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DIRECT DETERMINATION OF ΔpH IN CHLOROPLASTS, AND ITS RELATION TO THE MECHANISMS OF PHOTOINDUCED REACTIONS

Hagai ROTTENBERG, Tilly GRUNWALD* and Mordhay AVRON

Department of Biochemsitry, Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

The light-induced pH rise in chloroplast suspensions is generally interpreted as an acidification of the thylakoid compartment [1,2]. However, a quantitative deduction of the internal pH from the H^+ uptake is not possible due to insufficient data about the compartment size and, particularly, its buffer capacity.

We have developed a method for determining the ΔpH across the thylakoid membrane which is based on the uptake of methylamine by the chloroplasts. This method is similar, in principle, to the DMO technique employed in mitochondria [3]. However, in chloroplasts the DMO technique cannot be applied, since upon acidification DMO would be expected to move out of the thylakoid space, in contrast to amines, which should be taken up. It has been demonstrated [4,5] that the uptake of amines is driven by the pH gradient, since they penetrate in their neutral form. When equilibrium is reached the following relation holds:

$$[RNH_3^+]_{in} / [RNH_3^+]_{out} = [H^+]_{in} / [H^+]_{out}$$

Thus, a determination of the concentration ratio of methylamine inside to outside the thylakoid space provides a value identical to that of the proton concentration ratio.

* On leave from the B.C.P. Jansen Institute for Biochemistry, Amsterdam, Holland.

2. Methods and results

Chloroplasts from lettuce leaves (Lactuca sativa) were prepared by homogenizing 40 g of leaves in a blender in 150 ml of a medium containing 300 mM NaCl, 20 mM Na-ascorbate and 50 mM tris-Cl, pH 7.8. After filtration through cheese-cloth, the cell debris and other large particles were discarded through sedimentation at 200 *g* for 90 sec. The chloroplast fraction was collected by sedimentation at 1400 *g* for 7 min and washed once in the reaction medium.

To a chloroplast suspension of about 400 μg chlorophyll/ml the proper tracers were added: ^3H_2O for a water content determination and either ^{14}C-sorbitol (sorbitol concentration 5 mM) for the determination of the pellet non-osmotic water fraction, or ^{14}C-methylamine (methylamine concentration 10–20 μM). Small (0.4 ml) polyethylene microfuge tubes were filled with this suspension, at least in triplicate for each determination, and put into position in a Beckman 152 microfuge. When illuminated, a reflector flood lamp of 300 W was placed directly above the centrifuge with a water heat filter in between (maximum light intensity, 65,000 Lux). The samples were incubated for one minute and then centrifuged for 3 min (when illuminated, the illumination continued also throughout the centrifugation). 50 μl of the supernatant were taken, and a slice from the bottom of the pellet was cut with the plastic (containing about 1/3 of the pellet). Each was mixed with 2 ml acetone–water (80:20 v/v), and the pellet samples were vigorously shaken till the pellet was thoroughly suspended. The precipitated proteins (and the plastic tube ring) were sedimented in a clinical centrifuge,

Table 1
The effect of light and uncouplers on ΔpH

Conditions	Total pellet water (μl/mg chlorophyll)	Pellet sorbitol space	(^{14}C-methylamine)pellet / (^{14}C-methylamine)medium	(^{14}C-methylamine)osmotic space / (^{14}C-methylamine)medium	ΔpH
Dark	113	0.86	1.28	3	0.48
Light	74	0.79	12.4	54	1.73
+ Methylamine (100 μM)	83	0.80	11.5	53	1.72
+ Methylamine (5 mM)	112	0.75	5.08	17	1.23
+ Gramicidin (10 μM)	104	0.83	1.95	7	0.85
+ X-464 (1 mM)	150	0.83	1.25	2.5	0.40

The basic reaction mixture contained 100 mM KCl, 25 mM Na-tricine (pH 6.9), 25 μM pyocyanine, 5 mM sorbitol and 400 μg chlorophyll/ml; in addition the appropriate tracers were added (2 μg/ml of ^3H_2O and either 1 μg/ml of ^{14}C-methylamine or 2 μg/ml of ^{14}C-sorbitol). For other details see text.

Table 2
The dependence of the light-induced ΔpH on the external pH.

External pH	Pellet sorbitol space	(^{14}C-methylamine)pellet / (^{14}C-methylamine)medium	ΔpH
6.0	0.77	1.6	0.57
7.0	0.89	4.7	1.54
8.0	0.84	21.4	2.11
9.0	0.93	14.9	2.30
9.7	0.92	1.1	0.40

The basic medium was the same as described in table 1. Before the experiment aliquots of the same chloroplast suspension were adjusted to the indicated pHs with NaOH or HCl. For further details see text.

0.5 ml of phtalate buffer (pH 5.5, 50 mM) were added to each sample and the chlorophyll was extracted into 5 ml diethylether. The amount of chlorophyll in the pellet sample was determined spectroscopically from the extinction at 643 nm, using 32.5 as the millimolar extinction coefficient. A sample of the aqueous phase, 0.2 ml, was added to 10 ml Bray's solution and the ^{3}H- and ^{14}C-activities were simultaneously counted. Pellet water content per mg chlorophyll was calculated from the ^{3}H content of the pellet and that of the medium. Sorbitol permeable space in the pellet was calculated from the ratio of ^{14}C-sorbitol/^3H_2O in the pellet over that in the medium.

Methylamine distribution was similarly determined from ^{14}C-methylamine/^3H_2O in pellet and medium. A correction of this value was made, based upon the assumption that methylamine is concentrated only in the osmotic space, i.e. the non-sorbitol space.

Table 1 shows that methylamine is more concentrated in the pellet even in the dark, corresponding to a ΔpH of about 0.5. However, upon illumination there was a very large increase in the internal methylamine concentration to give a ΔpH of about 1.7. This ΔpH was not affected by the low concentration of methylamine employed, and thus the use of this compound in very small concentration does not affect the true

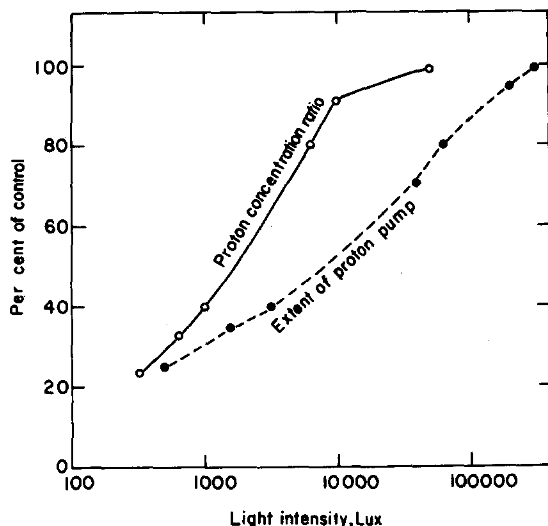

Fig. 1. The dependence of the proton concentration ratio and the extent of the proton pump on light intensity. The reaction mixture for the proton concentration ratio determination was as described under table 1. For the determination of the extent of the proton pump chlorophyll concentration was reduced to 75 μg chlorophyll/ml and the Na-tricine concentration to 0.5 mM (pH 6.9); the reaction mixture contained, in addition, 100 mM KCl and 25 μM pyocyanine. The extent of the proton pump was determined with a glass electrode. White light was used in both experiments. Both parameters are given as percentage of the maximum value, at full light intensity; 100% proton concentration ratio was 48 (ΔpH = 1.68) and 100% proton pump was 0.35 μmoles/mg chlorophyll.

ΔpH; at higher concentrations, of course, it exhibited its uncoupling effect. A much stronger effect on the ΔpH was seen with the nigericin-like antibiotic X-464 or gramicidin, which affected ΔpH at moderate concentrations.

Table 2 shows the dependence of the ΔpH on the medium pH. It is clear that a strong dependence exists. The highest ΔpH was observed around pH 8 to 9. This pH-optimum corresponds to the pH-optimum of the rate of electron transport and phosphorylation, but not to that of the proton pump [6].

Fig. 1 shows the dependence of both the proton concentration ratio and the extent of proton pump on light intensity at pH 6.9; it is observed that the proton gradient is built up and saturated at a lower light intensity than the extent of the proton pump.

3. Discussion

The finding that there is a light-dependent ΔpH increase in chloroplasts is, of course, not surprising. However, its quantitative estimation under various conditions gives an opportunity to relate this phenomenon to other measurable parameters, and to evaluate existing models of photophosphorylation.

The difference in pH-optimum as well as light intensity dependence of the proton pump is not a measure of the pH gradient. Indeed, if one compares the amount of protons taken into the intrathylakoid space (from the proton pump experiments) with the amount of free protons actually present there (from the ^{14}C-methylamine distribution and the non-sorbitol space), it is evident that at any given external pH most of the protons that enter the thylakoid must be buffered (more that 99.9%). Thus the extent of the proton pump must be basically a measure of the buffering capacity. According to this interpretation, the pH- and light intensity dependence of the proton pump and the ΔpH, all indicate a strong buffering region with an apparent pK of about 5.5.

It seems reasonable to assume that the coincidence in the dependence of phosphorylation, electron transport and ΔpH on the external pH is actually reflecting a strong dependence of the rate of electron transport on the *internal* pH. On the basis of this assumption, relating the electron transport rate to the internal pH, there seems to be a rather sharp optimum at about pH 6.0 (internally), with a steep drop in rate at low pH and a moderate decrease at higher pH.

With this hypothesis one is able to explain a number of hitherto not clearly understood phenomena: (i) It is generally observed that uncouplers, uncoupling conditions, damaging of chloroplasts etc., cause a shift of the electron transport pH optimum to a lower value [1,6–9]. This is predicted by the hypothesis simply as a reflection of the decrease in the light-produced ΔpH: when the ΔpH is reduced, the optimum of the curve (measured at the outside pH) will shift to a lower pH. (ii) The inhibition of electron transport by high uncoupler concentrations [10] can also be logically predicted. Thus, at neutral pH, a low concentration of an uncoupler stimulates electron transport, not only by its uncoupling action, but also by shifting the internal pH towards the optimum (say from 5.5 to 6). As the concentration of the uncoupler

is increased, the ΔpH is further decreased and the internal pH rises above the optimum so that inhibition of electron transport occurs. It follows that at very high pH (> 8.5), since the internal pH is initially above the optimum, uncouplers should only inhibit electron transport and this indeed was recently observed in several cases (9). (iii) The existence of an internal pH optimum for electron transport could also explain the difference in the initial kinetics of electron transport upon illumination at low and at high pH [11]. At low pH initially the internal pH is optimal, so that electron transport rate is fast, but once the ΔpH develops both the energy load and the lowering of the internal pH would tend to decrease the steady state electron transport rate. At high external pH, conversely, the internal pH is initially higher than the optimum, but as the ΔpH is built up, the effect of the energy load (tending to lower the rate) is compensated by the decrease in internal pH towards the optimal value; thus the steady state rate remains as high as the initial rate.

The large ΔpH observed at high external pH (between 8 and 9) coincides with the pH optimum of phosphorylation and is in agreement with those models that consider the ΔpH to be an important factor in energy conservation during photophosphorylation.

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