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Modes of action of uncouplers in thylakoids

I. Proton conduction characteristics

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A formal description of proton efflux from chloroplasts as a function of proton concentrations inside and outside the thylakoids, was used to analyze effects of uncouplers and energy transfer inhibitors on proton conduction in thylakoids. Plots of proton efflux vs ΔpH, obtained with NH_4Cl, matched simulated curves portraying increases of proton permeability. Effects of DCCD fitted those expected for blocking CF_0-CF_1 channels. Unexpectedly, effects of gramicidin or FCCP on proton conduction indicated that interference with proton efflux via CF_0-CF_1, and not stimulation of proton leakage, was the direct cause for inhibition of photophosphorylation by these uncouplers.

Chloroplast; Thylakoid membrane; Proton permeability; Proton conduction; Uncoupler; CF_0-CF_1; (Lettuce)

1. INTRODUCTION

The adequacy of the chemiosmotic theory [1] as a mechanism for photosynthetic phosphorylation is frequently claimed to depend on finding strict correlation between the ΔpH across the thylakoid membrane, and the rate of ATP synthesis. The ability of certain uncouplers, e.g. gramicidin, to inhibit ATP synthesis without dissipating the ΔpH, was interpreted as indicating that such uncouplers interrupt membrane-localized proton fluxes, which link electron transport directly with CF_0-CF_1 ([2] and [3] for review).

In the present work we used plots of proton efflux vs ΔpH to analyze complex effects of uncouplers and energy transfer inhibitors on proton conduction in the thylakoid membrane. Effects of gramicidin and FCCP on this proton conduction curve indicated that they inhibit ATP synthesis primarily by blocking proton efflux via CF_0-CF_1. Gramicidin was previously reported to act as an energy transfer inhibitor only at very low concentrations [2,4], and is commonly regarded and used as an uncoupler [5]. The results presented indicate that proton conduction curves can be used to characterize proton fluxes, in the same way that current vs voltage plots are used to portray the features of electronic components and circuits.

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Abbreviations: J_H, rate of proton efflux; P_H, proton permeability of the thylakoid membrane; $[H^+]_i$, $[H^+]_o$, internal and external proton concentrations, respectively; CF_0-CF_1, the thylakoid H^+-ATPase

2. MATERIALS AND METHODS

Envelope-free chloroplasts were isolated from lettuce leaves and photoreactions were assayed essentially as in [6]. A magnetically stirred glass cuvette, equipped with a glass combination electrode and an oxygen electrode, was installed in the sample compartment of a Jasco FP-550 spectrofluorometer. A projector-lamp equipped with a heat filter and red cut-off filters provided 1000 $\mu E \times m^{-2} \times s^{-1}$ of red actinic light ($\lambda > 600$ nm) at the position of the cuvette. The intensity was further attenuated by neutral density filters. This arrangement permitted simultaneous measurements of the internal pH via fluorescence changes of 9-aminoacridine [7], of electron transport, and of ATP synthesis via pH changes in the medium [8]. The reaction mixture contained in a final volume of 3 ml: 50 mM KCl, 2 mM

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$MgCl_2$, 2 mM Na_2HPO_4, 0.5 mM ADP, 0.1 mM methyl viologen, 2 mM NaN_3, 2 μM 9-aminoacridine and chloroplasts equivalent to 24 μg Chl/ml. The pH was adjusted to 8.0 with NaOH. Proton fluxes were calculated from measured rates of electron transport using a stoichiometry of $2H^+/e^-$ [9]. Parallel measurements of ΔpH and electron transport, at different light intensities, were used to obtain plots of proton efflux vs ΔpH as in [10].

3. RESULTS

Our previous analyses of the dependence of proton efflux from thylakoids (J_H) on the external and internal proton concentrations [10,11] can be summarized by writing J_H as a sum of three terms, representing three parallel efflux pathways.

$$J_H = P_H([H^+]_i - [H^+]_o) + K_1\frac{[H^+]_i}{[H^+]_o} + K_2\frac{[H^+]_i b}{[H^+]_o} \quad (1)$$

where $[H^+]_i$ and $[H^+]_o$ are the internal and external proton concentrations, P_H is the proton permeability of the membrane, and K_1, K_2 and b are constants. The first term is due to diffusion-mediated proton efflux, the second to an unidentified leakage pathway [10,11], and the third to proton efflux via CF_0-CF_1 [10,12]. Typical values for these parameters, experimentally determined as

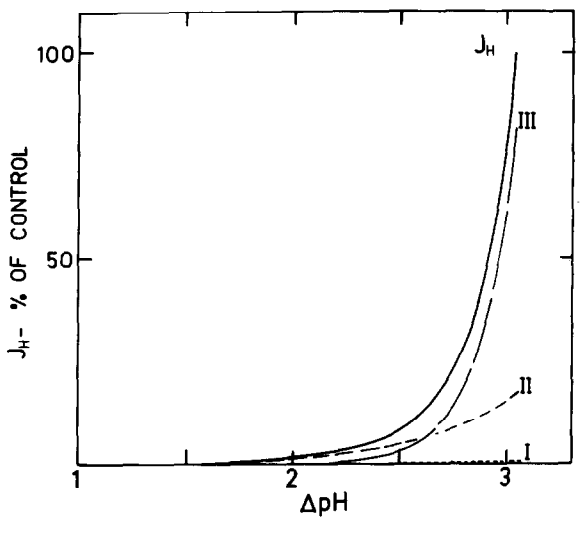

Fig.1. Dependence of J_H (proton efflux from thylakoids) and its three components on ΔpH. Fluxes were calculated by substituting in eqn 1 the following values: $P_H = 2.5 \times 10^{-5}$ cm/s, $K_1 = 4 \times 10^{-15}$ mol $\times$ cm$^{-2} \times$ s^{-1}, $K_2 = 2.6 \times 10^{-19}$ mol $\times$ cm$^{-2} \times$ s^{-1}, $b = 2.6$. Curves I–III correspond to the three terms on the right side of eqn 1.

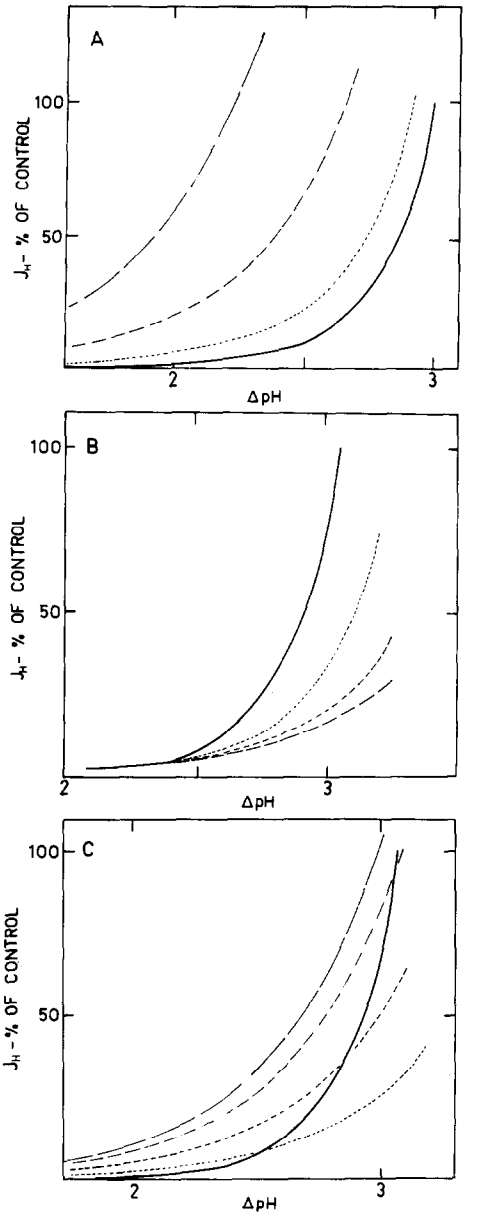

Fig.2. Simulation of effects of inhibitors of photophosphorylation on the dependence of J_H on ΔpH, by attenuation of parameters in eqn 1. Control curves (solid lines) were obtained as in fig.1. (A) Simulation of uncoupling activity. Increasing dash length represents increasing proton permeabilities. Values substituted for P_H are: 6×10^{-4}, 4×10^{-3} and 1.2×10^{-2} cm/s. The control is 2.5×10^{-5} cm/s. (B) Simulation of energy transfer inhibition, where increasing dash length represents diminishing b values. Values substituted for b are: 2.4, 2.1 and 1.9. The control is 2.6. (C) Mixed inhibition. All dashed curves have $b = 1.5$, compared to $b = 2.6$ in the control curve. Increasing dash length represents increasing P_H. Values substituted for P_H are: 10^{-3}, 1.9×10^{-3}, and 2.5×10^{-3} cm/s, compared with 2.5×10^{-5} cm/s in the control.

described in [10], were substituted into eqn 1 and the results are illustrated in fig.1.

The proton conduction curve of the thylakoid membrane thus obtained is distinctly nonlinear. Due to the low proton permeability of the membrane, the contribution of efflux by diffusion to J_H (curve I in fig.1) is evidently negligible under these conditions, whereas efflux by the second leakage pathway is somewhat more significant (fig.1, curve II). Proton efflux via CF_0-CF_1 becomes measurable only at relatively high ΔpH but then rises sharply due to its exponential dependence on $[H^+]_i/[H^+]_o$, and becomes the dominant component of proton efflux (fig.1, curve III).

Uncouplers known to stimulate passive proton leakage, are expected (in terms of eqn 1) to induce an increase in P_H, and thereby decrease ΔpH. Computer-simulated increases in P_H were evident as the conduction curve shifts upward, and to the left (fig.2A). Ammonium chloride was found to

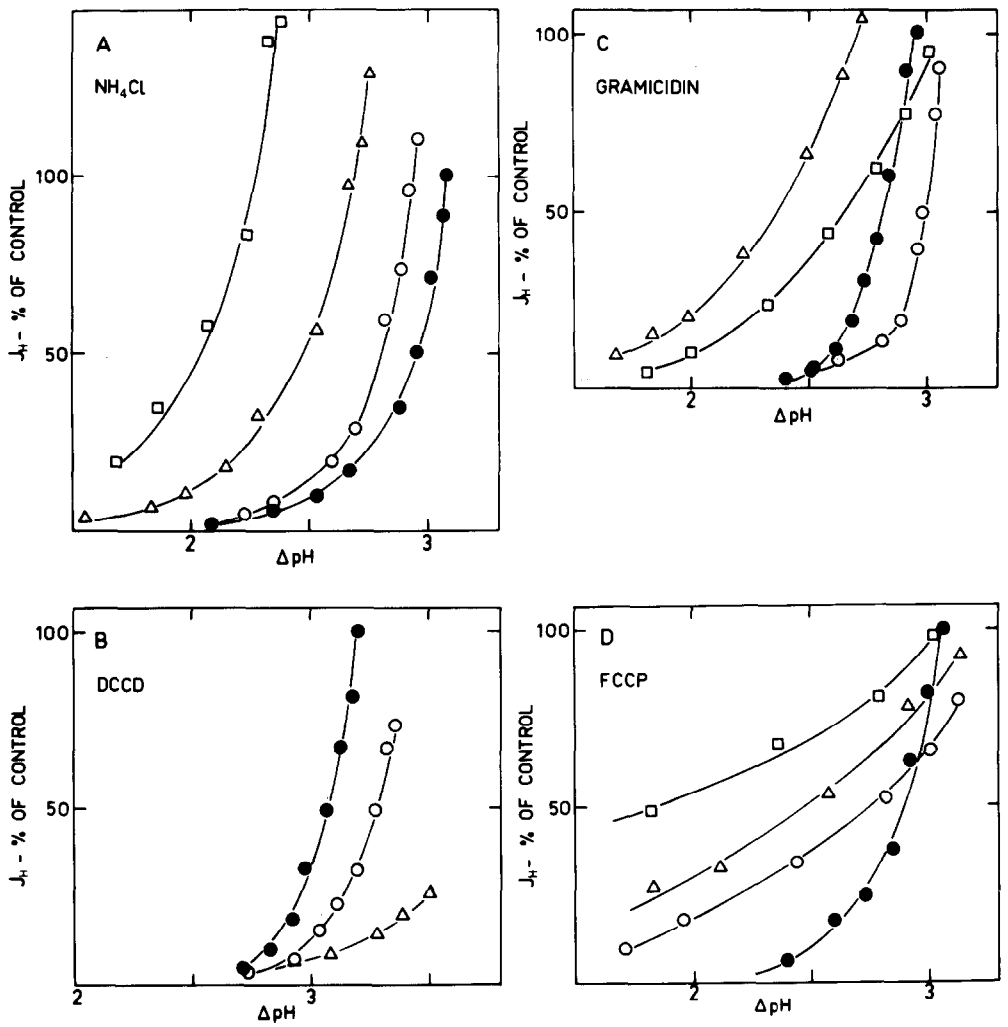

Fig.3. Effects of inhibitors of photophosphorylation on the dependence of J_H on ΔpH. Each data point represents simultaneous measurements of electron transport and ΔpH. Each line illustrates measurements taken at a single inhibitor concentration and different light intensities. Closed circles stand for control in all frames. (A) Effects of NH_4Cl: ○, 0.25 mM; △, 0.75 mM; □, 2.5 mM. (B) Effects of DCCD: ○, 10 μM; △, 30 μM. (C) Effects of gramicidin: ○, 50 nM; □, 160 nM; △, 5 μM. (D) Effects of FCCP: ○, 0.5 μM; △, 1 μM; □, 2 μM. Control rates of electron transport for A–D were 620, 600, 685 and 485, respectively. Inhibitor concentrations required for 50% inhibition of ATP synthesis were 1.5 mM NH_4Cl, 20 μM DCCD, 50 nM gramicidin, and 0.5 μM FCCP.

affect the proton conduction characteristics in the predicted manner (fig.3A). Increasing NH_4Cl concentrations, in parallel with inhibiting ATP synthesis, shifted the conduction curve as depicted, so that reductions in ΔpH were observed at all proton efflux levels. About 1.5 mM NH_4Cl was required for 50% inhibition of ATP synthesis under these conditions.

Specific inhibitors of proton efflux via CF_0-CF_1 [5], should in terms of eqn 1 induce decreases in either b or K_2. Simulated decreases of b resulted in typical reductions in the slope of the proton conduction curve (fig.2B). Reduction of K_2 yielded largely similar results. At any given efflux rate, such changes should result in increases in ΔpH. DCCD, an inhibitor of proton efflux via the CF complex, altered the conduction characteristics in a manner consistent with reductions in either b or K_2 (fig.3B), in parallel with inhibiting ATP synthesis, and induced increases of ΔpH at all proton efflux levels. 20 μM DCCD were required for 50% inhibition of ATP synthesis. Similar changes in the proton conduction curve were induced by phloridzin, another energy transfer inhibitor in thylakoids (not shown).

Gramicidin and FCCP affected the proton conduction curve in a manner suggesting simultaneous inhibition of proton efflux via CF_0-CF_1 and stimulation of passive proton leakage (fig.3C and D, respectively). Proton conduction curves obtained with these uncouplers were both left-shifted and with reduced slopes compared to the control curves. This conclusion is supported by the similarities between curves obtained with gramicidin or FCCP, and computer-simulated curves which illustrate combinations of reductions in b and stimulations of P_H (fig.2C). Note that curves obtained with these uncouplers tend to cross the control curves. Measurements taken at, or around the crossing points showed little or no effect of the uncoupler on the ΔpH in spite of the prominent changes in the proton conduction curve. As far as the ΔpH was concerned, blocking of proton efflux via CF_0-CF_1 channels was balanced by the increased efflux facilitated by gramicidin channels or FCCP molecules. ATP synthesis, however, which is coupled to proton efflux via CF_0-CF_1 was, of course, inhibited. 50 nM gramicidin or 0.5 μM FCCP reduced phosphorylation by about 50%, although the ΔpH was not diminished under these conditions. Much higher concentrations of gramicidin and FCCP were required to significantly reduce the ΔpH, and evidently it was the blocking of CF_0-CF_1 channels that inhibited ATP synthesis. Differences between cyclic and noncyclic photophosphorylation in response to uncouplers [2,5] were not investigated in the present study.

Valinomycin affected the proton conduction curve in a manner basically similar to FCCP and gramicidin, although the inhibition of the CF_0-CF_1 mediated efflux, in this case, was relatively more prominent than the increase in membrane permeability. Nigericin stimulated passive proton efflux in a manner largely similar to that of NH_4Cl but mild energy transfer inhibition was also indicated. High concentrations of DCCD induced uncoupling effects which were superimposed on the inhibition of CF_0-CF_1 mediated proton efflux.

4. DISCUSSION

The proton circuit in chloroplasts, as outlined in the chemiosmotic hypothesis, seems to be susceptible to 'short-circuits' at more than one location. It seems that beyond the requirement for a proton-impermeable membrane, the proton pumps involved also have to be 'proton-proof' in order to function properly. Compounds which shuttle protons across phospholipid bilayers, might also be capable of transporting protons across hydrophobic barriers, into and out of special domains in large multi-polypeptide complexes. According to this concept, both redox-mediated and ATPase-linked proton pumps might be inhibited by short-circuiting their active site to one of the bulk phases. Interference of protonophores with the activity of either CF_0-CF_1 or with redox-mediated proton pumps does not necessarily indicate a direct, membrane-localized, link between electron transport and ATP synthesis.

Several reagents tested affected both passive proton efflux and the CF_0-CF_1-mediated process. Classification of a reagent as an uncoupler is fully justified only where its effects on passive efflux are the direct cause of inhibition of ATP synthesis. Suppression of ATP synthesis by gramicidin or FCCP was found to be closer to energy transfer inhibition than to uncoupling.

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