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Characterization of an alkaline pH-dependent proton 'slip' in the ATP synthase of lettuce thylakoids

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An alkaline-pH-dependent increase in basal electron transport is analyzed to be due to a proton 'slip' through the ATP synthase of isolated lettuce thylakoids. The 'slip' was completely blocked by DCCD, unaffected by tentoxin and partially blocked by the presence of magnesium, ADP and phosphate or magnesium and ATP. All the stated components were required to observe the inhibition, suggesting that both the adenine and the γ-phosphate binding position on the ATP synthase must be occupied for the inhibitory effect. The nucleotides were effective in submicromolar concentrations, suggesting binding to a regulatory (non-catalytic) rather than a substrate (catalytic) site on the ATP synthase. The 'slip' modulated by phosphorylating reagents may serve an important regulatory role under physiological conditions. When phosphate availability limits ATP synthesis in the light, the 'slip' will be operative, allowing electron transport to proceed, and preventing damage due to excessive transthylakoid ΔpH. As soon as phosphate becomes available the 'slip' is avoided and normal coupled electron transport is resumed.

Introduction

The chemiosmotic hypothesis [1] postulates that during photophosphorylation ATP synthesis is coupled to electron transport via an electrochemical proton gradient. The rate of electron transport in the steady state is therefore determined by the rate of proton efflux from the thylakoid lumen. Protons can escape the thylakoid either through the membrane bilayer or through the ATP synthase. Evidence has accumulated suggesting that under non-phosphorylating conditions significant proton efflux can proceed through the ATP synthase, despite the lack of ATP synthesis [2–4]. This 'slip' through the ATP synthase, along with the proton efflux through the membrane, is presumably responsible for the observed rate of electron transport in what has been termed basal or non-phosphorylative electron flow.

The rate of basal electron flow is relatively low and constant, when measured in the pH range of 6–7.5 (about 1/5 to 1/10 the rate of uncoupled electron flow). Furthermore, it is unaffected by inhibitors of proton efflux through the ATP synthase, such as DCCD. However, this rate is markedly increased, to levels similar to uncoupled thylakoids, when the pH is increased to 8.5–9.0. That increase is clearly related to a proton 'slip' through the ATP synthase, since it is strongly inhibited by ATP synthase inhibitors such as DCCD, Dio-9 or triphenyltin [5,6]. Similarly, ATP or ADP inhibit basal electron transport only at high pH [7–11] and *N*-ethylmaleimide inhibits photophosphorylation only when its preincubation with thylakoids in the light is performed at high pH [12]. Similar phenomena were observed in mitochondria [13], where an increased rate of electron transport appears at high $\Delta\tilde{\mu}_{H^+}$ values. It is not clear at present whether the increased electron flow in the mitochondrial system is due to a 'slip' within the electron transfer system (i.e., electron flow in the absence of coupled transmembrane proton flow) or a 'slip' in the ATP synthase, or both.

Most authors attribute the 'slip' within the ATP synthase in thylakoids to the induction of a conformational change within the complex. However, some suggest that the change is caused by the high external pH [5,6], while others conclude that it is due to the high ΔpH which is maintained under these conditions [11,12,14]. Most studies concerning the 'slip' phenomenon were performed in the pH range of 8.0–8.2 where phosphorylation is maximal but the 'slip' is only partial.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; FeCy, ferricyanide.

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In this communication we report studies of the 'slip' phenomenon performed over a wide pH range utilizing a variety of physiological and artificial effectors. A possible physiological significance of the 'slip' is suggested.

Materials and Methods

Preparation of thylakoids

The procedure was carried out at 0–4°C. 12 g lettuce leaves (central vein removed) were cut into small pieces and suspended in 40 ml of a homogenizing solution containing 0.2 M sucrose, 0.1 M NaCl, 0.02 M ascorbate and 0.05 M Tricine (pH 8.0). They were blended in a 'Virtis-45' homogenizer at high speed for 5 s followed by low speed for 25 s. The homogenate was filtered through eight layers of cheese cloth and one layer of Miracloth, and centrifuged for 7 min at $1000 \times g$. The pellet was resuspended in 40 ml of the homogenizing solution lacking ascorbate, centrifuged for 7 min at $2000 \times g$, and the pellet resuspended in a small volume of a solution containing 0.2 M sucrose/0.1 M NaCl/0.5 mM Tricine (pH 8.0) to give the desired chlorophyll concentration. Chlorophyll was extracted with 80% acetone and determined as previously described [15].

Ferricyanide reduction

300 μl of the thylakoid suspension (0.1 mg Chl $\cdot$ ml^{-1}) were added to 2.7 ml of a reaction mixture containing 50 mM NaCl, 20 mM Taps, 20 mM Mops, 0.1 mM EDTA, preadjusted to the desired pH. 10 μl of 0.1 M pottasium ferricyanide was added prior to running the reaction. All measurements were made at room temperature.

Ferricyanide reduction was measured continuously in a DW-2 Aminco dual wavelength spectrophotometer set at 420–450 nm. The sample was illuminated with a slide projector filtered through a Schott OG550 filter. A corning C.S.4-96 filter combined with a Balzers interference filter (max. transmittance 437 nm, half-band width 85 nm) served to protect the photomultiplier from the actinic light. Unless otherwise indicated, light intensity at the cuvette was 130 W $\cdot$ m^2. The steady-state slope, representing the change in absorbance difference per unit time, was determined. The reduction of a known amount of ferricyanide served to calibrate the instrument.

Proton uptake

300 μl thylakoid suspension (0.5 mg Chl $\cdot$ ml^{-1}) were added to 2.7 ml of a reaction mixture containing 50 mM NaCl, 0.5 mM Taps, 0.5 mM Mops, 0.1 mM EDTA and 0.03 mM phenazine methosulfate preadjusted to the desired pH and placed in a measuring cell held at 20°C. Proton uptake was measured as the extent of illumination-induced pH rise with a Radiometer pH meter connected to a recorder with a GK 2321C electrode.

Fine tuning of the initial pH and calibration was done with fresh NaOH or H_2SO_4. Light (645–730 nm) filtered through a RG 645 Schott filter and a heat filter was provided by a slide projector at an intensity of 350 W $\cdot$ m^{-2}.

ATP synthesis

The formation of ATP from ADP and P_i was determined by following the accompanying rise in pH in a weakly buffered solution [16]. The reaction mixture contained in a total volume of 3.0 ml: 50 mM NaCl, 5 mM $MgCl_2$, 2 mM P_i, 0.5 mM ADP, 0.03 mM phenazine methosulfate, 1 mM Tricine, (pH 8.2) and thylakoids containing 30 μg Chl. pH measurement and calibration were as described under proton uptake. The pH of the reaction mixture was set to 8.2, where each ATP synthesized produces 0.98 OH^- [16].

Inhibitors

DCCD (10 mM) dissolved in ethanol was added at the indicated final concentrations to a thylakoid suspension containing 0.1 or 0.5 mg Chl $\cdot$ ml^{-1} at pH 8.0 and preincubated for 0.5 h. 300 μl of the suspension were then added to the reaction mixture and the reaction followed as described above. Tentoxin was used in the same way, although preincubation in this case is not required. The concentration required for inhibiting photophosphorylation was dependent on the thylakoid concentration. Thus, 50% inhibition was observed with 20 μM DCCD and 0.25 μM tentoxin in the preincubation medium when the chlorophyll concentration was 0.1 mg $\cdot$ ml^{-1} (as used in studies of electron transport) while 80 μM DCCD and 1 μM tentoxin were required to observe the same inhibition when the chlorophyll concentration was 0.5 mg $\cdot$ ml^{-1} (as used in studies of proton uptake). In the experiments reported herein the inhibitors were employed at concentrations which fully inhibited photophosphorylation at the given chlorophyll concentration.

Results

Agents affecting the pH dependence of basal electron transport

The marked increase in basal electron flow at alkaline pH is illustrated in Fig. 1. Furthermore, as previously reported [3,4], the increased electron transport at alkaline pH is completely inhibited by CF_0-directed inhibitors such as DCCD, suggesting that the increase in electron transport at alkaline pH is due solely to a proton 'slip' through the ATP synthase. However, tentoxin, a CF_1-directed inhibitor [17,18], at concentrations which fully inhibit photophosphorylation, had no effect on the pH dependence of non-phosphorylating

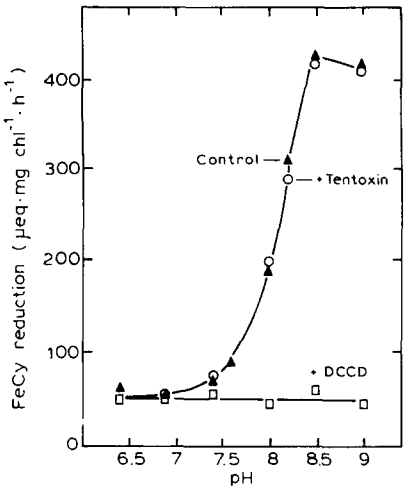

Fig. 1. pH-dependence of basal electron transport in the presence or absence of the energy transfer inhibitors tentoxin and DCCD. Thylakoids were preincubated with no addition (▲), 1 μM tentoxin (o) or 50 μM DCCD (□); and the steady-state rate of ferricyanide reduction was measured as described under Materials and Methods.

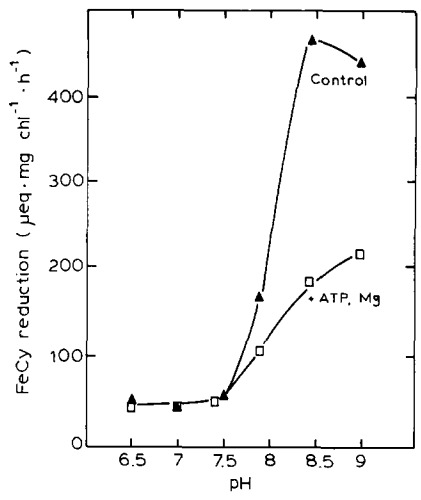

Fig. 3. Effect of ATP and Mg on basal electron transport in the pH range 6.5–9.0. Where indicated, ATP, 100 μM; and $MgCl_2$, 2 mM were added before the addition of the thylakoids. Other details as described under Materials and Methods.

electron transport. Thus, it is a specific inhibition of proton transport at the ATP synthase, rather than the inhibition of turnover of the ATP synthase, that prevents the proton 'slip' at high external pH. This observation enabled us to probe into the effect of the full complement of the phosphorylating reagents, ADP, P_i and magnesium, in the absence of any phosphorylation. Fig. 2 illustrates that in the presence of tentoxin, when no phosphorylation can occur, ADP, P_i and magnesium inhibited basal electron transport in a manner resembling the effect of DCCD. In the absence of tentoxin, the classical stimulation of electron flow by simultaneous phosphorylation was observed. ATP plus magnesium also inhibited the high pH induced increase in electron flow, but the effect was incomplete (Fig. 3).

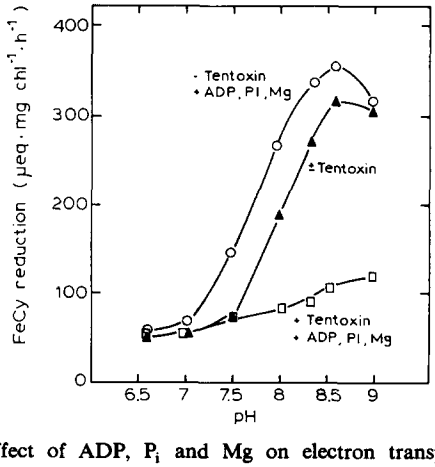

Fig. 2. Effect of ADP, P_i and Mg on electron transport in the presence and absence of phosphorylation in the pH range 6.5–9.0. Tentoxin in preincubation, 1 μM; ADP, 100 μM; $MgCl_2$, 2 mM; P_i, 2 mM. Other conditions as described under Materials and Methods.

Since the maximal effect of the 'slip' inhibitors occurred at pH 8.5 (Figs. 1–3) we tested the concentration dependence of several agents in this system at that pH. Fig. 4 illustrates that the inhibition of electron transport by ATP was completely Mg-dependent, as reported previously [10]. Half maximal activity required about 0.5 μM ATP and was unaffected by the presence or absence of tentoxin.

The inhibition of electron transport by ADP, Mg and P_i was dependent upon the presence of all three components (Fig. 5). Half maximal inhibition was again observed with about 0.5 μM ADP. The small inhibition observed at high ADP concentrations in the absence of

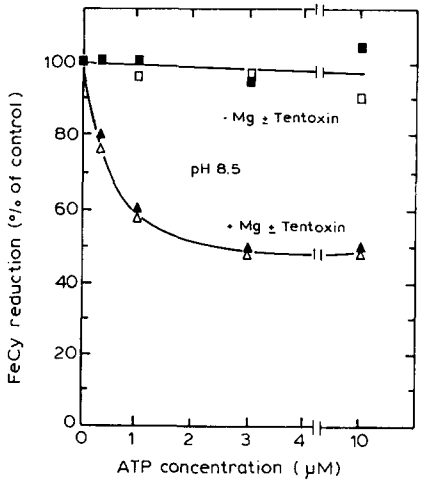

Fig. 4. Inhibition of electron transport as a function of ATP concentration at pH 8.5. Basal electron flow at pH 6.5 was subtracted from all measurements. 100% refers to the following rates in μequiv.·$MgChl^{-1}\cdot h^{-1}$: □, Mg, −tentoxin, 351; ■, −Mg, −tentoxin 342; △, +Mg, −tentoxin, 336; ▲, +Mg, +tentoxin, 324; where indicated tentoxin was 1 μM in the preincubation; Mg was 2 mM. Other conditions as described under Materials and Methods.

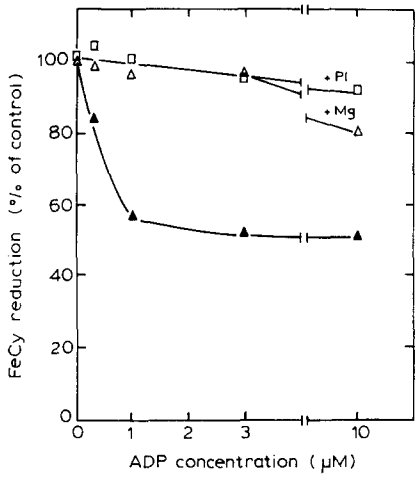

Fig. 5. Inhibition of electron transport as a function of ADP concentration at pH 8.5. 100% refers to the following rates in μequiv.·mgChl^{-1}·h^{-1}: □, +P_i (2 mM), 370; △, +Mg (2 mM), 360; ▲, +Mg (2 mM) +P_i (2 mM), 333; other conditions as in Fig. 4.

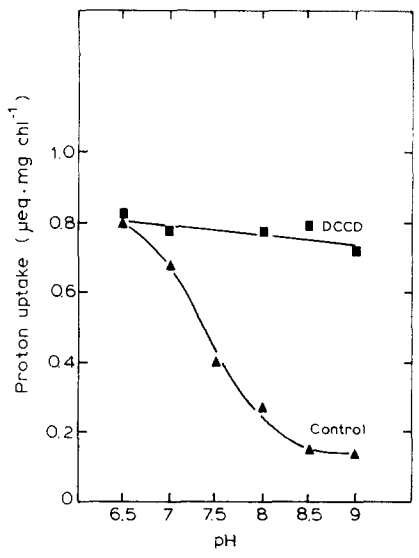

Fig. 6. The effect of DCCD on the pH-dependence of the extent of proton uptake. DCCD concentration during the preincubation was 200 μM. Other details as described under Materials and Methods.

P_i is probably due to contaminating ATP in the commercial ADP. Half-maximal inhibition with optimal ADP and magnesium required about 0.5 mM P_i. Arsenate could substitute for phosphate, and ADP did not release the inhibition exerted by ATP and magnesium (not shown). GTP could not substitute for ATP, in concentrations up to 10 μM.

The low concentration of ADP and ATP required for half-maximal effect and the ineffectivity of guanine nucleotides [8,10] clearly placed this nucleotide effect of inhibiting the 'slip', as a regulatory rather that a substrate type effect, since the latter requires concentrations around 50 μM [19].

Agents affecting the pH dependence of proton uptake

The increase in electron flow observed at alkaline pH is interpreted as due to an increased proton flux through the ATP synthase. Fig. 6 shows the pH-dependence of the extent of proton uptake. As has been observed previously [20], the extent of proton uptake decreases sharply with increased external pH. In analogy with electron transport (Fig. 1), the decrease at high pH was completely prevented by DCCD, but totally unaffected by tentoxin (not shown). This is in agreement with the interpretation that the decreased proton uptake at alkaline pH is due to an alkaline pH induced proton 'slip' through the ATP synthase.

ATP and magnesium (Fig. 7), or ADP, P_i and magnesium (not shown) increased proton uptake in a similar manner, but to a lesser degree than DCCD, as was the case with basal electron flow (Figs. 2, 3). ATP had its maximum effect at pH 8.5 and the effect was completely magnesium dependent and phosphate-independent (Fig. 7). However, ADP, magnesium and phosphate stimulation of proton uptake required P_i (not shown). A small stimulation of proton uptake was observed with magnesium and P_i alone, which correlated well with a small decrease in electron transport which was observed under the same conditions (not shown).

As indicated earlier, it is not clear at present whether the 'slip' is external-pH- or ΔpH-dependent [5,6,11,12]. An indication in this regard may be obtained by studying the rate of electron flow as a function of light intensity. If the 'slip' were external-pH-dependent, it might be expected that at pH 8.5, when the 'slip' is maximal, it would be effective independent of light intensity. However, if the 'slip' were ΔpH-dependent we might expect an upward break in the line depicting basal electron flow as a function of light intensity when ΔpH reaches the effective 'slip' inducing level. Fig. 8 shows the light intensity dependence of basal electron

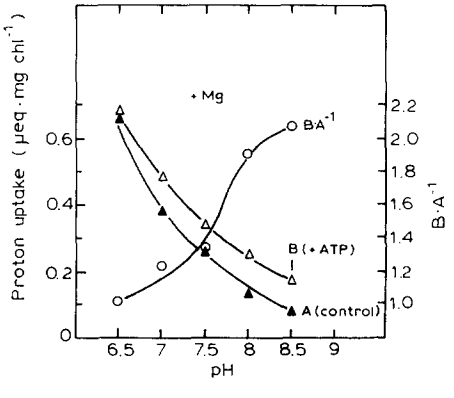

Fig. 7. Stimulation of proton uptake by ATP as a function of pH. Light-dependent proton uptake was measured as described under methods. ▲, $MgCl_2$, 2 mM. △, $MgCl_2$, 2 mM; ATP 10 μM. ○, The relative ATP stimulation of proton uptake at each pH value is illustrated in the curve designated B·A^{-1}.

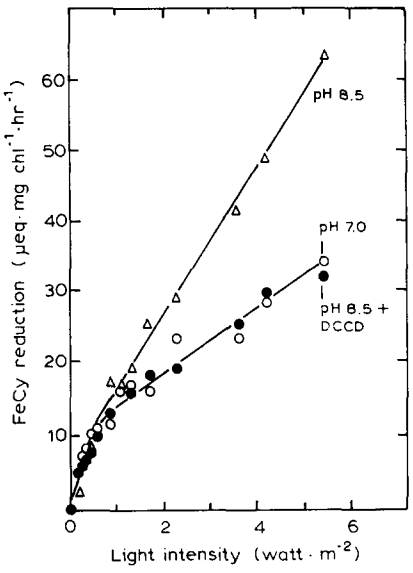

Fig. 8. The rate of ferricyanide reduction as a function of light intensity. Light intensity (560–730 nm) was varied with neutral-density filters. △, pH 8.5; ●, pH 8.5 + DCCD (50 μM in the preincubation medium); ○, pH 7 . Other conditions as described under Materials and Methods.

flow at pH 8.5, where the 'slip' is maximal, in the presence or absence of DCCD, and at pH 7.0 . As can be seen, no upward break is observed in the pH 8.5 curve. However, the expected downward break, due to the inhibition of electron flow by the limiting coupled proton flow through the ATP synthase, is clearly evident at pH 7.0 and in the DCCD-treated thylakoids at pH 8.5 . These data support external pH, rather than ΔpH, as the causative agent of the 'slip'.

Discussion

The observations reported herein, when coupled with previous reports on the subject [3,4,6,8], suggest that high external pH induces a proton 'slip' through the ATP synthase which is strongly regulated by the phosphorylating reagents. The observation that tentoxin, at concentrations which fully inhibit photophosphorylation, had no effect on the alkaline pH induced 'slip', permitted us to clearly define ATP with magnesium or ADP, phosphate and magnesium as the reagents which prevent the high-pH-induced 'slip'. This is at variance with some earlier reports, in which ADP and magnesium, by themselves, were reported to be effective [7,9–11]. However, we believe that this was due to the inability, in the absence of tentoxin and with the techniques employed, to prevent the formation of the very small amounts of ATP required for inhibition.

In order to measure the high-pH-dependent proton 'slip' accurately, it is essential to employ a continuously monitoring system for electron transport where the steady-state rate of electron transport can be determined unambiguously, as employed herein. In many of the previous studies on the subject, electron transport was measured only after a given period of illumination [10,12]. Since electron transport is not linear at the early times, particularly at the lower pH range [22], this can lead to considerable error in interpretation.

The available data suggest that the 'slip' induced by alkaline pH is affected by several, but not all, effectors which interact with some specific subunits of the ATP synthase. Thus, DCCD and triphenyltin, presumably affect the 'slip' by blocking the CF_0 portion and so preventing protons from reaching the CF, portion of the ATPase. However, the nucleotides at low concentrations affect the 'slip' by interacting with regulatory sites on the $\alpha + \beta$ subunits of the CF_1 portion, since these are the only subunits which seem capable of interaction with nucleotides. Also clearly the adenine, the γ-phosphate and the magnesium binding sites must all be occupied before inhibition of the 'slip' effect can be observed. Thus, ADP with magnesium, but in the absence of inorganic phosphate, is ineffective in preventing the 'slip'. Nevertheless tentoxin, which also interacts with the $\alpha + \beta$ subunits [18] and fully prevents ATP synthesis, is without effect.

The regulatory effects of ADP, phosphate and magnesium may have an important physiological role in the plant. It is well established [23] that the level of inorganic phosphate is regulated at a low level within the cytoplasm, so as not to deplete the chloroplast stroma of triose-phosphate through the P_i-triose phosphate translocator. Also, the stromal pH rises considerably in the light, reaching values of 8.2–8.5, well within the range where the 'slip' is observed. In the presence of adequate phosphate (and ADP plus magnesium) the 'slip' will be inhibited and coupled phosphorylation will proceed unabated. However, when inorganic phosphate is limiting, below about 0.5 mM which is the apparent K_m for both the P_i-triose phosphate translocator and the inhibition of the 'slip', excessive ΔpH could have developed across the thylakoids, slowing down electron transport and leading to damage to the thylakoid system. The operation of the 'slip' under these conditions, of high stromal pH and low phosphorylation due to limiting inorganic phosphate, permits electron-transport to proceed and avoids damage due to excessive ΔpH. The situation will, of course, immediately revert to normal coupled phosphorylation as soon as inorganic phosphate becomes available. Under physiological conditions it is inorganic phosphate, and not ADP, ATP or magnesium, which is most likely to limit the phosphorylation machinery. Indeed, during the preparation of this communication, a report was published [24] in which the authors conclude that, in intact chloroplasts, in the absence of inorganic phosphate, ΔpH is reduced due to 'a proton leak' through the ATP synthase.

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