The H⁺/ATP Coupling Ratio at the H⁺-ATP-Synthase of Spinach Chloroplasts is Four

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1. Introduction
The importance of the H⁺/ATP ratio, i.e. the number of H⁺ which have to be translocated across the ATP-synthase for each ATP molecule being synthesized or hydrolysed, has its origin in the threefold aspect of
(1) mechanistic consequences with respect to the coupling process,
(2) energetic consequences with respect to the attainable excess of ATP above ADP,
(3) stoichiometric consequences with respect to the overall process of CO₂ reduction.
In this paper two complete independent experimental approaches, namely the kinetic approach realizing H⁺ flux measurements and the energetic approach realizing transmembrane ΔpH measurements, prove the H⁺/ATP ratio at the chloroplastic ATP-synthase to be four.

2. Experimental Procedure
2.1 Materials and Methods.
The experiments were performed with suspensions of envelope-free chloroplasts isolated from spinach at 20 °C temperature. The initial pH was 8.0 and did never deviate more than 0.05 units. The intensity of the actinic light was 300 Wm⁻² unless otherwise stated. Changes of external pH (by glass electrode measurement) and of fluorescence (from the added indicator of internal pH) were monitored simultaneously. Changes of the ATP concentration were obtained from the change of external pH (0.94 H⁺ consumed per ATP formed). Light-induced electron flow (in the presence of ferricyanide as electron acceptor) was obtained from the decrease of external pH (1 H⁺ liberated per electron transported). Transmembrane ΔpH was obtained from the fluorescence quenching of N-(1-naphthyl)ethylenediamine (NED) or from the external pH jump after a pulse of 50 μM imidazole. The H⁺/e determination was performed as described in [1].

2.2 Specification of the Suspension Medium.
H⁺ flux measurements: 10 mM KCl, 3 mM MgCl₂, 1 mM tricine, 1 (or 0.1 as specified in the Table) mM Mg ATP, 2 μM NED, and chloroplasts containing 10 μM chlorophyll. Additionally 100 μM benzylviologen, 3 μM thioredoxin, 500 μM DTT for activation of ATP hydrolysis, which were replaced by 100 μM ferricyanide for realization of the e⁻-flow measurements. Addition of DCMU, uncoupler, and tentoxin as specified in the Table.
Measurement of energy balance: 50 mM KCl, 3 mM of free MgCl₂, 1 mM tricine, 2.5 μM diadenosinepentaphosphat, and chloroplasts containing 10 μM or 60 μM chlorophyll. The

e⁻-cofactor was 20 μM pyocyanin or 100 μM benzylviologen. Agents for activation of the ATP-synthase were 5 mM DTT (preillumination 5 min) or 3 μM thioredoxin, 500 μM DTT (preillumination for 70 s). Phosphorylation substrates were varied between 100 μM and 3 mM for ATP, 20 μM and 1 mM for ADP, 20 μM and 2.5 mM for phosphate.

3. Results
3.1 H⁺/ATP from Flux Measurements
We realized a two-step experiment. In the first instance we measured simultaneously the rate of ATP hydrolysis and the fluorescence quenching of NED which gives information on the transmembrane ΔpH. Then we replaced the agents for activation of the ATPase, namely benzylviologen, thioredoxin and DTT, by ferricyanide and measured simultaneously the rate of light-induced e⁻-flow and again the fluorescence quenching of NED. By addition of DCMU the electron transport system was forced to produce just the same value of fluorescence quenching as in the case of ATP hydrolysis. Both experiments were performed under conditions of identical H⁺ permeability of the thylakoid membrane, and therefore the H⁺ fluxes are identical, too.

If additionally the H⁺/e ratio is determined, the proton flux is calculable from the e⁻-flow and the H⁺/e ratio and may be compared to the rate of ATP hydrolysis. The results presented in the Table and Fig. 1 clearly demonstrate a H⁺/ATP ratio of four, irrespective of the change of the rate of ATP hydrolysis produced by addition of different uncouplers, addition of the ATPase inhibitor tentoxin or by the use of different ATP concentrations.

<table>
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<tr>
<th>No</th>
<th>Uncoupler</th>
<th>ATP</th>
<th>Tentoxin</th>
<th>Vₜₜ</th>
<th>ΔF/F₀</th>
<th>DCMU</th>
<th>Vₑ</th>
<th>H/e</th>
<th>H/ATP</th>
<th>No. of exps.</th>
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</table>

**Table**
Data of the rate of ATP hydrolysis on the one hand, of e⁻-flow and H⁺/e ratio on the other hand for conditions of identical transmembrane ΔpH measured by fluorescence quenching ΔF/F₀ of NED. ATP hydrolysis has been initiated by preillumination for 70 s in the presence of benzylviologen, thioredoxin, DTT and e⁻-flow was induced by steady illumination in the presence of ferricyanide. The light intensity was 200 Wm⁻².
3.2 H⁺/ATP from Energy Balance

Equilibrium at the H⁺-ATP-synthase is obtained, if the phosphorylation potential, \( \Delta G_p \), and the electrochemical potential of H⁺, \( \Delta \mu_{\text{H}^+} \), are related to each other according to

\[
\Delta G_p + n \Delta \mu_{\text{H}^+} = 0
\]

where \( n \) is the H⁺/ATP coupling ratio, \( \Delta G_p = \Delta G_p^o + 2.3 \, \text{RT} \, \lg \left[ \text{ATP} / (\text{ADP} \cdot \text{P}) \right] \), and \( -\Delta \mu_{\text{H}^+} = 2.3 \, \text{RT} \, \Delta \mu_{\text{H}^+} + F \Delta \psi \). By comparison of \( \Delta G_p \) and \( \Delta \mu_{\text{H}^+} \) the H⁺/ATP ratio is determinable. The crucial problem is the determination of \( \Delta \mu_{\text{H}^+} \). In the case of high salt concentration, which is realized here, the contribution of \( \Delta \psi \) during steady state conditions is negligible [2].

Trustworthy results on \( \Delta \mu_{\text{H}^+} \) are obtainable by both the electron transport method put forward by us twenty years ago [3] and the amine distribution method as realized by use of imidazole, introduced by Pick et al. [4]. A critical evaluation is outlined in [5]. The fluorescence quenching method introduced by Schuldiner et al. [6] is most easily handy but suffers from the wrong theory offered (for critical discussion see [5]). Fluorescence quenching has to be calibrated with respect to \( \Delta \mu_{\text{H}^+} \) by means of other methods (see Fig. 2).

Examples of finding out the equilibrium state at the ATP-synthase are given in Fig. 3. If such equilibrium state data are analysed it comes out that \( \lg \left[ \text{ATP} / (\text{ADP} \cdot \text{P}) \right] \) correlates linearly to \( \Delta \mu_{\text{H}^+} \) (Fig. 4). From the slope a H⁺/ATP ratio of four is read out. The intersection at the ordinate gives a \( \Delta G_p^o \) of 31.2 kJ/mol in full agreement with the value determined by Rosing and Slater [7].

4. Discussion

H⁺/ATP ratios as determined hitherto culminated in values around three. In our mind three reasons account for the deviation from the value of four reported here: (1) As far as H/e ratios have been taken as a basis [8] H/e values of two have been used instead of more reliable higher ones as shown in [1].
Fig. 3, left  Rate of ATP formation (consumption, respectively) in dependence on the light-induced transmembrane ΔpH for different substrate concentrations as indicated. ΔpH was varied by change of the light intensity and determined by the fluorescence quenching of NED using the calibration curve in Fig. 2.

Fig. 4, right  Logarithm of ATP/(ADP·P) in dependence on the light-induced transmembrane ΔpH for equilibrium conditions at the ATP-synthase.

(2) As far as ΔpH measurements have been taken as a basis [9], overestimation of ΔpH has been arisen from the use of a false fluorescence quenching interpretation. (3) Direct H⁺ flux measurements during ATP synthesis [10] may have suffered from a limited response time of the experimental setup for pH change measurement.

With respect to mechanistic consequences of the H⁺/ATP ratio of four we refer to a recent coupling model described in [11]. With respect to the overall process of photosynthesis we have to accept that on the basis of the H⁺/ATP ratio of four, taken together with the probable H/e ratio of 2.5 (see [1]), linear electron transport alone does not produce enough ATP to drive CO₂ reduction.

5. Literature