness following an illumination period as short as 100 ms. This capacity for ATP synthesis in darkness after short pre-illumination periods is decreased in the presence of imidazole, and this may account for the apparent lags reported in earlier studies which have used rapid flash photophosphorylation in the presence of permeant buffers. The results of the present study argue that in chloroplasts, initial ATP synthesis and post-illumination ATP synthesis are driven by distinct components of the proton motive potential.

The chemiosmotic hypothesis has been able to organize a great many observations in the field of bioenergetics by its concepts of vectorial proton movements, transmembrane proton and electrical potentials, and proton-translocating ATP synthetases. One set of observations which has been well explained is the effect of weak amine buffers on proton movement and phosphorylation in chloroplasts. Work by Nelson et al. (1) and Avron (2) have shown that pyridine, phenylendiamine, aniline, and imidazole not only increased the amount of proton uptake by chloroplasts in the light but also increased ATP synthesis during post-illumination phosphorylation. It was suggested that these buffers permeated the thylakoid membrane and increased the capacity of the inner thylakoid space to store protons. In later work, Ort et al. (3) reasoned, as a corollary, that these buffers should also delay the build-up of the proton gradient at the beginning of illumination. These workers studied the early events of photophosphorylation under conditions where the electrical component of the electrochemical gradient was abolished by the addition of valinomycin and KCl. An electronic shutter was employed to provide illumination in the form of a short flash which, by generating a burst of protons, drove ATP synthesis. To magnify the signal derived from a short flash, Ort et al. had used a train of short flashes where each single flash was followed by a long dark period (multiple flashes). The duration of the dark period was such as to allow any pH gradient formed by a single flash to be dissipated before a second flash occurred. Using this experimental protocol, Ort et al. demonstrated that although tris(hydroxymino)methane, orthophosphate, and bicarbonate permeated the inner thylakoid space, these buffers did not delay the onset of photophosphorylation beyond the 50-ms lag caused by valinomycin and KCl. These results were challenged by Vinkler et al. (4) and Davenport and McCarty (5). Using single short flashes, Vinkler et al. observed that pyridine, imidazole, and phosphate did yield an apparent increase in the lag of initial ATP synthesis. Davenport and McCarty found that if samples were illuminated by a single flash, imidazole appeared to increase the lag in initial ATP synthesis; whereas, if samples were illuminated by multiple flashes, imidazole did not appear to increase this lag. Vinkler et al. and Davenport and McCarty postulated that Ort et al.'s failure to observe the imidazole-dependent lag in initial ATP synthesis was the result of the experimental design and in specific that multiple flashes caused the progressive build-up of a transmembrane proton gradient. This would lead to a situation where each successive flash would yield progressively more ATP, and the average yield of ATP/flash would increase as the length of the train of flashes increased, which would result in an underestimation of lags. However, Ort et al. had measured photophosphorylation under single flashes also and had observed essentially the same results as with multiple flashes of equal energy input.

In the above mentioned experiments, the investigators graphed the yield of ATP versus the duration of the light flash and derived curves indicating lags in initial ATP synthesis. It should be noted that the rapid flash technique uses an electronic shutter to begin and end the photophysical events of photophosphorylation with great precision. However, the chemical events cannot be controlled with equal precision because after illumination, the reaction is ended by manual injection of acid. Thus, light-independent chemical transformations are likely to continue for unaccounted periods of time beyond the extinction of the light. Our laboratory has recently used the technique of rapid mixing and quenching to study photophosphorylation (6), and one advantage of this technique over short flash methods is that the interval of darkness between the end of illumination and perchloric acid termination can be precisely controlled. Using this technique, we have obtained evidence that after illumination intervals as short as 100 ms, significant ATP synthesis continues in the

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dark and that imidazole, as a permeant buffer, inhibits this pre-steady state post-illumination ATP synthesis but not initial light-driven ATP synthesis.

**EXPERIMENTAL PROCEDURES**

Rapid-mix, acid-quench experiments were carried out using the apparatus described by Froehlich et al. (7). Mixers, illumination tubing, and illumination have been described (8). In the following experiments, which measured initial rates of ATP synthesis, chloroplasts were kept in darkness until the instant of mixing by using a stainless steel tube to carry the sample from the syringe block to the first mixer. Once in the first mixer, the sample path leading to the mixing T-junction was stainless steel tubing set in the plexiglass mixer. Using this arrangement, the chloroplasts were illuminated just as they were mixed with ADF and P. Illumination continued as the sample flowed through clear Teflon tubing, and the reaction was ended under illumination as the sample mixed with perchloric acid in a clear plexiglass mixer. Reaction time was controlled by varying the lengths of the clear tubing, and in this protocol the illumination time and the total reaction time were the same. To simulate a short light flash and the following period of darkness before perchloric acid addition, the clear illumination tubing was connected to stainless steel tubing through which the sample flowed in darkness until the reaction was quenched by mixing with perchloric acid in a dark mixer. In this protocol, illumination time was controlled by the length of clear Teflon tubing, and the time in darkness was controlled by the length of stainless steel tubing. Chloroplasts were isolated from fresh market spinach and were washed in a hypotonic medium to deplete them of residual potassium according to Ort and Dilly (6). Chloroplasts were preincubated in STNM (0.2 M sucrose, 20 mM Tricine·NaOH, pH 8, 10 mM NaCl, 2 mM MgCl2) containing permeant buffers for 1 h at 0°C in darkness. Before rapid mixing, samples were diluted 10-fold in the same medium containing either 1 mM methyl viologen or 50 μM PMS, 1 mM ascorbate with or without 10 mM KCl at 18°C, and valinomycin in ethanol was added to 0.65 μM, 0.5% ethanol. Samples were rapidly mixed with an identical medium which was supplemented with 2 mM ADP and 2 mM NaF; pH 8 (10,000 cpm of 32P, nmol). Reactions were quenched by mixing with 1 M perchloric acid containing 50 mM NaH2PO4. Formation of labeled ATP and ADP was followed by removing 10 μl of sample to end the reaction. We found that in the presence of PMS and ascorbate. Photophosphorylation at pH 8 in the presence of either 10 mM bicarbonate or 2 mM imidazole showed that these two buffers did not extend the valinomycin-KCl lag (Fig. 3) even at increased concentrations of valinomycin and KCl (Fig. 3, inset). Imidazole did decrease the initial rate of ATP synthesis, but because imidazole also decreased the rate of steady state photophosphorylation (Fig. 4), this decrease was not considered as evidence that imidazole delayed initial proton gradient formation. However, the lag was not dependent on osmolarity and hence upon the osmolarity of the medium: If the internal volume were enlarged, the amount of permeant buffers inside would be increased, and any lag in building up the proton gradient would be exaggerated. It was previously shown by Rottenberg et al. (10) and Ort et al. (3) that the chloroplast thylakoids behave as osmotically sensitive species. Fig. 5 shows the results of one of our experiments in which the osmolarities of the 0.2 and 0.01 M sucrose reaction mixtures differed by nearly 10-fold, and yet the initial lags in the presence or absence of imidazole were the same. Moreover, if the 0.01 M sucrose curve was normalized to the 0.2 M curve, the two imidazole curves could be superimposed. Lags in initial rates in the presence or absence of imidazole were not dependent upon osmolarity and hence upon the internal volume of the thylakoids.

To reconcile our results with those of Vinkler et al. (4) and Davenport and McCarty (5), we set up the rapid-mix, acid-quench machine to simulate a single short light flash followed by a defined period of darkness before perchloric acid was added to end the reaction. We found that in the presence of valinomycin and KCl, there was significant post-illumination

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1 The abbreviations used are: Tricine, N-tris(hydroxymethyl)methylglycine; PMS, phenazine methosulphate; CP, coupling factor 1 of chloroplasts; CP1, hydrophobic intramembrane segment of the ATP synthetase of chloroplasts.
Initial ATP Synthesis by Chloroplasts

**Fig. 2.** Photophosphorylation in the presence of PMS/ascorbate, valinomycin, and KCl. Without preincubation, chloroplasts were suspended at 0.11 mg of chlorophyll ml\(^{-1}\) in STNM containing 50 \(\mu\)M PMS, 1 mM sodium ascorbate, and with (○) or without (□) 10 mM KCl, and valinomycin in ethanol was added. The PMS/ascorbate control rate was 9.18 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) of chlorophyll.

**Fig. 3.** Photophosphorylation in the presence of PMS/ascorbate, valinomycin, and KCl. Prior to rapid mixing, chloroplasts were preincubated at 1.02 mg of chlorophyll ml\(^{-1}\) in STNM or STNM containing 10 mM KCl, 10 mM NaHCO\(_3\)-HCl, pH 8; or 100 mM KCl, 2 mM imidazole-HCl, pH 8. The PMS/ascorbate control rates in the presence of imidazole, NaHCO\(_3\), and no permeant buffer were 0.88, 3.85, and 4.72 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) of chlorophyll, respectively.

**Fig. 4.** Imidazole inhibition of steady state photophosphorylation with methyl viologen and PMS/ascorbate. Without preincubation, chloroplasts were suspended at 0.20 mg of chlorophyll ml\(^{-1}\) in 0.2 M Tricine-NaOH, pH 8.0, 2 mM MgCl\(_2\), and aliquots were taken for assay of steady state photophosphorylation using either 1 mM methyl viologen or 50 \(\mu\)M PMS, 1 mM sodium ascorbate at the indicated concentrations of imidazole-HCl, pH 8, the maximum rates of which were 0.57 and 7.05 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) of chlorophyll, respectively.

**Fig. 5.** Internal volume effects on PMS/ascorbate photophosphorylation with valinomycin, KCl, and imidazole. Chloroplasts were preincubated at 0.92 mg of chlorophyll ml\(^{-1}\) in 20 mM Tricine-NaOH, pH 8.0, 5 mM MgCl\(_2\), containing either 0.2 or 0.01 M sucrose and either with or without 2 mM imidazole-HCl, pH 8. Prior to rapid mixing, chloroplasts were diluted 10-fold in the same medium containing 10 mM KCl, 50 \(\mu\)M PMS, 1 mM sodium ascorbate, and valinomycin in ethanol was added. PMS/ascorbate control rates without imidazole were 4.01 and 3.95 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) of chlorophyll for 0.2 and 0.01 M sucrose, respectively.

**Fig. 6.** Photophosphorylation after illumination for 100 ms. In fact, after a subsequent 100 ms of darkness, the amount of ATP more than doubled. The addition of imidazole inhibited photophosphorylation as we have shown and also decreased...
In short flash experiments, illumination time is precisely more accurately interpret the results of those earlier studies. With the results presented here, we believe that we can now well within the limits of resolution of our technique. This delay in initial ATP synthesis was as pronounced. Without imidazole, post-illumination synthesis continued for 180 ms, while in the presence of imidazole, post-illumination synthesis stopped after 110 ms. In the absence of imidazole, 190 ms of light yielded approximately 10 nmol of ATP mg\(^{-1}\) of chlorophyll in post-illumination synthesis, an increase from the 4.5 nmol after 110 ms of illumination. In the presence of imidazole, 110 and 190 ms of light both yielded the same amount of post-illumination synthesis (3 nmol of ATP mg\(^{-1}\) of chlorophyll).

**DISCUSSION**

In photophosphorylation catalyzed by either methyl viologen or PMS/ascorbate, the presence of imidazole did not increase the lag in initial ATP synthesis over that caused by valinomycin and KCl alone (Figs. 1 and 3). Moreover, if the osmolarity of the medium was decreased (which has the effect of increasing the internal volume of the thylakoids), the presence of imidazole did not extend the valinomycin-KCl lag (Fig. 5). Assuming an internal volume of 30 \(\mu\)g of chlorophyll (3), equal internal and external concentrations of imidazole, and a proton internalization rate of 2000 \(\mu\)mol mg\(^{-1}\) of chlorophyll h\(^{-1}\) (3), 2 mM imidazole should have delayed the initiation of ATP synthesis for 117 ms. This was well within the limits of resolution of our technique. This proton internalization rate is very close to the rate calculated from the steady state rates observed in our controls using the value of 3 H\(^+\) for every ATP (11).

Studies which have been interpreted to indicate the presence of initial lags in ATP synthesis due to permeant buffers (4, 5) have used short flash photophosphorylation techniques. With the results presented here, we believe that we can now more accurately interpret the results of those earlier studies. In short flash experiments, illumination time is precisely controlled by an electronic shutter, but the termination of the reaction is carried out by manual injection of 1–2 ml of acid from a 3-ml syringe into the 1-ml sample. Although the injection is begun less than 100 ms after the flash (4), the addition is not instantaneous, nor is the mixing of acid with the sample. The time span required to terminate the chemical transformations has never been determined in those experiments. However, even if it takes as little as 100 ms, which is unlikely, significant post-illumination ATP synthesis can take place in that time, as Fig. 6, a and b, has shown. Thus, the ATP synthesized under a short flash experiment is the sum of the ATP made in the light plus the ATP made in darkness before the added perchloric acid can completely quench the reaction. Any buffer, such as imidazole, which inhibits post-illumination ATP synthesis would lower the yield of ATP synthesized under such an experimental protocol, and this could be interpreted as a lag. The rapid quench technique does not share this weakness in that the continuous flow of reactants makes it possible to control not only illumination time but also substrate addition and acid termination with great precision. Using this technique, imidazole appears to slow the development of the capacity for pre-steady state post-illumination ATP synthesis and not to affect initial light-driven ATP synthesis.

Both Ort et al. (3) and Vinkler et al. (4) have shown that imidazole and pyridine slow the development of the capacity for post-illumination ATP synthesis while increasing the ultimate capacity if illumination is long enough (this concept is illustrated by Fig. 7). This is consistent with the results presented here, although the instability of methyl viologen-mediated photophosphorylation and the inhibitory effects of imidazole upon steady state photophosphorylation call for further work to clarify this issue. However, if we assume that the effect of imidazole on the development of the capacity for post-illumination ATP synthesis is due to its action as a permeant buffer, an interesting suggestion can be made. ATP
The buffering capacity of the permeant buffer would be saturated, and the internal pH would drop. The chloroplasts would now become competent for post-illumination synthesis, and the extra protons stored by the permeant buffer would serve to increase the final post-illumination yield of ATP as observed by Avron (3) and Nelson et al. (1). Thus, a permeant buffer could have little effect on initial rates of ATP synthesis during illumination and yet could exert major effects on post-illumination yield of ATP.

If every proton must first pass into the inner thylakoid space before being utilized for ATP synthesis by coupling factors, as required by the chemiosmotic coupling hypothesis (Fig. 7), permeant buffers should affect the availability of protons generated either by methyl viologen or by PMS in the same manner. If permeant buffers do cause lags in initial ATP synthesis, the lags should be present in either cyclic or noncyclic photophosphorylation. Since we have found that imidazole does not appear to extend the valinomycin-KCl lag in initial ATP synthesis, we therefore must concur with Ort et al. (3) that initial ATP synthesis and possible steady state as well may be driven by a pool of protons distinct from the transmembrane gradient which powers post-illumination synthesis.

When properly considered, this interpretation emphasizes the element of time in the overall process of proton utilization for ATP synthesis. According to this concept, a given "mobilized" proton does not have to obligatorily migrate into the lumen of the membrane vesicle before it can be utilized by the machinery of the coupling factor. However, there exists a significant probability for a given proton to leave the intramembrane domain and enter the lumen space before it can find its way back to the CF1-CF0 complex. The establishment of a transmembrane pH gradient is thus viewed as a consequence of the relative conductance of intramembrane protons through the CF, and to the lumen. Because of the reversibility of the overall process, some of the protons that have accumulated in the thylakoid lumen can and eventually will travel through the CF1 to generate ATP.

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