Kinetics of dark proton efflux in chloroplasts

Mordechay Schönfeld and Bente Sloth Kopeliovitch

The Hebrew University of Jerusalem, Department of Agricultural Botany, PO Box 12, Rehovot, Israel

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The rate constant for dark proton efflux from preilluminated chloroplasts, was found to be proportional to the proton permeability, and inversely proportional to the 'proton binding capacity' of the thylakoids (the ratio between bound and free protons). Dark proton efflux was resolved into two kinetically distinct components: a diffusion-mediated process which was predominant below pH 7, and a separate leakage pathway, characterized by linear dependence on the internal to external proton concentration ratio, which was predominant above pH 7. An increased proton-binding capacity accompanied the increased proton permeability associated with this second pathway.

(Lettuce chloroplast) *Thylakoid membrane* *Proton efflux* *Proton permeability* *Proton binding* *H^+-ATPase*

1. INTRODUCTION

It is now well established, as a result of extensive studies in the last 2½ decades, that ATP synthesis in chloroplasts is coupled to proton efflux driven by a light-induced proton gradient [1]. However, the mechanisms of proton efflux and its coupling to ATP synthesis have not yet been elucidated. The purpose of this study was to investigate further the process of proton efflux from thylakoids.

During steady-state illumination, the efflux of protons equals their influx rate, so neither one can be measured. With that, this cyclic proton flux can be monitored indirectly, via measurements of electron transport which is stoichiometrically coupled to it [1]. Alternatively, one can monitor directly 'dark' proton efflux, on cessation of illumination.

A previous study in our laboratory [2] was based on measurements in the light of steady-state electron transport and $[H^+]_i$. It indicated that proton efflux from illuminated thylakoids could be resolved into 3 kinetically distinct components: efflux associated with the chloroplast H^+-ATPase; a special leakage component characterized by a linear dependence on $[H^+]_i/[H^+]_o$; and diffusion-mediated leakage.

Here, we used the alternative approach for monitoring proton efflux: we examined the dark decay of the proton gradient by direct measurements of pH changes inside and outside the thylakoids. The 2 proton-leakage pathways observed in the light were also evident in the dark and values obtained in the dark for P_H, the proton-permeability, were similar to those obtained in the light [2]. On the other hand, proton efflux, typically associated with the H^+-ATPase in the light, was not evident in the dark.

Abbreviations: J_H, initial rate of proton efflux; P_H, proton permeability of the thylakoid membrane; C_H, proton-binding capacity of thylakoids; H_x, amount of protons accumulated by thylakoids; $[H^+]_i$, $[H^+]_o$, internal and external proton concentrations; K_D, rate constant for dark proton efflux

2. MATERIALS AND METHODS

Chloroplasts were prepared from lettuce leaves and photoreactions were assayed essentially as in [2,3]. Measurements of $[H^+]_i$ through changes in fluorescence of 9-aminoacridine were carried out with a Jasco FP-550 spectrofluorometer, according to Schuldiner et al. [4]. External pH changes were simultaneously measured with a glass

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combination electrode in the same magnetically stirred cuvette. The pH changes were calibrated by addition of aliquots of standard HCl solutions. Actinic illumination from a projector lamp, filtered through a heat filter and red cut-off filters ($\lambda > 600$ nm), provided 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the position of cuvette. The light intensity was attenuated with neutral density filters.

3. RESULTS AND DISCUSSION

J_H, the initial rate of proton efflux from chloroplast thylakoids, was measured in the dark after a steady-state proton gradient was established by illumination. Fig.1a illustrates the relationship between J_H, and the $[H^+]_i$ measured in parallel. Attenuating $[H^+]_i$ and J_H by the light intensity in the preillumination stage resulted in a linear relationship between the 2 parameters. The slope of the line was not affected by external pH changes between 6 and 7. The results indicate that in this range, dark proton efflux took place predominantly by simple diffusion, as observed for proton efflux in the light [2], and thus was given by $J_H = P_H([H^+]_i - [H^+]_o)$. As under the prevailing conditions $[H^+]_i \gg [H^+]_o$, this was reduced to

$$J_H = P_H[H^+]_i \quad (1)$$

The value of P_H, as calculated from the slope of the line in fig.1a according to eqn 1, was 2.6×10^{-5} cm/s. This was similar to values obtained for proton permeability in the light [2].

Proton efflux from preilluminated thylakoids decays with apparent first-order kinetics with time in the dark [5]. The rate of proton efflux, at any moment after turning the light off, was accordingly found to be proportional to H_x, the total amount of protons accumulated by the thylakoids at that time, (not shown). Therefore,

$$J_H = K_D H_x \quad (2)$$

where K_D is the first-order rate constant for proton efflux in the dark.

The experiments thus indicated on the one hand that J_H was linearly proportional to $[H^+]_i$, and on the other that it was proportional to H_x. Parallel measurements of H_x and $[H^+]_i$ carried out at different light intensities (fig.1b), indicated indeed (as

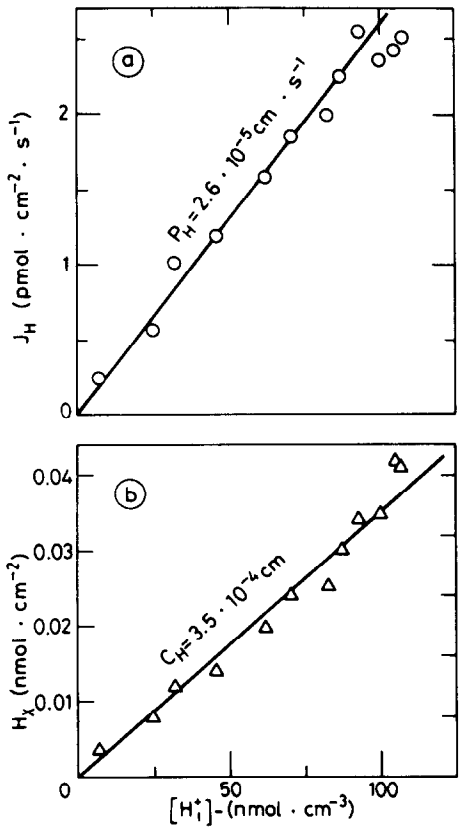


Fig.1. Dependence of J_H, the initial rate of proton efflux (a), and H_x, the extent of proton uptake (b), on $[H^+]_i$. The reaction mixture contained in a final volume of 3 ml: 50 mM KCl, 0.2 mM methyl viologen, 1 mM NaN_3, 2 μM 9-aminoacridine and chloroplasts equivalent to 24 μg/ml. The mixture was brought to pH 6.6 with HCl. A value of 2.2 nm^2 was assumed for the membrane area occupied by a chlorophyll molecule [7] to obtain the different parameters in the standard units used here.

reported [6]) that a constant ratio existed under these conditions between H_x and $[H^+]_i$. As more than 99% of the protons taken up are apparently bound by the thylakoids, this ratio, denoted here by C_H, is a measure for 'the proton binding capacity of the thylakoids'.

$$C_H = H_x/[H^+]_i \quad (3)$$

C_H was accordingly obtained from the slope of the line in fig.1b.

A combination of eqns 1-3 yielded a simple relationship between K_D, C_H and P_H.

$$K_D = P_H/C_H \quad (4)$$

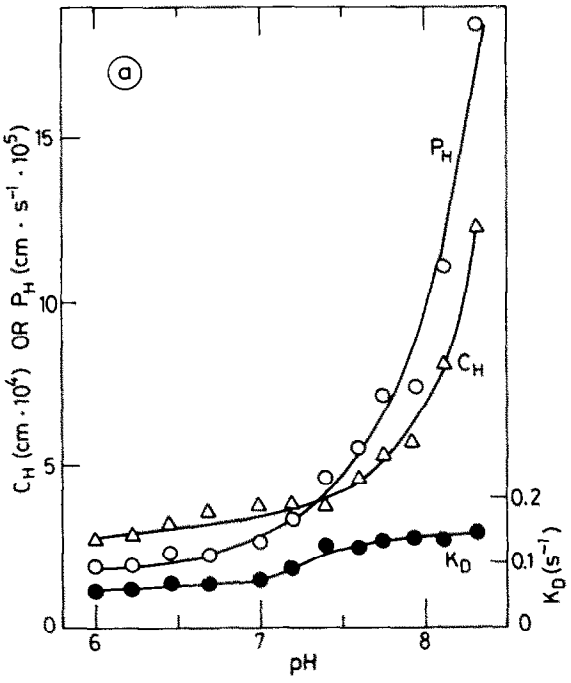
K_D, usually treated as a measure for proton permeability, is indeed seen to be proportional to P_H. It is however also inversely proportional to C_H, the proton binding capacity of the thylakoids. Changes in proton permeability would be directly reflected in K_D only if C_H was not affected. This description is formally similar to the discharging process in an electric RC circuit. The 'rate of discharge' in that case is proportional to the conductance of the pathway, and inversely proportional to the capacitance.

The experiments described so far were conducted below pH 7. More complex relationships were obtained above it. Fig.2a illustrates the effects of pH on K_D, P_H and C_H. P_H and C_H increased with pH in a largely parallel manner, while changes in K_D were relatively limited and even negligible in many experiments. As P_H proper should be independent of pH, its apparent increase above pH 7 actually indicated the presence of an additional leakage pathway. The data of the same experiment were used to plot the ratio of $J_H/[H^+]_i$ vs $[H^+]_o^{-1}$. This resulted in a straight line as shown in fig.2b. Dark proton efflux, for the whole pH range examined, was accordingly given by,

$$J_H = P_H[H^+]_i + k[H^+]_i/[H^+]_o \quad (5)$$

In addition to the diffusion-mediated leakage characterized here by its dependence on $[H^+]_i$, dark proton efflux evidently contained a second leakage pathway characterized by a linear dependence on $[H^+]_i/[H^+]_o$. This second component was negligible below pH 7 (i.e at high $[H^+]_o$), but became significant and eventually dominant, above pH 7, where the contribution of diffusion declined, with the declining $[H^+]_i$. Values for P_H proper can be obtained from the intercept at the ordinate in fig.2b, (or from eqn 1 for pH<7).

The results of fig.2a seem to indicate that significant proton binding in the thylakoids was involved in the second leakage pathway. A mechanism for this process, might involve a 'natural protonophore', which would bind protons inside the thylakoids and transport them outside. If a linear relationship was established between the concentration of the protonated form of such protonophore in-



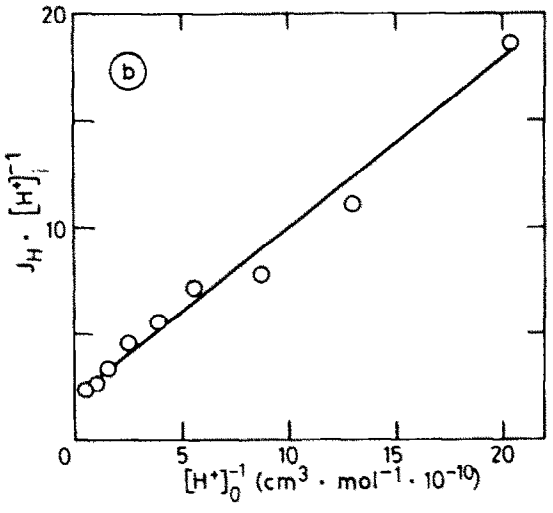


Fig.2. Effect of pH on dark proton efflux from preilluminated thylakoids. H_x, $[H^+]_i$, and K_D were measured at different pH values, and J_H, C_H and P_H were calculated using eqn 2,3 and 1, respectively, as described in the text. (a) Effect of pH on K_D, C_H and P_H. (b) Dependence of the ratio of $J_H/[H^+]_i$ on $[H^+]_o^{-1}$. Reaction conditions as in fig.1. The pH was adjusted with HCl or NaOH as required. The units in the ordinate are $cm \cdot s^{-1} \times 10^5$, i.e. the same as used for P_H.

side the thylakoids, and $[H^+]_i/[H^+]_o$, this would account for changes in C_H and P_H, such as those illustrated in fig.2.

The 2 components into which dark proton efflux was resolved here conform to 2 of the 3 components of proton efflux under steady-state illumination. These included the diffusion-mediated efflux [2], and the pathway characterized by a linear dependence on $[H^+]_i/[H^+]_o$. This second component was evident in the light above pH 7, in the presence of ATP [1,2]. The third 'light' component, characterized by an exponential dependence on the proton concentration ratio [1,2], involved proton efflux via the H^+-ATPase. In the light, this component was dominant above pH 7 during ATP synthesis, but also where ADP was omitted (unpublished). The absence of this component from the dark efflux seems to indicate that the dark decay of this light-activated efflux pathway is fast compared to the decay of the proton gradient.

Fig.3 illustrates the effects of nigericin on P_H, C_H and K_D. As expected, P_H increased with increasing concentration of the protonophore. On the other hand, the proton binding capacity of the thylakoids was not affected by nigericin under these conditions, and therefore the changes in P_H were also reflected as changes of equal extent in K_D. A similar pattern was observed with gramicidin, but not with ammonium chloride or alkyl amines which exhibited more complex effects (not shown). Changes in P_H were only partially reflected in K_D as they were offset to a varying extent by changes in C_H (see eqn 2). These results (and those of fig.2) indicate that changes in K_D should not be interpreted as changes in proton permeability, unless it is first established that proton binding capacity is not affected.

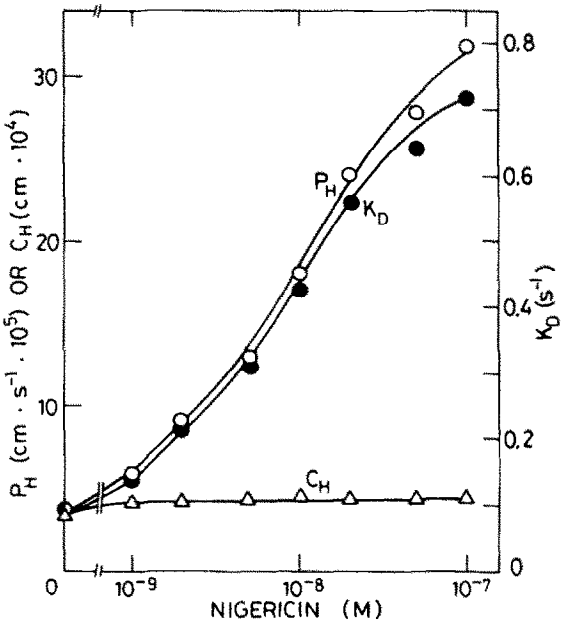


Fig.3. Effects of nigericin on K_D, C_H and P_H. Reaction conditions as in fig.1.

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