ATP SYNTHESIS IN CHLOROPLASTS INDUCED BY A TRANSMEMBRANE ELECTRIC POTENTIAL DIFFERENCE AS A FUNCTION OF THE PROTON CONCENTRATION

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1. Introduction

According to the chemiosmotic hypothesis, ATP synthesis is coupled to the translocation of protons through the membrane-bound ATPase, the latter being driven by the electrochemical potential gradient [1]. Experimental evidence for this mode of action of ATPase in photosynthesis is outlined in [2]. The proton translocation takes place through the hydrophobic component (CF_o) of the ATPase. The hydrophilic component (CF_1) is attached at one end of the proton-conducting channel (CF_o) and contains the catalytic sites where ATP synthesis and ATP hydrolysis occur.

The molecular mechanism of (a) proton transport through the ATPase and (b) coupling between the proton translocation and the catalytic reaction is a matter of discussion, and several models have been proposed [3]. The rate of ATP synthesis, which is coupled to the proton efflux, may depend on the protonation state of functional groups. The latter may be involved in the translocation of H^+ or the reaction at the catalytic site. Therefore, conclusions concerning the mechanism may be drawn from the dependence of the rate of ATP synthesis on the pH value, provided that other parameters controlling the rate (e.g., the extent of the transmembrane electrochemical proton gradient) can be kept constant. The problem of the pH dependence is further complicated by the fact that the ATPase complex, coupling proton translocation from the internal phase to the external phase with the formation of ATP, is exposed to both phases. The enzyme activity, therefore, will depend on the pH value of the internal phase (pH_{in}) and on that of the external medium (pH_{out}).

The rate of phosphorylation measured in continuous light (linear electron transport) depends strongly on the pH_{out} value and shows an optimum around pH_{out} 8.4 [4,5]. As electron transport and formation of the pH gradient are functions of pH_{out} [5,6], the observed pH dependence reflects the superposition of the pH dependence of both energization and phosphorylation. Especially between pH_{out} 7 and pH_{out} 8.5 the increase in the pH gradient is very similar to that in the rate of phosphorylation [6]. These measurements were made under continuous light using conditions for cyclic electron transport [6].

By excitation with periodic light flashes a steady state for ΔpH can be obtained and can be changed independently of the initial flash-induced transmembrane electric potential difference ($\Delta\psi$). Therefore, a constant energization can be maintained at different pH_{out} values. Under these experimental conditions it was found that the rate of ATP synthesis does not depend on pH_{out} between pH_{out} 7–9 [7].

This work analyses the dependence of the rate of ATP synthesis on pH_{out} under conditions where ATP formation is induced by a transmembrane electric potential difference ($\Delta\psi$) only ($\Delta pH \approx 0$) and where the magnitude of $\Delta\psi$ is kept constant at the different pH values between pH_{out} 5–9. To realize these conditions, we adopted two different experimental approaches:

(1) ATP synthesis generated by external electric field pulses [8] was measured at pH values between pH_{out} 5–9. This method was used for the following reasons:

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(i) Electron transport from H_2O to $NADP^+$ and formation of a pH gradient between the aqueous bulk–bulk phases can be excluded ($pH_{out} = pH_{in}$);
(ii) The magnitude of the transmembrane electric potential difference generated artificially by the external electric field pulses does not depend on pH_{out} in this range [9].

(2) ATP synthesis generated by saturating light pulses of 20 ms duration was measured over the same pH_{out} range. The experiments were carried out in the presence of permeable buffers (section 4) to prevent the generation of a pH gradient, i.e., also in this case $\Delta pH \approx 0$ and ATP formation is driven by the light-induced $\Delta\psi$, only.

2. Materials and methods

Broken chloroplasts were prepared as in [4] from spinach grown either in a phytocell or obtained from the local market. Additionally, 10 mM ascorbate was added during grinding. The freshly prepared chloroplasts were stored in an ice bath and used within 3 h after preparation.

(1) In experiments with external voltage pulses the chloroplasts were suspended in a cuvette between 2 flat platinum electrodes (2 mm electrodes; 5 cm^2 area) as in [8]. Voltage pulses of 220 V with a duration of 30 ms were applied. Experiments with 2, 4 and 6 voltage pulses were performed in the dark with the voltage polarity being changed after each pulse. The cuvette was maintained in an ice bath throughout each experiment. The time interval between the pulses was chosen to be long enough to allow reversal of the heating of the solution generated by the voltage pulse. The principle of the external electric field method has been described in [9]. The reaction medium contained 10^{-4} M KCl, 5×10^{-4} M $MgCl_2$, 5×10^{-4} M K_2HPO_4, 3×10^{-4} M ADP, 5 μCi ^{32}P/ml and chloroplasts from the suspension medium giving 4×10^{-4} M chl. Buffer was 5×10^{-3} M. The following buffers were used:
At pH 5.0 and 5.5, succinic acid (pK 5.57);
At pH 6, 2-(*N*-morpholino)-ethane sulfonic acid (pK 6.15);
At pH 6.5, maleic acid (pK 6.26);
At pH 7–9, *N*-Tris(hydroxymethyl)methylglycine (pK 8.15);
At pH 9.5 and 10.0, glycine (pK 9.6) adjusted to the corresponding pH values with NaOH or HCl.
The reaction volume was 1 ml.
The suspension medium contained: 5×10^{-4} M $MgCl_2$, 0.4 M sucrose, and 5×10^{-4} M tricine adjusted to pH 8 with NaOH.

(2) In photo-shutter experiments saturating light pulses of 20 ms duration were used. The dark time between the pulses was 30 s. The reaction medium contained: 10^{-2} M buffer adjusted to the respective pH values with NaOH or HCl, 10^{-2} M KCl, 10^{-2} M sucrose, 5×10^{-3} M $MgCl_2$, 10^{-4} M benzylviologen, 5×10^{-3} M K_2HPO_4, 5 μCi ^{32}P/ml, 3×10^{-4} M ADP, and chloroplasts from the suspension medium giving 2×10^{-4} M chl. The buffers were the same as those used in the external electric field experiments. The suspension medium contained: 10^{-2} M KCl, 5×10^{-3} M $MgCl_2$, 0.4 M sucrose, 5×10^{-4} M tricine (pH 8). Illumination of 1 ml reaction medium was carried out in an optical cell (optical pathlength: 1.4 mm, white light, light intensity 10^6 erg . cm^{-2} . s^{-1}). To keep the formation of a pH gradient during light pulses as small as possible, the chloroplasts were preincubated in the reaction medium containing a high concentration of permeable buffers for ~20 min before illumination. ATP formation was measured by the incorporation of ^{32}P as in [10,11].

(3) The accelerated charge efflux of ions under phosphorylating conditions was measured as in [12–14]. The suspension and reaction media used in these experiments were the same as those used in the photo-shutter experiments. Illumination of 1 ml reaction medium was achieved by flash groups. Each group consisted of 5 or 8 saturating single turnover flashes (~20 μs flash duration; 2 ms dark time between flashes; 10–30 s between the flash groups; 610–730 nm wavelength). The transmembrane electric potential difference induced by the flash groups was measured by the electrochromic absorption changes at 515 nm [12] in a flash spectrophotometer [15]. The electric potential difference was calculated using the relation:

$$\Delta\psi = \frac{\Delta A_{515}}{{}^{1}\Delta A_{515}} \; 50 \text{ mV}$$

where ${}^{1}\Delta A_{515}$ denotes the absorption change which is induced by a single saturating short flash of light

[2,16]. The ion efflux, which can be calculated from the decay rate of the absorption change, was determined under phosphorylating (+ADP, $+P_i$) and under non-phosphorylating (–ADP, $-P_i$) conditions. The difference in efflux under the 2 regimes reflects the additional, accelerated ion efflux which can be correlated to phosphorylation. Integration of this separated ion efflux with respect to time yields the number of ions correlated to phosphorylation.

3. Results

Fig.1 shows the relative amount of ATP generated by external electric field pulses as a function of pH_{out}. The duration of the external electric field pulses was 30 ms. The ATP yields in each set of experiments were standardized relative to the amount of ATP generated at $pH_{out} = 8$. This was done to eliminate scattering resulting from differences between chloroplast preparations. The ATP yield does not depend on pH_{out} between pH 6.5 and pH 8.6. Above pH_{out} 8.6 and below pH_{out} 6.5 the ATP yield decreases. Half maximal ATP yield has been observed at pH_{out} 5.8 and pH_{out} 9.4, respectively.

Under the experimental conditions, the average value of $\Delta\psi$ has been estimated to be ~200 mV [8]. By applying external electric field pulses to a chloroplast suspension, the formation of a pH gradient between the aqueous bulk–bulk phases can be excluded, i.e.,

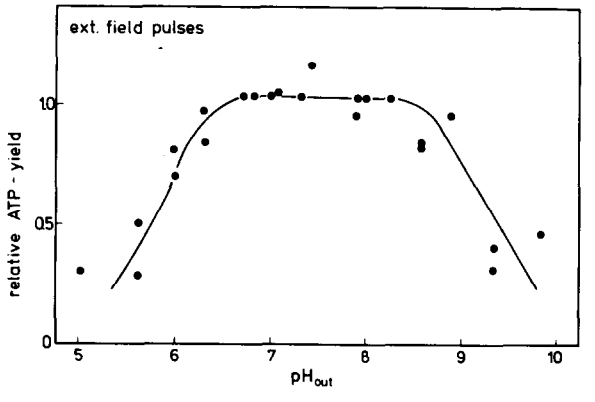

Fig.1. Relative amount of ATP generated by external electric field pulses as a function of pH_{out}. The data are standardized relative to the ATP yield obtained in one external electric double pulse of 30 ms duration at $pH_{out} = 8$ (average value of different measurements: 9.0×10^{-4} ATP/chl pulse). External electric field strength: 1100 V/cm.

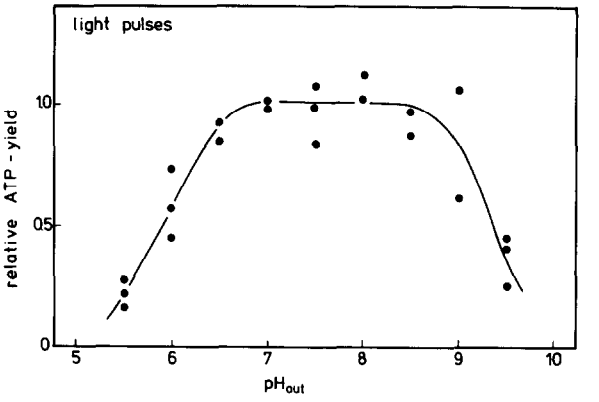

Fig.2. Relative amount of ATP generated by light pulses of 20 ms duration as a function of pH_{out}. The data are standardized relative to the ATP yield obtained in one light pulse at $pH_{out} = 8$ (average value of different measurements: 8.0×10^{-4} ATP/chl pulse).

$pH_{out} = pH_{in}$. The ATP-yield of 2 external electric field pulses should be compared to the ATP-yield of one light pulse. The reason for this is that the transmembrane electric potential difference generated by an external electric field has the right polarity for phosphorylation only in one-half of each vesicle (positive inside and negative outside).

Fig.2 shows the relative amount of ATP generated by photo-shutter light pulses of 20 ms duration as a function of pH_{out}. The ATP yield in each set of experiments has been standardized as in fig.1.

The light pulses induce a maximum transmembrane electric potential difference of ~150 mV as measured by the field-indicating absorption changes at 515 nm [17]. The light pulse experiments were carried out in the presence of permeable buffers (section 2) to prevent the generation of a light-induced pH gradient, i.e., also in this case, $pH_{out} \approx pH_{in}$ (see section 4). Similarly, as in fig.1, the ATP yield does not depend on pH_{out} between pH 6.5 and pH 8.6. Half-maximal ATP yield was observed at about pH_{out} 5.9 and pH_{out} 9.4.

Table 1 shows, as a function of pH, the stoichiometric relationship between the accelerated efflux of monovalent ions and phosphorylation. These results were obtained by flash group experiments. The number of ions per ATPase and flash group (upper line) is approximately independent of pH_{out} between pH 7 and pH 9. Furthermore, the number of ATP molecules per ATPase and flash group (centre line) is constant

Table 1
The ion/ATP ratio as a function of pH_{out}

pH_{out}	6	7	7.5	8	8.5	9
ions translocated / ATPase flash group	2.3	3.1	2.6	3.0	3.0	3.7
ATP generated / ATPase flash group	0.4	1.0	0.8	1.0	1.0	1.2
ions / ATP	5.7	3.1	3.2	3.0	3.0	3.1

Top: Number of positive monovalent ions correlated to phosphorylation as a function of pH_{out}

Center: Amount of ATP generated per ATPase and flash group as a function of pH_{out}

Bottom: Ratio between the number of translocated ions and the number of generated ATP molecules as a function of pH_{out}

between pH 7 and pH 9 and decreases to about half at $pH_{out} = 6$. This result is comparable to those obtained by other techniques (fig.1,2). The ratio between the number of translocated ions and the number of generated ATP molecules is ~3 between pH_{out} 7 and pH_{out} 9 and increases by a factor of ~2 at pH_{out} 6.

4. Discussion

Our results concerning the pH-dependence of phosphorylation were obtained under experimental conditions where ATP formation was induced by a transmembrane electric potential difference ($\Delta\psi$). More specifically, using the external electric field method, the transmembrane electric field was generated artificially and the pH gradient between the aqueous bulk–bulk phase was zero, i.e., $pH_{out} = pH_{in}$. Furthermore, the magnitude of the membrane potential induced by the external electric field is independent of the pH between pH 5.0–9.0 [9]. To test the results obtained by artificial energization, we have performed light-pulse experiments. In this case, the maximum light-induced transmembrane electric potential difference measured by the field-indicating absorption changes at 520 nm is constant between pH_{out} 6.0 and pH_{out} 9.0 and amounts to ~150 mV [17]. The light-induced formation of the membrane potential is always coupled to a proton translocation to the inner phase, i.e., with the generation of a pH gradient. To minimize the generation of a ΔpH, the chloroplasts were pre-incubated for ~20 min in the reaction medium containing permeable buffers. Assuming that the buffer concentrations of the internal and external phase are about equal, the ΔpH can be estimated to be <0.2, i.e., in this case too, $pH_{in} \approx pH_{out}$.

The results presented in fig.1,2 indicate that ATP synthesis does not depend on pH_{out} between 6.5 and 8.6 provided that a constant electric potential difference is set up across the membrane. This implies that, within this pH range, $\Delta\psi$ alone and not the absolute proton concentration determines the rate of ATP synthesis.

The maximum ATP yield of 9.0×10^{-4} ATP/chl . pulse induced by the external electric field pulse of 30 ms duration corresponds to a rate of 30×10^{-3} ATP/chl . s. About the same rate can be estimated for the light-pulse experiments. Whether the pH_{out}-dependence of phosphorylation observed here for linear electron transport (fig.2) is also realised for cyclic electron transport [18,19] is not known.

We are not yet able to give a detailed interpretation of the observed pH_{out} independence of ATP synthesis in the range of pH 6.5–8.6, because the result may be a superposition of several pH-dependent partial reactions:

(1) The standard free enthalpy change of ATP synthesis, ΔG_p^o, rises with increasing pH from pH 6–9, by ~12 kJ/M [20].

(2) The H^+-translocating activity of the proton conducting channel (CF_o) may depend on the pH value. The passive H^+-conductivity has been investigated using vesicles reconstituted from the ATPase-proton channel (TF_o) of the thermophilic bacterium, PS 3, and phospholipids. The pH dependence of the H^+-conductivity indicates a monoprotic proton binding site in TF_o ($pK_a \approx 7$) [21].

(3) The preceding activation of the membrane-bound ATPase [11,22] may depend on pH_{out}.

(4) The protonation state of the substrates as well as that of groups involved in binding the substrates may affect the apparent K_m values (referring to all

ionization states of the substrates). For example, if $Mg-ADP^-$ and $Mg-P_i$ are the active form of the substrates of phosphorylation [23], their apparent K_m value will decrease with increasing pH from pH 5.0–8.0.

(5) There might be a change in the ionization state of groups involved in the catalysis of the ATPase reaction.

Investigations are being prepared in order to separate the influence of these different effects.

The decrease of the ATP yield above pH_{out} 8.6 is most probably caused by an inactivation of the enzyme. The degree of inactivation depends on the quality of the chloroplasts and the incubation time at the corresponding pH (M. R., unpublished). The decrease below pH_{out} 6.5 cannot be explained by inactivation alone. However, considering the results in table 1, one might suggest that below pH_{out} 6.5 the coupling between the vectorial proton transport through the membrane-bound ATPase and the catalytic reaction is affected. Assuming that the number of ions correlated to phosphorylation (see table 1, top) corresponds to the number of H^+ translocated through the ATPase pathway, an increase of the H^+/ATP ratio by a factor of 2 at pH_{out} 6 results. Furthermore, the participation of 2 functional groups with $pK \approx 5.9$ and 9.4 may be responsible for the decrease of the ATP yield above pH_{out} 8.6 and below pH_{out} 6.5.

A comparison of these results with the pH dependence of the rate of phosphorylation measured in continuous light should be made mainly to point out 2 differences in the experimental conditions:

(1) The strong pH_{out} dependence with the optimum around pH_{out} 8.4 may reflect mainly the pH_{out} dependence of the energization, as the light-induced ΔpH and the rate of phosphorylation [6] depend on pH_{out} in a similar way. In contrast, in the present work the pH_{out} dependence was analyzed using conditions where the magnitude of the energization ($\Delta\psi$) was kept constant at different pH values between pH_{out} 5 and pH_{out} 9.

(2) In continuous light phosphorylation is mainly driven by the pH gradient; whereas, in our experiments, ATP synthesis was induced by a transmembrane electric potential difference only. The rate of ATP synthesis induced by an acid–base jump has been studied at a fixed ΔpH by changing both the acidic (pH_{in}) and basic (pH_{out}) pH values [24]. Although the magnitude of energization has been kept constant, a strong pH_{out} dependence was observed [24]. This result indicates that the rate of ATP synthesis induced by a pH gradient is not only determined by the magnitude of the ΔpH but is also regulated by pH_{in} and pH_{out}. These conditions are remarkably different from those used in this work where ATP synthesis is driven by $\Delta\psi$ only.

These results may prove important for selecting models to describe the mechanism of phosphorylation. Such a model should accommodate the pH-independent range (pH 6.5–8.6) of the overall ATPase reaction.

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References

[1] Mitchell, P. (1966) Biol. Rev. 41, 445–502.
[2] Witt, H. T. (1979) Biochim. Biophys. Acta 505, 355–427.
[3] Boyer, P. D., Chance, B., Ernster, L., Mitchell, P., Racker, E. and Slater, E. C. (1977) Annu. Rev. Biochem. 46, 955–1026.
[4] Winget, G., Izawa, S. and Good, N. E. (1965) Biochem. Biophys. Res. Commun. 21, 438–443.
[5] Gould, J. M. and Izawa, S. (1973) Biochim. Biophys. Acta 314, 211–223.
[6] Pick, U., Rottenberg, H. and Avron, M. (1974) FEBS Lett. 48, 32–36.
[7] Gräber, P. and Witt, H. T. (1976) Biochim. Biophys. Acta 423, 141–163.
[8] Witt, H. T., Schlodder, E. and Gräber, P. (1976) FEBS Lett. 69, 272–276.
[9] Schlodder, E. and Witt, H. T. (1980) FEBS Lett. 112, 105–113.
[10] Sugino, Y. and Nigoshi, Y. (1974) J. Biol. Chem. 239, 2360–2364.
[11] Schlodder, E. and Witt, H. T. (1981) Biochim. Biophys. Acta 635, 571–584.

[12] Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 244–254.
[13] Rumberg, B. and Siggel, U. (1968) Z. Naturforsch. 23b, 239–243.
[14] Junge, W., Rumberg, B. and Schröder, H. (1970) Eur. J. Biochem. 14, 575–581.
[15] Rüppel, H. and Witt, H. T. (1969) Methods Enzymol. 16, 316–380.
[16] Schliephake, H., Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 1571–1578.
[17] Schlodder, E. and Rögner, M. (1978) Biophysiktagung Ulm, F3.
[18] Avron, M. (1960) Biochim. Biophys. Acta 40, 247–272.
[19] Rumberg, B. and Heinze, Th. (1980) Ber. Bunsenges. Phys. Chem. 84, 1055–1059.
[20] Rosing, J. and Slater, E. C. (1972) Biochim. Biophys. Acta 267, 275–286.
[21] Sone, N., Hamamoto, T. and Kagawa, Y. (1981) J. Biol. Chem. 256, 2873–2877.
[22] Gräber, P., Schlodder, E. and Witt, H. T. (1977) Biochim. Biophys. Acta 461, 426–440.
[23] Komatsu, M. and Murakami, S. (1976) Biochim. Biophys. Acta 423, 103–110.
[24] Yamamoto, T. and Tonamura, Y. (1975) J. Biochem. 77, 137–146.