EFFECT OF PHOSPHORYLATION ON THE SIZE OF THE PROTON GRADIENT ACROSS CHLOROPLAST MEMBRANES

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1. Introduction

The coupling between photophosphorylation and proton uptake in chloroplasts [1, 2] requires that ATP formation should create a drain on the steady state electrochemical potential formed during electron transport. In isolated chloroplasts most of this potential seems to exist in the form of a pH gradient, and therefore, a decrease in the size of this gradient is to be expected when phosphorylation is initiated. Several methods for measuring the ΔpH formed in the light across the thylakoid membrane were recently developed in this laboratory [3–5].

Using one of them, the fluorescence quenching of fluorescent amines in the light, we show here that phosphorylation or arsenolysis conditions indeed lead to a decreased steady state ΔpH in the light.

2. Methods

Chloroplasts from lettuce leaves were prepared essentially as previously described [6], except for the final centrifugation and resuspension which were in 0.2 M sucrose, 0.1 M KCl.

Fluorescence of 9-aminoacridine was measured in an Eppendorf fluorimeter as previously described [4].

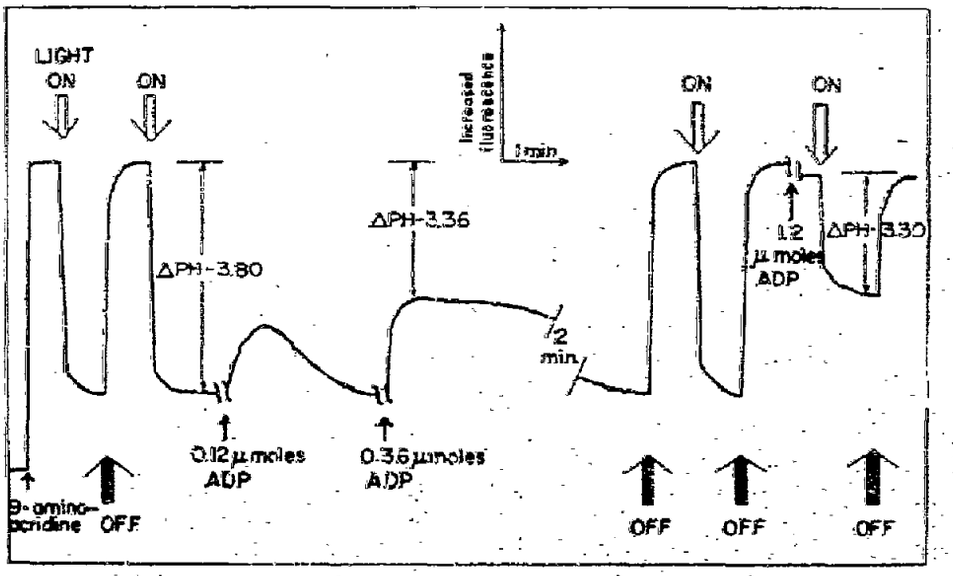

Fig. 1. Decrease in the light induced fluorescence quenching of 9-aminoacridine under phosphorylation conditions. The reaction mixture contained in a total volume of 3.0 ml: Tricine-glycine-NaOH, pH 8.25, 50 mM; KCl, 50 mM; pyocyanine, 5 μM; $MgCl_2$, 5 mM; phosphate, 2.3 mM; 9-aminoacridine, 1 μM; and chloroplasts containing 45 μg of chlorophyll.

Table 1
Phosphorylation components needed for the decrease in ΔpH.

Experiment No.	Component absent	Component added	ΔpH measured
I	$MgCl_2$	None	3.72
		5 mM $MgCl_2$	3.50
		5 mM $CaCl_2$	3.68
II	Arsenate	None	3.74
		5 mM Arsenate	3.24
		2.3 mM Phosphate	3.23
III	ADP	None	3.81
		0.4 mM ADP	3.41
		0.4 mM ATP	3.76

The complete system contained in a total volume of 3.0 ml Tricine-glycine-NaOH, pH 8.0, 50 mM; KCl, 50 mM; $MgCl_2$, 5 mM; arsenate, pH 8.0, 5 mM; ADP, 0.4 mM; pyocyanine, 5 μM; and chloroplasts containing 41 μg of chlorophyll.

Measurements and calculations of the osmotic volumes of chloroplast fragments from the distribution of [^{14}C]Sorbitol and [^{3}H]H_2O were done as described [3]. ΔpH values were determined from the osmotic volumes and fluorescence quenching values according to Schuldiner et al. [4].

3. Results and discussion

A typical experiment demonstrating the effect of phosphorylation conditions on the steady state ΔpH formed in the light is shown in fig. 1. The reversible light induced fluorescence quenching of 9-aminoacridine was markedly reduced after the addition of ADP to an otherwise complete phosphorylation system. When low concentrations of ADP were added in the light, a smaller steady state quenching ratio was formed within 15–30 sec which in the light slowly returned to its original level, indicating the conversion of all the ADP to ATP. The higher the amount of ADP added, the longer it took to return to the high quenching in the light. When the rate of photophosphorylation was estimated from the time necessary to consume the ADP added, values comparable to those estimated by other methods were obtained.

Determination of the osmotic spaces of chloroplasts did not reveal any significant difference in the presence or absence of ADP. These results rule out shrinkage of the thylakoids as the cause for the decreased fluorescence quenching ratios under phosphorylation conditions. In a typical experiment, in the absence and presence of ADP, osmotic spaces of 12.1

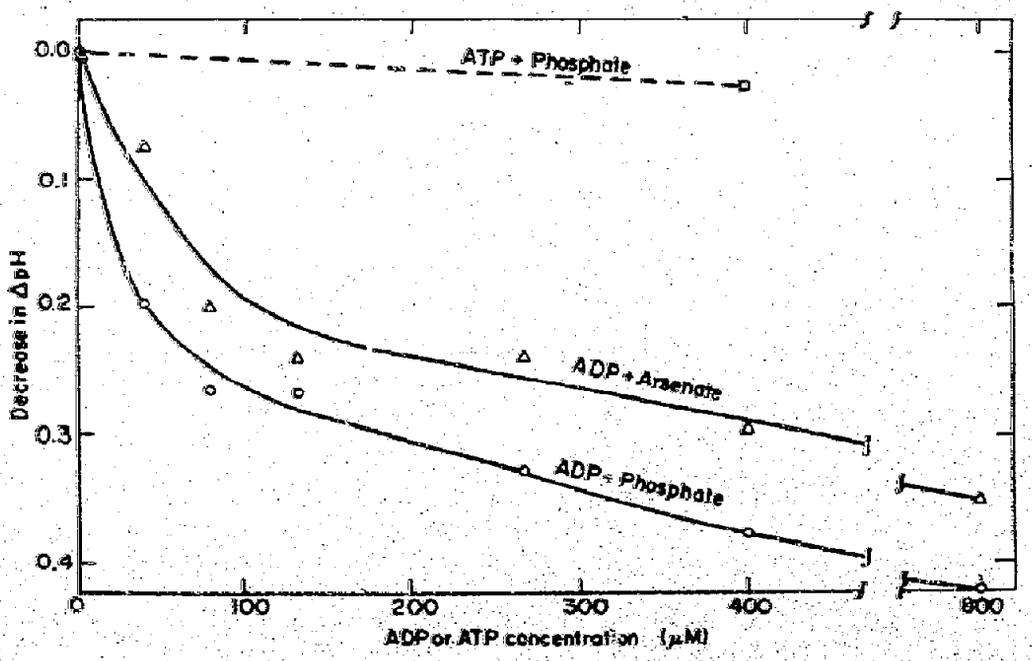

Fig. 2. ADP concentration dependence of the decrease in ΔPH with phosphate or arsenate. The procedure was similar to that illustrated in fig. 1. The decrease in ΔpH was derived from the two quenching ratios. The ΔpH with no ADP added was 3.7 units, and the osmotic water 15 μl × mg chlorophyll^{-1}. The reaction mixture contained in a total volume of 3.0 ml: Tricine-glycine-NaOH, pH 8.0, 50 mM; NaCl, 20 mM; $MgCl_2$, 5 mM; pyocyanine, 5 μM; phosphate or arsenate, 5 mM; 9-aminoacridine, 2 μM; ADP or ATP at the indicated concentrations, and chloroplasts containing 40 μg chlorophyll.

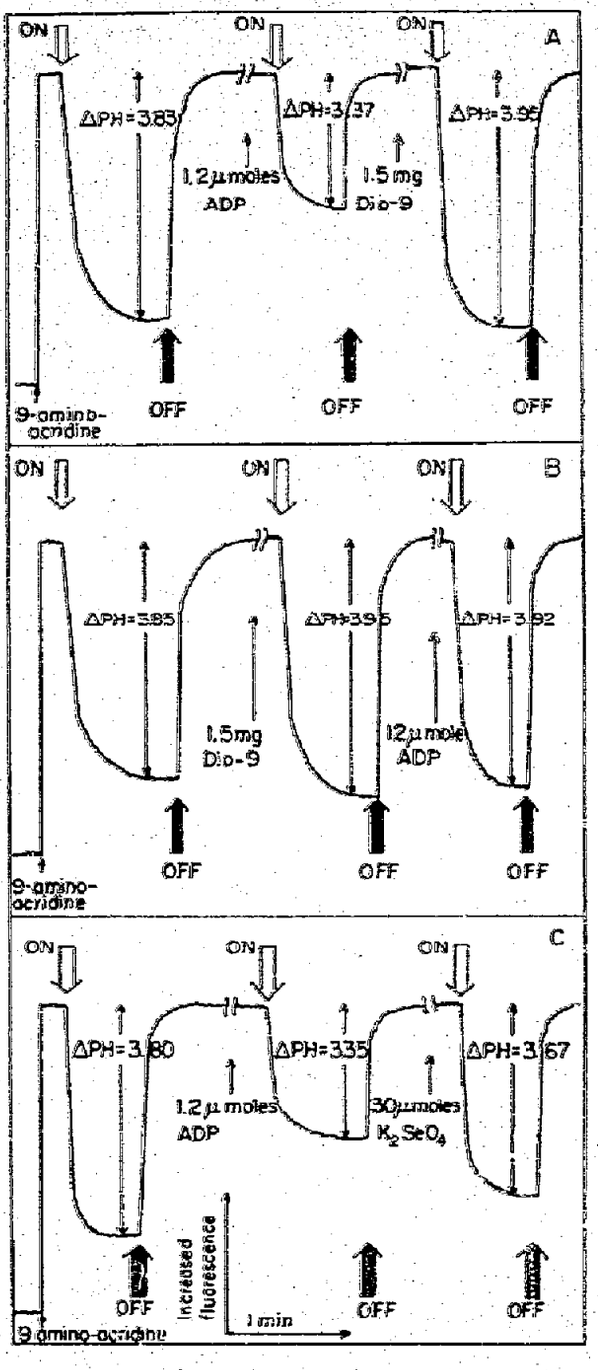

Fig. 3. Effect of energy transfer inhibitors on the decrease in ΔpH due to phosphorylation. The reaction mixture contained in a total volume of 3.0 ml: Tricine-glycine-NaOH, pH 8.65, 50 mM; KCl, 20 mM; $MgCl_2$, 5 mM; arsenate 1 mM; pyocyanine, 5 μM; 9-aminoacridine, 1 μM and chloroplasts containing 30 μg of chlorophyll. Where indicated, ADP, 0.4 mM; Dio-9, 0.5 mg/ml; or K_2SeO_4, 10 mM, were added.

and 12.8 μl H_2O × mg chlorophyll^{-1}, and ΔpH values of 3.54 and 3.12 were determined, respectively.

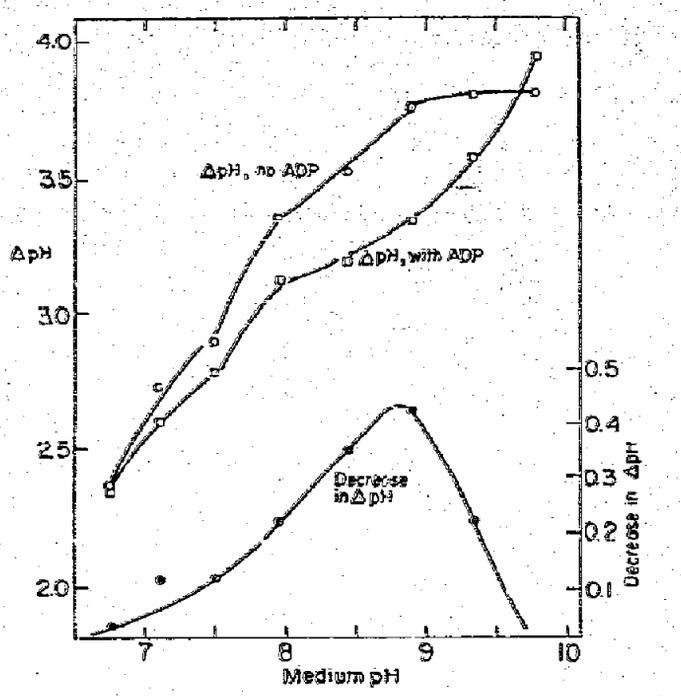

Fig. 4. pH dependence of the decrease in ΔpH under phosphorylation conditions. The reaction mixture contained, in a total volume of 3.0 ml: tricine-maleate-NaOH, (at the indicated pH between 6.85–8.0), or tricine-glycine-NaOH (pH 8.45–9.8), 50 mM; KCl, 50 mM; $K_3Fe(CN)_6$, 0.1 mM; $MgCl_2$, 5 mM; phosphate, 2.3 mM; 9-aminoacridine 1–2 μM; and chloroplasts containing 36 or 72 μg of chlorophyll. Where indicated, ADP, 0.4 mM, was added.

Table 1 shows that all the components needed for photophosphorylation (or arsenolysis), were also needed to obtain the decrease in fluorescence quenching in the light. Either phosphate or arsenate were needed, with a half maximal effect observed around 1 mM and maximal effect beyond 10 mM (not shown). ADP could not be replaced by ATP, and Mg^{2+} could not be replaced by Ca^{2+}. The Mg^{2+} effect was the smallest, probably due to the endogenous Mg^{2+} in the preparation.

Fig. 2 demonstrates the dependence of the decrease in ΔpH on the concentration of ADP. A half-maximal effect was observed around 50 or 80 μM ADP with phosphate or arsenate, respectively. ADP concentrations beyond 1 mM had to be avoided since at these concentrations ADP by itself caused some quenching of the fluorescence of 9-aminoacridine. These results correlate well with the dependence of photophosphorylation on ADP concentration [7]. ATP did not lead to a similar decrease in ΔpH under the same conditions.

Further evidence relating the decrease in ΔpH to photophosphorylation was obtained by testing the effect of energy transfer inhibitors. Fig. 3 shows that Dio-9 [8], when added after ADP, completely restored the high fluorescence quenching obtained in the absence of ADP, and even increased the ΔpH a little above its original value in the light [9]. Also, addition of ADP after Dio-9 had no effect on the increased ΔpH. Selenate, recently found to act as an energy transfer inhibitor of photophosphorylation [10], led to a similar effect when present in excess over arsenate (probably due to competition between them).

The pH dependence of the decrease in ΔpH under phosphorylation conditions is shown in fig. 4. The effect was obvious only within the pH range of 7.5–9.5 in either noncyclic ferricyanide dependent photophosphorylation or with cyclic photophosphorylation with pyocyanine (not shown). Maximal effect was observed between pH 8.5–9.0, similar to the dependence of photophosphorylation on medium pH [11].

In conclusion: i) Photophosphorylation causes a significant decrease in the ΔpH formed in the light across the thylakoid membrane under steady state conditions. This observation is in agreement with the suggestion that phosphorylation accelerates the efflux of the protons pumped in. ii) Arsenate can replace phosphate, in this effect. iii) Dio-9 prevents this effect of phosphorylation, and reverses the effect if added after the phosphorylation reagents.

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