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The rate of ATP-synthesis as a function of ΔpH and $\Delta\psi$ catalyzed by the active, reduced H^+-ATPase from chloroplasts

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The H^+-ATPase from chloroplasts was brought into the active, reduced state. Then, an electrochemical potential difference of protons across the thylakoid membranes was generated by an acid–base transition, ΔpH, combined with a K^+/valinomycin diffusion potential, $\Delta\psi$. The initial rate of ATP synthesis was measured with a rapid-mixing quenched-flow apparatus in the time-range between 20–150 ms. The rate of ATP synthesis depends in a sigmoidal way on ΔpH. Increasing diffusion potentials shifts the ΔpH-dependencies to lower ΔpH values. Analysis of the data indicate that the rate of ATP synthesis depends on the electrochemical potential difference of protons irrespective of the relative contribution of ΔpH and $\Delta\psi$.

Chloroplast; H^+-ATPase; Enzyme-kinetics

1. INTRODUCTION

Upon illumination, chloroplasts can synthesize ATP from ADP and phosphate. According to the chemiosmotic theory [1,2] the light-driven electron transport leads to the generation of a transmembrane electric potential difference, $\Delta\psi$, and a transmembrane pH difference, ΔpH. This electrochemical potential difference of protons, $\Delta\tilde{\mu}_{H^+}$, gives rise to a proton flux across the membrane, and the membrane-bound H^+-ATPase can use the energy from this 'down-hill' proton flux for synthesis of ATP.

ATP synthesis in chloroplasts can be driven by an artificially generated ΔpH (acid–base-transition [3]) and $\Delta\psi$ (external electric field [4]). In this work, we investigated quantitatively the influence of ΔpH and $\Delta\psi$ on the rate of ATP-synthesis.

The H^+-ATPase was brought into the active, reduced state, E_a^{red}, by illumination in the presence of dithiothreitol. Then, thylakoids were incubated in an acidic medium until all ions were completely equilibrated across the membrane. Rapid mixing with a second (basic) medium changed the external concentrations rapidly, whereas the internal concentrations remained constant for a short time. Therefore, pH_{out} and pH_{in} can be measured by glass electrodes and $\Delta\psi$ can be calculated from the well-known ionic composition of the internal and external phase. The only problem is that ATP synthesis must be measured in a short time interval after mixing so that the initial conditions remain constant during the reaction time. Here, we measured the initial rate of ATP synthesis with a rapid-mixing quenched-flow apparatus [5]. Preliminary results were reported in [6].

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2. MATERIALS AND METHODS

Class II chloroplasts were prepared from spinach as described elsewhere [5]. Reduction of the enzyme was performed as follows: the chloroplasts were illuminated for 5 min at pH 8.2 in a medium which contained 4.6 mM tricine, 5 mM NaH_2PO_4, 2 mM $MgCl_2$, 10 mM dithiothreitol, 100 μM methylviologen, 135 μM chlorophyll, 8.5 mM KOH, 51.5 mM KCl (reaction condition A, see below). In order to obtain lower K^+-concentrations (reaction conditions B and C) the reduction medium contained instead of KOH and KCl, 8.5 mM NaOH and 10 mM sorbitol.

Immediately after illumination, when the enzyme is in the form E_a^{red}, chloroplasts were subjected to an acid–base transition. Combination of an acid–base transition with a change in K^+ concentration (in the presence of valinomycin) leads to the generation of ΔpH and $\Delta\psi$ which both can be varied independently from each other. The thylakoid membranes were subjected to the acid base transition as follows:

(a) Acidic stage: 250 μl reduced chloroplasts in the reduction medium were mixed with the same volume of the acidic medium and incubated for 30–40 s. The composition of the solution after mixing with the reduction medium is given in Table I for reaction conditions A, B and C. The pH in the acidic stage was varied between 4.7 and 8.0.

(b) Basic stage: after the acidic stage the chloroplasts were mixed with an equal volume of the basic medium. The composition of the solutions during the basic stage is given in Table I. The pH was adjusted so that after mixing with the acidic medium the final pH was always 8.2 ± 0.05. The reaction was stopped by the addition of an equal volume of 4% trichloroacetic acid.

Short reaction times (20–150 ms) were achieved with a rapid-mixing quenched-flow system (Durrum D 133) as described earlier [5]. ATP was measured by luciferin/luciferase [5].

The ATP concentration at reaction-time zero, ATP_0, resulted from

Table 1

Composition of the media during acidic incubation and during the basic stage for reaction conditions A, B and C

	Reaction condition (acidic/basic)		
	A	B	C
Tricine (mM)	1.5/100.8	1.5/100.8	1.5/100.8
DTT (mM)	3.3/ 1.7	3.3/ 1.7	3.3/ 1.7
MV (μM)	33.3/ 16.7	33.3/ 16.7	33.3/ 16.7
Valinomycin (μM)	0.7/ 0.3	0.7/ 0.3	0.7/ 0.3
DCMU (μM)	10.0/ 10.0	10.0/ 10.0	10.0/ 10.0
Chlorophyll (μM)	44 / 22	46 / 23	50 / 25
Sorbitol (mM)	– / –	3.3/ 1.7	3.3/ 1.7
Succinic acid (mM)	20.0/ 10.0	6.7/ 3.3	6.7/ 3.3
NaOH (mM)	– / 23–10	8–18/ 6–4	8–18/ 6–4
KOH (mM)	12–43/ 56–72	– / 60–58	– / 60–58
KCl (mM)	88–57/ 44–28	6.0/ 3–5	0.6/ 0.3–2.3
$MgCl_2$ (mM)	2.0/ 2.0	2.0/ 2.0	2.0/ 2.0
NaH_2PO_4 (mM)	5.0/ 5.0	5.0/ 5.0	5.0/ 5.0
K-ADP (μM)	– /100	– /100	– /100
ATP_0 (nM)	– /118±32	– /118±30	– /121±32

ATP present in the chloroplasts and ATP present in commercially available ADP.

Usually, the rate of ATP synthesis is based on the chlorophyll concentration. However, the concentration of the H^+-ATPase, CF_0F_1, is relevant for ATP synthesis, and the ratio chlorophyll/CF_0F_1 can vary considerably [7]. In order to allow a better comparison between different chloroplast preparations, the ratio chlorophyll/CF_0F_1 was determined by rocket immune electrophoresis [7] and all the data in this work were based on the concentration of CF_0F_1. The ratio of chlorophyll/CF_0F_1 varied between 600 and 1200. All measurements were carried out at room temperature.

3. RESULTS

Fig. 1 shows the ATP yield as a function of the reaction time after an acid–base transition. Measurements were carried out in the time range between 20 and 150 ms where a linear increase of the amount of synthesized ATP with the reaction time is observed. During this time the ΔpH and $\Delta\psi$ remain constant. The slopes give directly the rate of ATP synthesis and the numbers at the slopes give the rate in ATP/(CF_0F_1·s). The curves are displaced arbitrarily along the ordinate for a clearer presentation of the data. Actually, they all start at the same initial ATP concentration (ATP_0, see Table I). In Fig. 1A, measurements at different ΔpH values are shown for reaction condition A, i.e. when any diffusion potential is eliminated by high internal and external K^+-concentrations in the presence of valinomycin. Fig. 1B and C show measurements at different ΔpH values for reaction conditions B and C, respectively, when diffusion potentials of about 55 mV or 90 mV, respectively (see below) were superimposed.

Fig. 2 shows the rate of ATP synthesis as a function of ΔpH for different diffusion potentials (data from Fig. 1 and additional sets of experiments). In all three cases a sigmoidal dependence of the rate of ATP synthesis on ΔpH is observed. The maximal rate of ATP synthesis is (380 ± 20) ATP/(CF_0F_1·s) and the same rate is observed for all three diffusion potentials. However, the addi-

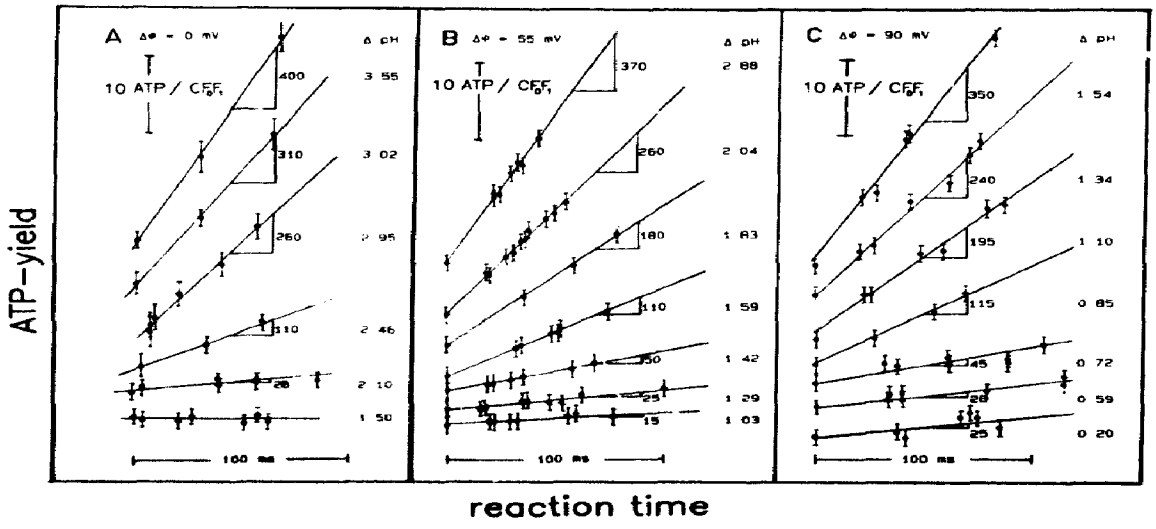

Fig. 1. ATP yield as a function of the reaction time. The enzyme was brought into the active, reduced state and then an acid–base jump was carried out, followed by denaturation with trichloroacetic acid after the time intervals indicated in the figure. The pH_{out} was always 8.2 ± 0.05, the internal pH was changed and the resulting ΔpH is given in the figure. The numbers at the slope give the rate of ATP synthesis in ATP per CF_0F_1 per s. (Panel A) Reaction conditions A (no $\Delta\psi$); (Panel B) reaction conditions B ($\Delta\psi$ ≈55 mV); (Panel C) reaction conditions C ($\Delta\psi$ ≈90 mV).

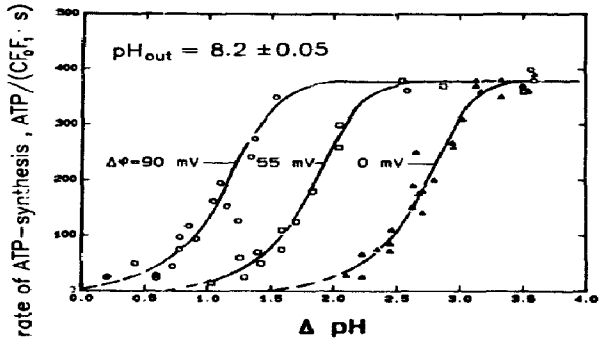

Fig. 2. The rate of ATP synthesis as function of ΔpH at different superimposed diffusion potentials. Data was taken from Fig. 1 and additional sets of experiments.

tional diffusion potential shifts the curve to a lower ΔpH value. The half-maximal rate of ATP synthesis is reached for reaction condition A at ΔpH=2.7, for reaction condition B at ΔpH=1.8 and for reaction condition C at ΔpH=1.1. The data for conditions A–C can be fitted with the same function if it is displaced along the ΔpH axis by a certain value. These values are listed in Table II (column 4).

Since the diffusion potential under reaction condition A is about zero, we can calculate experimental values for the diffusion potentials from the ΔpH displacement of the curves B and C. With 59 mV for 1 pH unit (T=298 K), we obtain for reaction condition B $\Delta\psi_{exp}$=53 mV and for reaction condition C $\Delta\psi_{exp}$=95 mV.

With $\Delta\psi$ calculated from the Goldman equation as described in [5], and the experimental ΔpH, the electrochemical potential difference of protons, $\Delta\tilde{\mu}_{H^+}$, is calculated. The data from Fig. 2 are replotted as a function of $\Delta\tilde{\mu}_{H^+}$ and of the proton motive force, $P = \Delta\tilde{\mu}_{H^+}/F$ (F = Faraday constant) (Fig. 3). All the data can now be described by a single function. This result shows that for the present conditions ΔpH and $\Delta\psi$ are kinetically equivalent in driving ATP synthesis, i.e. a change of ΔpH by 1 unit changes the rate by the same factor as a change of 59 mV in $\Delta\psi$. The smallest rate which can be measured reliably in the time range of our experiments is about 10–20 ATP/(CF$_0$F$_1$·s). Therefore, the curves in Figs. 2 and 3 below this value are dashed.

Table II

Calculated diffusion potentials for the reaction conditions A, B and C, and experimental data from Fig. 2

Reaction condition	$\Delta\psi$ (mV)		
	Nernst	Goldmann	From ΔpH shift
A	0	0	0 (0)
B	59	55	53 (0.9)
C	118	90	95 (1.6)

Figures in parentheses indicate shift in ΔpH.

4. DISCUSSION

The data presented here show that all rate measurements can be described by a single function when the data for reaction conditions B and C are shifted by a constant ΔpH value. For a quantitative evaluation the absolute value must be known. Whereas ΔpH can be easily measured with a glass electrode, the magnitude of $\Delta\psi$ must be calculated from the ionic composition of the internal and external aqueous phase. The concentration of the different substances in the acidic and the basic stage are listed in Table I. We assume that during acidic incubation a complete equilibration across the membrane is reached. The external concentrations arise from the mixing of the acidic and basic medium.

In a first approximation $\Delta\psi$ was calculated from the Nernst equation, i.e. it was assumed that the membrane is semipermeable for K^+. The resulting $\Delta\psi$ is listed in Table II. In a second approximation, $\Delta\psi$ was calculated from the Goldmann equation using the same permeability coefficients as described earlier [5]. In this approximation only H^+, Na^+, Cl^- and the K^+/valinomycin complex are taken into account. The influence of other monovalent ions and of all divalent ions is neglected. Furthermore, neither the phosphorylation-coupled proton flux nor the effect of surface potentials is taken into account. The resulting $\Delta\psi$ is listed in Table II.

For the comparison of the calculated diffusion potentials with the experimental data (as estimated from the ΔpH shift, see Table II) we can draw the following conclusions: (1) below 55 mV there is no significant difference between the $\Delta\psi$ from the ΔpH shift and that calculated from Nernst or Goldmann equation; (2) at higher $\Delta\psi$ the Nernst potential is significantly higher than the Goldmann potential, however, the Goldmann potential agrees with the experimental $\Delta\psi$ from the ΔpH

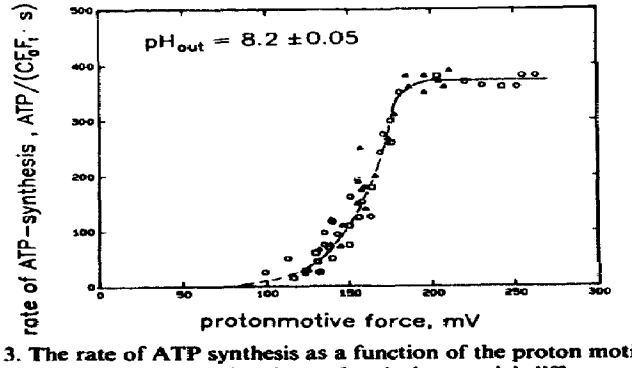

Fig. 3. The rate of ATP synthesis as a function of the proton motive force (bottom scale) and the electrochemical potential difference of protons (top scale). Data was taken from Fig. 2.

shift. Therefore, the Goldmann equation gives an appropriate description for the diffusion potential at thylakoid membranes under these conditions; (3) within the limits of this approximation, we can conclude that ΔpH and $\Delta\psi$ are kinetically equivalent, i.e. a change of pH by 1 unit changes the rate of ATP synthesis by the same factor as a change of $\Delta\psi$ by 59 mV.

The energetic equivalence of ΔpH and $\Delta\psi$ at equilibrium is an essential feature of the chemiosmotic theory [1,2]. The kinetic equivalence far from equilibrium is not required by the chemiosmotic theory. There are several mechanisms by which the enzyme can sense the transmembrane field and which can give rise to a kinetic equivalence of ΔpH and $\Delta\psi$.

(1) The transmembrane $\Delta\psi$ is converted into an intramembrane ΔpH by a proton well [8]. This requires an electrochemical equilibrium between protons in the internal aqueous bulk phase and protons in the F_0-part of the enzyme. Additionally, the F_0-part must be selectively permeable for protons.

(2) One of the rate constants in the reaction cycle of the enzyme can be changed by $\Delta\psi$ according to the Butler–Volmer equation [9]. The kinetic equivalence of $\Delta\psi$ and ΔpH imposes some restrictions for the models and the rate constants [10,11]. The conditions under which kinetic equivalence between ΔpH and $\Delta\psi$ can be observed were discussed by Hansen et al. [10]. This behaviour is expected when the rate constants for charge translocation and for protonation are much larger than those of the other steps of the cycle.

(3) The transmembrane electric field can also interact directly with the dipole moment of the membrane-bound enzyme thereby changing at least one rate constant in the reaction cycle ('electroconformational coupling' [12,13]).

As discussed in a previous paper [6] measurements of the rate of ATP synthesis immediately after generation of the active, reduced enzyme, E_a^{red}, are not kinetically controlled by a preceding activation process. The curves in Figs. 2 and 3 reflect, therefore, the dependence of the catalytic reaction on $\Delta\tilde{\mu}_{H^+}$, i.e. the data indicate a kinetic equivalence of ΔpH and $\Delta\psi$ for driving ATP synthesis.

Also, when the enzyme is, at the beginning of the rate measurements (i.e. before the acid–base transition), in the state E_i^{ox}, kinetic equivalence of ΔpH and $\Delta\psi$ was found [5]. However, these curves reflect mainly the preceding activation process of the enzyme. Therefore, one must conclude that ΔpH and $\Delta\psi$ are equivalent for the catalytic reaction as well as for the activation process.

For ATP synthesis in chromatophores from *Rhodospirillum capsulatus* driven by artificially induced ion gradients, a kinetic equivalence between ΔpH and $\Delta\psi$ was found [14]. In addition such a kinetic equivalence for the H^+-ATPase from *Rhodospirillum rubrum*, reconstituted into asolectin liposomes, was reported [15].

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