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Relationships between rates of steady-state ATP synthesis and the magnitude of the proton-activity gradient across thylakoid membranes

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Rates of ATP synthesis by illuminated spinach chloroplast thylakoids in the steady state have been compared to the magnitude of the pH difference across the thylakoid membrane (ΔpH). The apparent dependence of the rate of photophosphorylation on ΔpH was not affected by the method used to vary these parameters. ΔpH generated by electron flow through each photosystem alone or through both photosystems was equivalent in its ability to drive ATP synthesis. The uncouplers, gramicidin and carbonylcyanide *m*-chlorophenylhydrazone, affected the relationship between phosphorylation and ΔpH in the same way as alteration in the rate of electron transport by changes in light intensity. Also, *N,N'*-dicyclohexylcarbodiimide, an inhibitor of phosphorylation, increased ΔpH as phosphorylation is inhibited. The K_m of ADP for photophosphorylation was previously reported to increase when uncouplers were used to decrease the rate, but to decrease when the light intensity was lowered (Vinkler, C. (1981) Biochem. Biophys. Res. Commun. 99, 1095–1100). We show that higher uncoupler concentrations decrease the K_m for ADP and provide an explanation for the increase in the K_m caused by low uncoupler concentrations. These results are consistent with the concept that steady state ATP synthesis in thylakoids is driven by delocalized pH gradients.

Introduction

Photosynthetic electron transport causes proton release at the inner surface of thylakoid or chromatophore lumen, thus generating a high transmembrane electrochemical proton gradient. The efflux of protons down this gradient drives ATP synthesis. This view of the coupling between photosynthetic electron flow and ATP synthesis was first proposed by Mitchell more than 20 years ago [1,2]. Since Mitchell's first suggestions, these concepts have received broad experimental support. Electrochemical proton gradients generated artificially, either by imposing membrane potentials or pH differentials, are capable of driving ATP synthesis. The kinetic [3,4] and thermodynamic [5,6] competence of transmembrane gradients have been established. In addition, the characteristics of the coupling between electron flow [7,8] and ATP synthesis [9] by thylakoids in the steady state can be satisfactorily explained by a minimal scheme, involving the transmembrane proton-activity gradient, a reversible proton-translocating ATPase, and a proton leak through as yet uniden-

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Abbreviations: ΔpH, pH of the medium minus that of the thylakoid lumen; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DBMIB, dibromomethylisopropylbenzoquinone; P_i, inorganic phosphate.

tified pathways. A detailed analysis of steady-state proton fluxes through these pathways revealed that the maximum phosphorylation efficiency for linear electron flow through both photosystems should be 4/3 [8]. This value is close to the ratio of the proton-to-electron stoichiometry for proton translocation to that of the proton to ATP stoichiometry for the H^+-ATPase of thylakoids.

Other investigators, however, have concluded that bulk phase electrochemical H^+ gradients do not behave as if they powered ATP synthesis. In this paper we present data on the relation of ATP synthesis rates by thylakoids to the size of ΔpH and on the effects of uncouplers on the K_m for ADP in photophosphorylation. In contrast to some previous reports, our data are consistent with the chemiosmotic model for the coupling of electron flow and ATP synthesis.,

Experimental procedures

Phosphorylation, ΔpH and electron transport were routinely assayed in freshly prepared spinach chloroplast thylakoids by procedures outlined elsewhere [8,10]. Unless indicated otherwise, the reaction mixtures contained 20 mM Tricine-NaOH (pH 8.0)/50 mM NaCl/5 mM $MgCl_2$/1 mM $K_3Fe(CN)_6$/4 mM potassium phosphate (plus $2 \cdot 10^5$ cmp $^{32}P_i$ in phosphorylation experiments), 1 mM ADP, 0.04 mM EDTA and thylakoids equivalent to 0.1 mg of chlorophyll per ml. Hexylamine (12.5–25 μM) was present in each case. When ΔpH ($pH_{out} - pH_{in}$) was to be determined, ^{32}P was omitted and [^{14}C]hexylamine ($3 \cdot 10^3$ cpm) was included. $^{32}P_i$ incorporation was determined according to Lindberg and Ernster [11] and chlorophyll content, using the extinction coefficients of Arnon [12]. Ferrocyanide production was used to estimate the rate of electron flow [13]. ATPase activity was determined from the release of $^{32}P_i$ from $AT^{32}P$ (4 μCi/μmol) in the dark after exposure of thylakoids to dithiothreitol in the light [9]. All assays were performed in duplicate. Duplicate determinations agreed within less than approx. 0.05 pH unit for ΔpH measurements and were generally within approx. 5% for photophosphorylation and ATPase determinations, except at very low rates (less than 5 μmol per h per mg chlorophyll) where the error was up to approx. 20%.

Although ΔpH values were found not to vary with illumination time over the span of several seconds to several minutes, a slight change in these values was detected as the thylakoid preparation aged. This slow change in ΔpH may reflect an increase in the internal volume, which was not routinely measured in these experiments. Since over 1 h was needed to complete all of the incubations, and since the rates of ATP synthesis were invariant over this time period, the samples in which ΔpH was measured were incubated first, followed by those in which ATP synthesis or hydrolysis was to be determined.

Results

The dependence of the rate of ATP synthesis on ΔpH should not reflect the manner in which ΔpH is generated, if bulk phase proton activity gradients drive ATP synthesis. Although previous results [14] from this laboratory suggested that this is the case, more recent results [15] have challenged the idea that protons released into the thylakoid lumen by the oxidation of water and those released through the operation of Photosystem I are equivalent in promoting ATP synthesis. To provide a further test, phosphorylation and ΔpH, supported by various mediators of electron flow, were assayed as a function of light intensity. In contrast to the findings of other investigators [15], we find a nearly exact correspondence between phosphorylation rate and ΔpH when ATP synthesis is driven by Photosystem I or by Photosystem II electron transport alone and when electron transport through both photosystems is responsible for ΔpH generation (Fig. 1). Moreover, the relationship between phosphorylation and ΔpH, obtained by varying the concentration of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, was identical to that observed by varying the light intensity (not shown).

Uncouplers increase the proton permeability of membranes, and, thus, decrease ΔpH and the rate of ATP synthesis. Proton ionophores would not be expected to alter the relationship between ΔpH and ATP synthesis rate, provided transmembrane pH gradients drive ATP synthesis. This appears to be the case. The dependence of phosphorylation rate on ΔpH was the same, whether these parame-

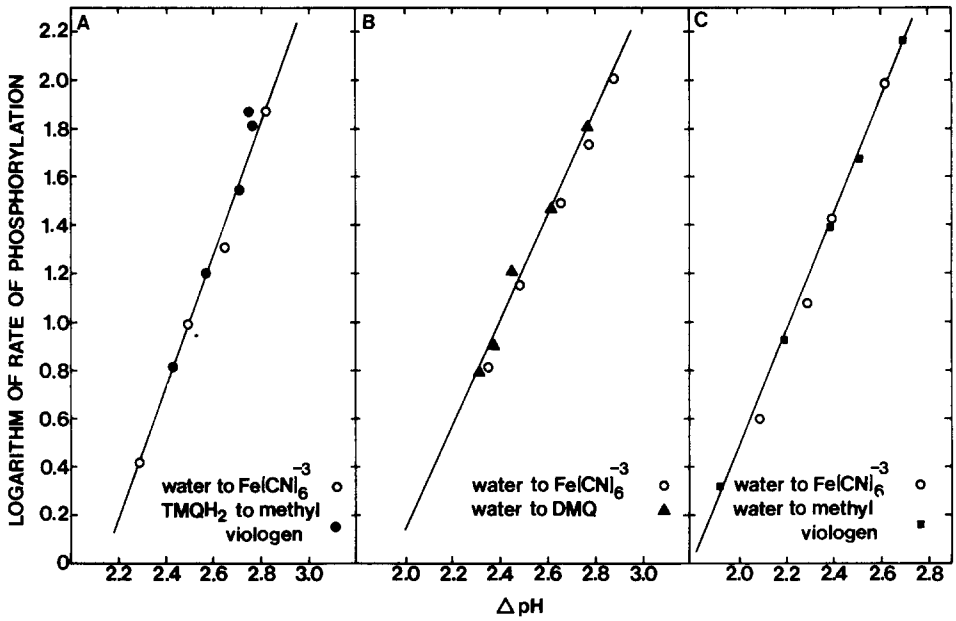

Fig. 1. Relationship between phosphorylation rate and ΔpH supported by different mediators of electron flow. ΔpH and ATP synthesis were determined using the reaction mixture given under Experimental Procedures. Photosystem-I-supported phosphorylation and ΔpH ($TMQH_2$ to methyl viologen, panel A) were assayed in the presence of 2 μM dichlorophenyl-1,1-dimethyl urea/0.8 mM tetramethylquinol ($TMQH_2$)/0.5 mM methyl viologen. Photosystem-II-supported phosphorylation and ΔpH (water to DMQ, panel B) were assyed in the presence of 10 μM nitrofluorfen and 0.8 mM dimethylquinone (DMQ). Electron flow through both photosystems was supported by either 1 mM $Fe(CN)_6^{3-}$ or 0.5 mM methyl viologen. The light intensities were varied from 10 to 100 $J \cdot m^2 \cdot s^{-1}$. Rates of ATP synthesis are expressed as μmol P_i incorporated per h per mg chlorophyll. The three experiments were performed on different days.

ters were varied by light intensity or by the protonophores, gramicidin or carbonylcyanide *m*-chlorophenylhydrazone (Fig. 2).

Phosphorylation decreases the magnitude of the bulk phase ΔpH, as expected if ΔpH drives ATP synthesis [8,10,14]. The addition of the energy-transfer inhibitor, *N*,*N*′-dicyclohexylcarbodiimide, which blocks the flow of protons through the hydrophobic portion of the H^+-ATPase, also inhibits ATP synthesis. As phosphorylation becomes progressively more inhibited by the carbodiimide, ΔpH approaches the value that it achieves in the absence of ATP synthesis (Table I). Similar results are obtained with another phosphorylation inhibitor, 4′-deoxyphlorizin [14], or when ATP synthesis rates are altered by limiting P_i concentrations [16].

The apparent dependence of the rate of phosphorylation on ΔpH is not changed when the pH of the medium is varied from 7 to 8.5. This is illustrated for the pH range 7.7 to 8.4 in Fig. 3. Alkaline lability of the thylakoid membrane and the probable underestimation of ΔpH at acidic pH values [8] precluded extension of this pH range. At high light intensities, phosphorylation and ΔpH are limited by the maximal rate of electron flow and phosphorylation has a pH optimum in the range of 8.0–8.5. At subsaturating light intensities, ΔpH and ATP synthesis are independent of external pH over the pH range of 7 to 8.5. Since ΔpH at a given light intensity is constant over this external pH range [8] the internal pH must be 1 pH unit lower at an external pH of 7.0 than at an external pH of 8.0, even though the ATP synthesis rate is the same. Clearly, ΔpH, rather than internal proton concentration, controls phosphorylation rates. Takabe and Hammes [17] showed that the V_{max} for ATP synthesis by phospholipid vesicles co-reconstituted with bacteriorhodopsin and the chloroplast H^+-ATPase is the same at pH 6.6 as it is at 8.0.

If thylakoid membranes are illuminated in the

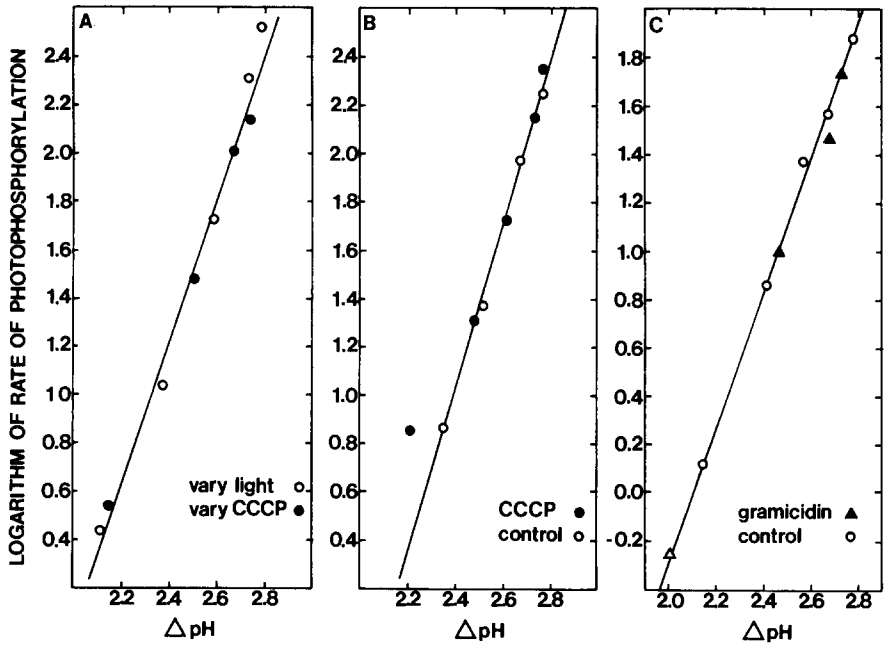

Fig. 2. Effect of uncouplers on the relationship between phosphorylation rate and ΔpH. Photophosphorylation and ΔpH were assayed in the presence of either 1 mM methyl viologen (panel A) or of 1 mM $K_3Fe(CN)_6$ (panels B and C). Light intensities were varied over the range 5 to 500 $J \cdot m^{-2} \cdot s^{-1}$. In A, light intensity (open circles) and CCCP concentration (closed circles) were varied. A fixed light intensity (5000 $J \cdot m^{-2} \cdot s^{-1}$) was used when CCCP (1–8 μM) was added. At 1 μM CCCP the phosphorylation rate was inhibited by 58% and at 8 μM, by 98%. In B, light intensity was varied in the presence (closed circles) and absence (open circles) of 0.25 μM CCCP. The inhibition of ATP synthesis by CCCP was 22% at 1000 $J \cdot m^{-2} \cdot s^{-1}$ and increased to 70% at 20 $J \cdot m^{-2} \cdot s^{-1}$. In C, light intensity was varied in the presence (closed triangles) and absence (open circles) of 0.01 μM gramicidin. Gramicidin inhibited ATP synthesis by 43% at 1000 $J \cdot m^{-2} \cdot s^{-1}$ and by 95% at 20 $J \cdot m^{-2} \cdot s^{-1}$. Phosphorylation rates are expressed as μmol P_i incorporated per h per mg chlorophyll.

TABLE I

EFFECT OF DCCD ON PHOSPHORYLATION, ELECTRON FLOW AND ΔpH

Aliquots of methanolic DCCD solutions were diluted 1:100 into thylakoid suspensions (2.5 mg chlorophyll/ml in 0.4 M mannitol, 0.02 M Tricine-NaOH (pH 8.0), 0.01 M NaCl) at 0°C. The final DCCD concentrations ranged from 0.25 to 1.0 mM. An equivalent amount of methanol was added to the controls. After 30 min at 0°, 0.01 ml aliquots of the thylakoid suspensions were diluted to 0.25 ml with assay mixture and ΔpH, phosphorylation and electron flow (ferrocyanide production) assayed. The concentrations of DCCD shown are those in the assay mixtures. The light intensity 50 $J \cdot m^{-2} \cdot s^{-1}$. Internal proton concentrations ($[H^+]_{in}$) were calculated from ΔpH values, assuming an external pH of 8.0.

DCCD (μM)	Electron transport (μmol $Fe(CN)^{4-}$ per h per mg chlorophyll)	Phosphorylation (μmol P_i per h per mg chlorophyll)	ΔpH	$[H^+]_{in}$ (μM)
0	185	79	2.66	4.6
10	173	53	2.74	5.6
20	129	30	2.90	7.9
30	111	12	2.98	9.5
40	106	6	3.04	10.9

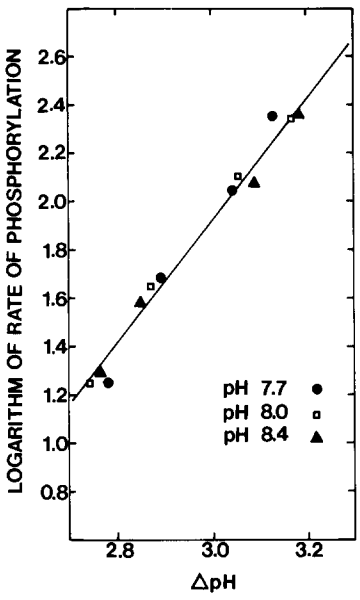

Fig. 3. Lack of effect of external pH on the relationship between phosphorylation and ΔpH. Phosphorylation and ΔpH were determined in the presence of 1 mM $Fe(CN)_6^{3-}$ as usual, except that the external pH was varied with 20 mM Tricine-NaOH buffers at pH 7.7 (closed circles), 8.0 (open squares) and 8.4 (closed triangles). The light intensity was from 20 to 200 $J \cdot m^{-2} \cdot s^{-1}$.

presence of sulfhydryl compounds, the ATPase complex is converted into a stable active state that can cleave ATP in the dark [18]. The hydrolysis of ATP is coupled to proton translocation into the thylakoid lumen [8,19]. The resulting pH gradient created by ATP hydrolysis inhibits further ATP hydrolysis as uncouplers stimulate ATPase activity [20] and light inhibits [21]. Part of the light inhibition is due to re-esterification of released phosphate and ADP and can be prevented by addition of pyruvate kinase and phosphoenol pyruvate or masked by addition of unlabeled phosphate [22]. The inhibition by illumination that persists after addition of phosphate is due to inhibition of hydrolysis by ΔpH. Dewey and Hammes [23] examined the dependence of the rate of ATP hydrolysis by vesicles reconstituted with bacteriorhodopsin and the H^+-ATPase on ΔpH. We have extended these studies to intact thylakoids (Fig. 4). The experiment is technically difficult because the reaction mixtures are transferred to microcentrifuge tubes after activation and addition of ATP, but before illumination. The slope of the plot of the logarithm of the rate of hydrolysis versus ΔpH is similar in magnitude, but opposite in sign to the slope of the corresponding plot for the synthetic reaction. Several such experiments

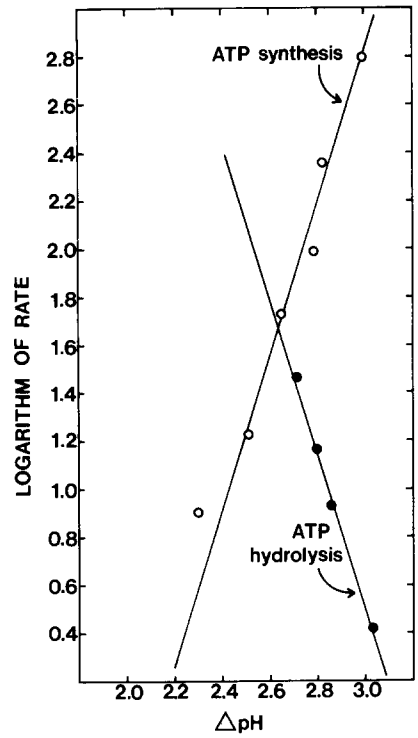

Fig. 4. Dependence of ATP synthesis and hydrolysis on ΔpH. The ATPase activity of thylakoids (12.5 μg of chlorophyll) was activated by illumination (2000 $J \cdot m^{-2} \cdot s^{-1}$) of suspensions in 0.05 ml of a medium containing 20 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.04 mM EDTA, 0.02 mM P_i, 0.025 mM pyocyanine and 10 mM dithiothreitol. After 1 min, 200 μl aliquots of the reaction mixtures for either ΔpH or ATPase activity were added. The samples for ΔpH assay contained 12.5 μM [^{14}C]hexylamine in addition to 0.5 mM ATP. The samples for ATPase assay contained 0.5 mM ATP containing 2 μCi [γ-^{32}P]ATP/μmol and 1 mM $Na_2H_2P_2O_7$ (pH 8.0). Both reaction mixtures contained Tricine, NaCl, $MgCl_2$, EDTA and pyocyanine at the concentrations given above. Light intensity (0 to 500 $J \cdot m^{-2} \cdot s^{-1}$) was used to vary ΔpH and ATPase activity. Photophosphorylation was assayed in the same reaction mixture, except that ATP was omitted and 1 mM ADP and and 4 mM P_i containing 3 μCi ^{32}P/μmol were added. Thylakoids were not illuminated in the presence of dithiothreitol prior to assay. Light intensities used varied 10 to 500 $J \cdot m^{-2} \cdot s^{-1}$. Rates are expressed as either μmol P_i incorporated per h per mg chlorophyll (ATP synthesis) or as μmol P_i released per h per mg chlorophyll (ATP hydrolysis).

show that the rate of ATP hydrolysis can be inhibited by light to rates equal to those found in non-activated, DCCD-poisoned controls, i.e., by at least 90%.

Our results show that the rate of ATP synthesis at saturating ADP and P_i concentrations depends on ΔpH and not upon the electron-transport pathway used to generate ΔpH or upon the treatment used to vary the parameters. The question arises as to whether the K_m values for nucleotide and P_i also vary with ΔpH. Although the predicted relationship between K_m values and ΔpH depends upon the reaction mechanisms chosen, the chemiosmotic hypothesis indicates that if changes in K_m values do occur, they are related to the magnitude of ΔpH rather than to the rate of electron flow. In thylakoids the K_m for ADP for phosphorylation at a given P_i concentration was found to increase in an approx. linear fashion with the V_{max} for ATP synthesis [24,16]. At low light intensity, ΔpH limits the rate of phosphorylation until the ADP concentrations approach levels far lower than those that would limit at higher light intensities. In other words, ΔpH and V_{max} for ATP synthesis are exponentially related, but K_m ADP is linearily related to ΔpH. However, Vinkler [25] reported that uncouplers increase the K_m for ADP, while decreasing ΔpH and phosphorylation rate, and suggested that the increase in K_m is controlled by electron flow, not ΔpH. However, we found that the K_m for ATP hydrolysis in the dark by light- and dithiothreitol-treated thylakoids was increased from approx. 30 μM to 150 μM by 4 mM NH_4Cl. In view of the similarity of the response of the K_m of photophosphorylation and ATPase activity for adenylates to uncoupler, a role of electron transport in this phenomenon must be questioned.

The interpretation of measured K_m values for ATP synthesis is not straightforward. Protons are substrates for ATP synthesis, and the K_m for ADP should be measured at constant internal proton concentration. At a constant, high light intensity, phosphorylation decreases ΔpH. As the ADP concentration is increased the rate of ATP synthesis also increases, causing a decrease in the internal proton concentration. Since the rate of phosphorylation depends strongly on ΔpH [7,8, 14], the decrease in ΔpH at higher ADP concentrations would lower the velocity of ATP synthesis relative to that expected if ΔpH were unchanged. This phenomenon would lead to an underestimation of the K_m for ADP. The increase in the measured K_m for ADP by uncouplers could be the result of a diminution of the effect of phosphorylation on ΔpH. If this is the case, low concentrations of uncouplers should increase the K_m and then decrease the K_m at higher concentrations as ΔpH falls. This interpretation is supported by the data shown in Table II in which both NH_4Cl and nigericin were used as uncouplers. If a value for the K_m ADP for ATP synthesis is assumed to be 2 μM + 0.10 V_{max}, where V_{max} is given as μmol P_i incorporated /h per mg chlorophyll, application of a model [26] describing photophosphorylation in terms of ΔpH yields the observed behavior of K_m for ADP on uncoupler concentration.

In passing, we note that the increase in K_m for ATP hydrolysis induced by uncouplers is probably not achieved by removing interactions between

TABLE II

UNCOUPLER-INDUCED CHANGES IN THE K_m OF PHOTOPHOSPHORYLATION FOR ADP

Reaction mixtures (0.5 ml) contained 100 mM Tricine-NaOH (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 0.4 mM $^{32}P_i$ (40 μCi/μmol), 0.05 mM methyl viologen, 0.04 mM EDTA, 0.005–0.08 mM ADP, uncoupler as indicated, and thylakoids equivalent to 5 μg of chlorophyll. Samples were illuminated ($1.75 \cdot 10^3$ J·m^{-2}·s^{-1} of white light) for 1 to 60 s. The K_m for ADP and V_{max} values were calculated from plots of [ADP]/phosphorylation rate vs. [ADP] concentration.

Uncoupler	K_m (μM)	V_{max} (μmol P_i incorporated per h per mg chlorophyll)
None	14	303
1 mM NH_4Cl	18	228
2 mM NH_4Cl	11	133
4 mM NH_4Cl	10	83
10 mM NH_4Cl	7	36
20 mM NH_4Cl	6	12
0.04 μM nigericin	17	263
0.08 μM nigericin	12	183
0.15 μM nigericin	11	91
0.70 μM nigericin	6	24
None, light intensity 10 J·m^{-2}·s^{-1}	3	20

ΔpH and the rate of ATP hydrolysis. Rather, it most likely arises from stimulation of the turnover rate of the ATPase, which seems to be the factor which controls the K_m values. This stimulation in turnover rate is much larger than the increase in V_{max} indicates, since Sherman and Wimmer [22] have shown that even as uncoupler stimulates overall ATPase activity it inactivates a substantial portion of the ATPase complexes.

Discussion

In this paper we have shown that phosphorylation by thylakoids depends on bulk phase ΔpH values and is independent of the source of the protons. Previously [9] we demonstrated that the synthesis of ATP by thylakoids in the dark, using ATP-dependent proton translocation as an energy source (ATP-P_i exchange), also depends on ΔpH in a manner identical to that of photophosphorylation. In our hands, protons released in the thylakoid lumen by water oxidation, plastoquinone oxidation, and by the operation of artificial cyclic electron flow around Photosystem I are equivalent with respect to their use in ATP synthesis.

In contrast to our results, De Kouchkovsky's laboratory reports that protons from Photosystem II have hindered access to the ATPase. Haraux and De Kouchkovsky [27] interpreted complex and subtle effects upon electron flow of substitution of ^2H_2O for ^1H_2O as indicating a substantial barrier to proton diffusion along the membrane surface. To lend credence to this proposal they presented theoretical calculations indicating that resistance to lateral proton diffusion along the membrane was not much smaller than resistance to proton transport across the membrane through the ATPase. These calculations are flawed by the assignment of a proton conductivity to the intact ATPase that is equivalent to an 0.8 nm pore through the membrane. Although this may be an adequate model for proton conductance through the membrane sector of the ATPase when no CF_1 is bount to it, the proton leak through the enzyme is normally low unless ATP is synthesized or hydrolyzed. Finally, in experiments similar to those reported in this paper, but using 9-aminoacridine to measure ΔpH, Haraux et al. [15] found that the relationship between ΔpH and phosphorylation using Photosystem II electron flow was distinctly different from that using electron transport through both photosystems. We believe this result to be artefactual. Haraux et al. [15] made no account of potential changes in internal volume. As mentioned in the Experimental Procedures section, this is important. Although quenching of 9-aminoacridine fluorescence is clearly related to ΔpH, the quenching is not a reliable quantitative measure ΔpH, especially at high ΔpH values [28]. In contrast, determination of ΔpH from hexylamine accumulation, estimated by silicone oil centrifugation, has been vindicated by a number of criteria. Dibromethylisopropylbenzoquinone (DBMIB), used in many of the experiments in question, reacts with amines [27]. The product of the reaction accumulates in the thylakoid lumen or membrane, causing an overestimation of ΔpH that increases with time [29]. In this regard, it is interesting to note that Haraux et al. [15] found that a higher apparent ΔpH was required to support a given rate of phosphorylation when electron flow from water to DBMIB was substituted for electron flow from water to ferricyanide. In our experiments we avoided use of DBMIB.

Inhibitors of electron transport and uncouplers of ATP synthesis in mitochondria were reported to affect the relationship between ATP synthesis rate and $\Delta\tilde{\mu}_{H^+}$ in a differential manner [30,31]. Very recently, however, Sogato et al. have reevaluated this claim [32]. They find that malonate and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone affect ATP synthesis and $\Delta\tilde{\mu}_{H^+}$ in submitochondrial particles equivalently. They attribute the failure of others to obtain this result to underestimation of $\Delta\tilde{\mu}_{H^+}$ in the presence of uncouplers. Also as pointed out by Portis and McCarty [14], very small changes in $\Delta\tilde{\mu}_{H^+}$ result in large changes in phosphorylation rate. These small changes in $\Delta\tilde{\mu}_{H^+}$ are often difficult to detect.

If uncouplers decrease bulk phase ΔpH, without a direct effect on local gradients [33], ΔpH should drop before the rate of phosphorylation is reduced during an uncoupler titration. Our data do not show such an effect. Uncouplers could also interact only with local gradients [34]. We cannot rule this out. However, large and relatively immobile

uncouplers (gramicidin and ATPase modified by cross linking within the γ subunit [8] or by removal of CF_1 [35]) uncouple many electron-transport chains, indicating that even if protons do not equilibrate with the bulk aqueous phases, they at least equilibrate along the membrane surface. Thus coupling, if not the gradients, is delocalized.

The dependence of the rate of phosphorylation upon ΔpH instead of pH_{in} per se is not surprising. Our results are consistent with those of analogous experiments performed with either light flashes [36,37] or electric-field pulses [38]. In these previous experiments $\Delta\Psi$ is the main component of $\Delta\tilde{\mu}_{H^+}$ instead of ΔpH as in the present experiment. All of these experiments are at odds with simple versions of Mill's and Mitchell's dual pH optimum description of ATPase activity [39]. The approximate linearity of time-courses in which $\Delta\Psi$ is replaced by ΔpH [40] and the interchangeability of $\Delta\Psi$ and ΔpH in experiments in which phosphorylation is driven by artificially imposed gradients [41] both indicate that $\Delta\Psi$ and ΔpH are equivalent as driving forces of phosphorylation. This is consonant with control of phosphorylation rate by ΔpH instead of internal pH. The importance of gradients instead of absolute concentrations indicates that the rate of phosphorylation is controlled by proton translocation.

The slope of the logarithmic plot of rate of ATPase activity vs. ΔpH in thylakoids is much steeper than that found by Dewey and Hammes in a reconstituted system [23]. This could reflect the different techniques used to measure ΔpH. It may be the result of the different range of rates, as at the low rates used by Dewey and Hammes activation of ATPases may occur as ΔpH is increased, partially masking the inhibitory effect of ΔpH upon turnover of already active complexes. The kinetic and regulatory properties of the reconstituted system need not be identical to those of the enzyme in its native membrane. In line with this, Dewey and Hammes [23] found the K_m values for ADP for ATP synthesis by reconstituted vesicles to be independent of ΔpH. Finally, we have measured ATPase activity and ΔpH in the presence of P_i (from slow pyrophosphate hydrolysis), i.e., not under initial rate conditions. Rates of ATP hydrolysis are not changed by substituting pyruvate kinase and phospho*enol*pyruvate for P_i [22,16], but the same may not be true for ΔpH. This may be significant, as our data are not readily reconciled with the modified Haldane relation developed by Dewey and Hammes [23].

The increase in the rate of ATP synthesis with increasing ΔpH may be caused by an increase in the turnover rate of each individual ATPase. Data supporting this view are (a) the decrease in intermediate ^{18}O exchange as light intensity is increased [22]; (b) the linear increase in K_{ADP} with V_{max} as light intensity is increases * [24].

However, the ATPase is thought to assume an inactive conformation at low $\Delta\tilde{\mu}_{H^+}$. This concept is based upon: (a) the evidence for massive conformational changes upon energization; (b) the inability of thylakoid membranes to catalyze significant ATP hydrolysis in the dark; (c) the ability of mild proteolysis or reduction by thiols to reveal the latent dark ATPase; (d) the inactivation of this ATPase activity by extremely low $\Delta\tilde{\mu}_{H^+}$, i.e., by uncouplers [20]. On the basis of a linear relationship between the extent of release of tightly bound ADP in the light and the associated rate of ATP synthesis, Gräber [4] has concluded that release of bound ADP measures activation and that the entire increase in the rate of ATP synthesis with ΔpH is due to an increase in the number of active ATPases, and not to an increase in the turnover rate per ATPase. In this view, dithiothreitol activation results from a considerable reduction in the $\Delta\tilde{\mu}_{H^+}$ required to activate a given fraction of the ATPases. Fig. 4 shows that the dependence of rate of ATP hydrolysis upon ΔpH is equal but opposite to that of synthesis. This is difficult to reconcile with Gräber's description [4], but the observation needs to be extended to a wider range of conditions. More discriminating tests of the role of activation can be obtained from quantitative studies of relaxation kinetics after changes in

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* Bickel-Sandkötter and Strotmann [24] measured K_{ADP} at a series of constant light intensities. The K_{ADP} should be measured at constant ΔpH, a more difficult experiment. However, the results in Table II suggest a similar relationship under conditions in which increases in [ADP] should not decrease ΔpH. Our experiments indicate that K_{ADP} (and K_{GDP}) approach limiting values at near-zero rates of phosphorylation, as the data, but not the discussion, in Bickel-Sandkötter and Strotmann [24] indicate.

$\Delta\tilde{\mu}_{H^+}$, redox potential (i.e., dithiothreitol) and [ADP] as functions of these same variable. Despite some reservations we regard Gräber's formulation as a useful framework for discussion of the kinetics of the ATPase.

In conclusion, the relationships between ΔpH, ATP synthesis and hydrolysis and electron flow described in this and previous papers [7–10,14] provide strong support to the concept that steady-state ATP synthesis in thylakoids is driven by transmembrane, delocalized proton gradients. We have also shown here that the effects of light intensity and of uncouplers on apparent K_m values for ADP for phosphorylation are not, as claimed previously [25], inconsistent with the chemiosmotic view of coupling. Moreover, the double-inhibitor titration approach [34], often cited as evidence against delocalized gradients, has been shown [16,41] to produce results consistent with either localized or delocalized gradients. There is a tendency among investigators to interpret observations which at first glance do not fit into the chemiosmotic framework as proof that local proton-activity gradients drive ATP synthesis. Since the coupling between steady-state electron flow and ATP synthesis in thylakoids is best described in quantititative as well as qualitative terms in relation to ΔpH, the chemiosmotic viewpoint should be given more serious consideration.

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