The permeability of the thylakoid membrane for protons

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Received 30 November 1983

Parallel measurements of light-induced proton gradients and of electron transport rates were used to characterize proton efflux in lettuce chloroplasts. Proton efflux mediated by the chloroplast H^+-ATPase was resolved from nonspecific leakage, through its dependence on the ratio of internal to external proton concentrations; proton leakage was proportional to the difference between these two concentrations. The proton permeability of the thylakoid membrane was found in these experiments to be 2×10^{-5} cm/s. In spite of this rather high permeability, proton leakage accounted for only ~1% of the total proton flux, due to the low internal proton concentration.

Proton permeability *Lettuce chloroplast* *Electron transport* *Proton gradient* *H^+-ATPase* *9-Aminoacridine*

1. INTRODUCTION

One of the basic postulates in the chemiosmotic hypothesis is a low permeability for protons of the 'coupling membrane' [1]. The experiments reported here were designed to measure the permeability for protons of the chloroplast thylakoid membrane and to assess its significance in the context of the energy conversion processes involved.

Light-induced electron transport in chloroplasts results in the establishment of a pH gradient across the thylakoid membranes [2,3]. Proton efflux from illuminated thylakoids occurs by 3 parallel pathways [4]: (i) transport facilitated by the CF-complex in a reaction coupled to ATP synthesis, (ii) noncoupled proton transport through the CF-complex, and (iii) nonspecific diffusion through the thylakoid membrane, which is proportional to the permeability of this membrane for protons. Under nonphosphorylating conditions, only the last two pathways operate, and the distinction between them is not trivial. It is usually assumed that the nonspecific leakage is given by the residual proton efflux, measured in the presence of ATP or some other CF-specific agent, which supposedly inhibits completely proton efflux facilitated by the CF-complex.

Diffusion-mediated proton efflux was resolved in this work from transport facilitated by the CF-complex on a kinetic basis. Permeability coefficients were derived for the diffusion mediated process by correlating steady-state proton fluxes and proton gradients. The permeability of the thylakoid membrane for protons was found to be $\sim 2 \times 10^{-5}$ cm/s.

Abbreviations: CF-complex, the chloroplast H^+-ATPase; J_{H^+}, proton flux; P_{H^+}, proton permeability; $[H_i^+]$ and $[H_o^+]$, internal and external proton concentrations; pH_i and pH_o, internal and external pH

2. MATERIALS AND METHODS

Chloroplasts were prepared from lettuce leaves and photoreactions were assayed essentially as in [5]. Measurements of ΔpH through fluorescence changes of 9-aminoacridine were carried out with a Jasco FP-550 spectrofluorometer and oxygen uptake was simultaneously measured with an oxygen electrode in the same, magnetically stirred, cuvette. Actinic illumination from a projector

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00145793/84/$3.00

lamp, filtered through a heat filter and red cutoff filters ($\lambda > 600$ nm), provided an intensity of 40 mW/cm^2 at the position of the cuvette. This intensity was further attenuated by neutral density filters, as necessary.

3. RESULTS

Proton efflux driven by a concentration gradient and carried out by nonspecific diffusion may be written as:

$$J_{H^+} = P_{H^+} ([H_i^+] - [H_o^+]) \quad (1)$$

providing that a membrane potential does not exist. In chloroplasts under conditions of steady-state electron transport, the membrane potential is negligible [6] and $[H_i^+] \gg [H_o^+]$. If proton efflux under these conditions was mediated by simple diffusion, it was expected to be given by: $J_{H^+} = P_{H^+} \cdot [H_i^+]$ and to be practically independent of $[H_o^+]$.

Fig.1A shows plots of J_{H^+} *vs* $[H_i^+]$ measured under phosphorylating conditions. To obtain such a plot, electron transport and $[H_i^+]$ were measured simultaneously. At the steady state, proton efflux equals proton influx. J_{H^+} was therefore calculated from the steady-state rates of electron transport using a stoichiometry of $2H^+/e^-$ [3].

At a fixed pH_o, J_{H^+} was an exponential function of $[H_i^+]$ rather than a linear one, as shown by the slopes of the lines. Lines obtained at different pH_o values were shifted in proportion to the changes in pH_o, indicating a dependence of J_{H^+} on this parameter also. Fig.1B shows indeed that most of these data fit a single line, where J_{H^+} is plotted *vs* $[H_i^+]/[H_o^+]$ rather than *vs* $[H_i^+]$. Steady-state proton efflux coupled to ATP synthesis was accordingly characterized by an exponential dependence on $[H_i^+]/[H_o^+]$.

Fig.1C shows that in the presence of ATP, under nonphosphorylating conditions, J_{H^+} was a linear function of $[H_i^+]$, as reported in [7]. However, the plots are again seen to shift in proportion to changes in pH_o. Also, where J_{H^+} is plotted *vs* $[H_i^+]/[H_o^+]$ (fig.1D), we see that the data obtained at different light intensities, and different pH_o values between 8.7 and 7.0, fit in a straight narrow band about a single line.

Fig.1C,D shows in addition that the situation

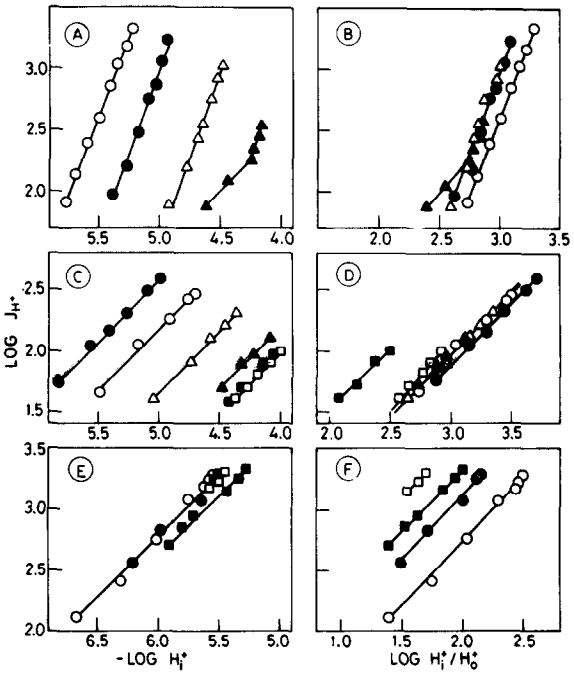

Fig. 1. Logarithmic plots of proton flux (J_{H^+}) *vs* either $[H_i^+]$ (A,C,E) or *vs* $[H_i^+]/[H_o^+]$ (B,D,F). Different lines were obtained for different pH_o values and different points on each line at different light intensities. The reaction mixture contained in a final volume of 3 ml: 30 mM NaCl, 3 mM Na_2HPO_4, 2 mM $MgCl_2$, 0.2 mM methyl viologen, 1 mM NaN_3, 20 mM Tricine and 20 mM Mes brought to the required pH_o with NaOH, and chloroplasts equivalent to 24 μg chlorophyll/ml. In addition, the reaction mixture contained in A and B, 0.5 mM ADP; in C and D, 0.1 mM ATP; and in E and F, it contained 2.5×10^{-7} M gramicidin. pH_o values: (A,B) ○, 8.5; ●, 8.0; △, 7.5; ▲, 7.0; (C,D) ●, 8.7; ○, 8.2; △, 7.7; ▲, 7.2; □, 6.9; ■, 6.5; (E,F) ○, 8.1; ●, 7.7; ■, 7.25; □, 7.1.

was different for experiments carried out at $pH_o < 7.0$. In this range J_{H^+} was independent of pH_o but still a linear function of $[H_i^+]$.

These results indicate that over most of the pH_o range examined (i.e. $pH_o > 7.0$), nonspecific diffusion does not contribute significantly to proton efflux under nonphosphorylating conditions. Proton efflux under these conditions seems to be predominantly a facilitated transport process characterized by a linear dependence on $[H_i^+]/[H_o^+]$.

Diffusion-mediated proton efflux, characterized by a linear dependence on $[H_i^+]$ and independence

of $[H_o^+]$, becomes evident and eventually predominant as pH_o is lowered below 7.0. The shift from facilitated transport to nonspecific diffusion is evident by $[H_i^+]$ becoming constant and independent of further increases in $[H_o^+]$, while $[H_i^+]/[H_o^+]$ decreases in proportion to these increases in $[H_o^+]$ (see also fig.2).

The pH_o-independent values of J_{H^+} and $[H_i^+]$, measured in parallel below pH_o 7.0 where eq. 1 was found to hold true, were used to calculate P_{H^+}. A value of 2.2 nm^2 was used for the area occupied by one chlorophyll molecule in the thylakoid membrane [8]. The permeability for protons of the thylakoid membrane according to these measurements was 2×10^{-5} cm/s.

Further evidence for this interpretation was provided by effects of gramicidin which increases the permeability of the thylakoid membrane for protons. The increase in permeability gradually broadened the pH_o range dominated by the diffusion-dependent efflux towards more alkaline values, while the range dominated by the facilitated transport process was accordingly narrowed (see also fig.2).

Fig.1E shows that in the presence of 2.5×10^{-7} M gramicidin J_{H^+} was a linear function of $[H_i^+]$ and was largely independent of $[H_o^+]$ for pH_o values up to 8.1. The independence of $[H_o^+]$ is further stressed in fig.1F where J_{H^+} is plotted as before *vs* $[H_i^+]/[H_o^+]$, but where in the case of gramicidin, separate lines were obtained for different pH_o values.

Fig.2 summarizes the effects of gramicidin at saturating light intensities on the pH dependence of electron transport, $[H_i^+]$, and ΔpH ($\Delta pH = \log [H_i^+]/[H_o^+]$). In the absence of gramicidin and in the pH_o range where proton efflux was predominantly mediated by the CF-complex, changes in pH_o induced similar changes in pH_i so that ΔpH was largely independent of pH_o. Below pH_o 7.0 where proton efflux by diffusion dominated, the situation was reversed: ΔpH decreased with pH_o while pH_i was largely independent of pH_o. In the presence of gramicidin, this second range was broadened, so that ΔpH changed in parallel with pH_o over most of the range, keeping pH_i largely invariant. Gramicidin was accordingly much more effective in reducing ΔpH at pH_o 7.0 than it was at pH_o 8.0, as it brought pH_i to the same level in both cases.

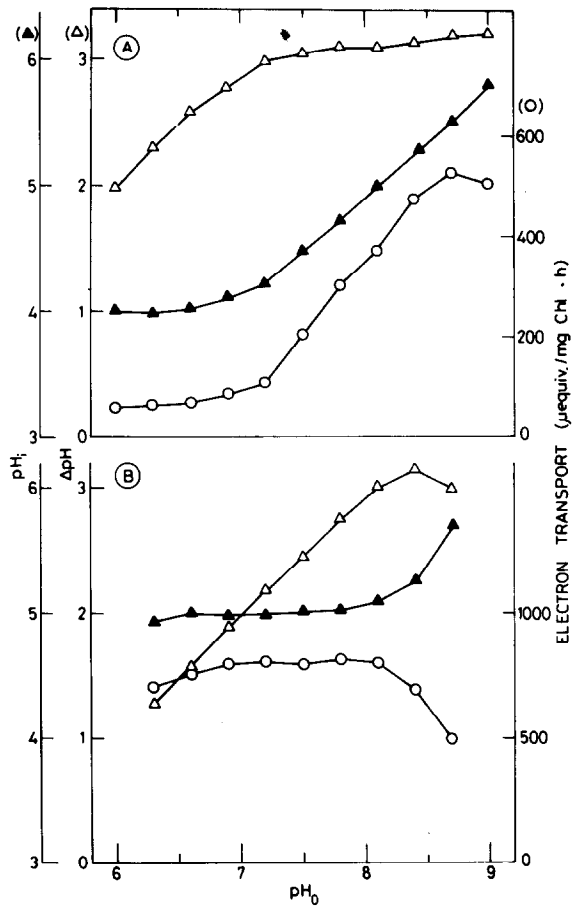

Fig. 2. Dependence on pH_o of electron transport (○), ΔpH (△) and pH_i (▲), in the absence (A) and presence (B) of 2.5×10^{-7} M gramicidin. Reaction conditions as in fig.1.

Electron transport, as a rule, followed changes in pH_i through most of the examined range and varied with pH_o (similar to the variations of pH_i!) in the absence of gramicidin, but was largely independent of pH_o in the presence of gramicidin.

The linear dependence of J_{H^+} on $[H_i^+]$ in the presence of gramicidin, enabled us to use eq. 1 to calculate its effect on the permeability of the thylakoid membranes for proton as a function of gramicidin concentration. Fig.3 shows that the permeability was linearly proportional to gramicidin concentration, and that the proportionality persisted over 2–3 orders of magnitude of changes in the two parameters. Extrapolation of this line shows that the intrinsic permeability of the

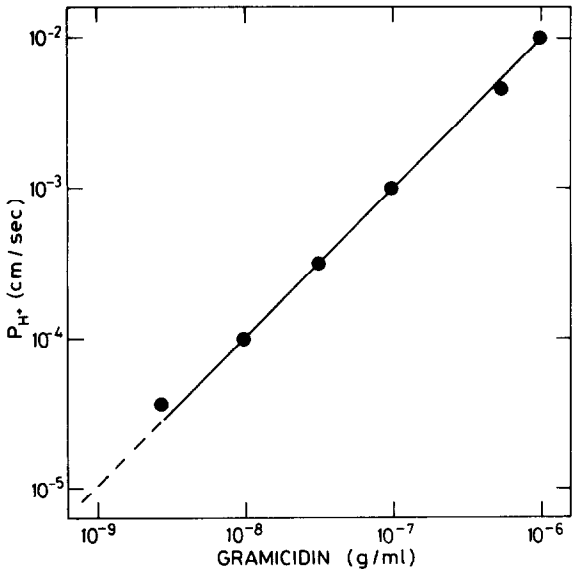

Fig. 3. Dependence of the permeability of the thylakoid membrane for protons on gramicidin concentration. Reaction conditions as in fig.1. Permeability coefficients were obtained using eq. 1 and the pH_o-independent values of J_{H^+} and $[H^+_{in}]$ measured at different gramicidin concentrations.

membrane for protons was similar to that induced by $\sim 10^{-9}$ M gramicidin.

4. DISCUSSION

A value of 2×10^{-5} cm/s for the permeability of the thylakoid membrane for protons seems rather high (although higher proton permeabilities, as high as 10^{-4} cm/s, were reported for pure phospholipid vesicles [9]). A calculation using eq. 1 will show however that under optimal conditions for ATP synthesis, such a leak would be insignificant as it amounts to only about 1% of the total proton flux, due to the low values of $[H_i^+]$ encountered. Nonspecific leakage therefore should not affect the efficiency of utilizing the proton gradient for ATP synthesis.

The bulk of proton efflux was characterized by its dependence on $([H_i^+]/[H_o^+])^b$. The exponent b can be obtained from the slope of a plot of log J_{H^+} *vs* log $[H_i^+]/[H_o^+]$ such as in fig.1. The value of b was ~ 2.5 under phosphorylating conditions and dependent to some extent on pH_o. It was ~ 1 under nonphosphorylating conditions in the presence of ATP. Under phosphorylating conditions proton efflux is known to be facilitated by the CF proton channels and this is probably also true for the residual proton efflux measured in the presence of ATP (for $pH_o > 7.0$), as proton efflux under these conditions was shown above to be kinetically distinct from nonspecific leakage.

The dependence of proton efflux on $[H_i^+]/[H_o^+]$ could be resolved from effects of pH of proton influx. The dependence on pH of electron transport and therefore on proton influx was evident in fig.1 through the different maximal values of J_{H^+} and ΔpH which were obtained at saturating light intensities at the different pH_o values. This did not interfere with the dependence of proton efflux on $[H_i^+]/[H_o^+]$ which was observed over a wide range of light intensities. A dependence of J_{H^+} on $([H_i^+]/[H_o^+])^b$, largely similar to that described here, was previously reported by authors in [10] who used periodic flashes to induce electron transport and also limited their measurements to $\Delta pH < 2.7$.

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