

The H⁺/ATP coupling ratio of the ATP synthase from thiol-modulated chloroplasts and two cyanobacterial strains is four

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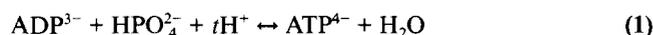
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Abstract In this paper the authors emphasise that the proton translocating ATP synthase from thiol-modulated chloroplasts and two cyanobacterial strains has a coupling ratio of 4 protons per ATP synthesised or hydrolysed. This ratio is determined by several thermodynamic studies at equilibrium between phosphate potential (ΔG_p) and proton gradient ($\Delta\bar{\mu}_{H^+}$), and is confirmed by measurement of proton flux during ATP hydrolysis. Ratios lower than 4 H⁺/ATP that have been published in the past have predominantly been determined with the oxidised chloroplast enzyme. Errors in these measurements will be discussed.

Key words: ATP synthase, H⁺; Energy transduction; Proton translocation; Proton gradient; H⁺/ATP ratio; Enzyme regulation; Chloroplasts; Cyanobacteria

1. Introduction

The reversible H⁺-ATP synthase (F₁F₀) catalyses the synthesis and hydrolysis of ATP coupled to the translocation of protons [1]. The reactions involved are:



At pH 8 the factor t in reaction 1 is close to 1 (Chance and Nishimura [2], see also Krab and Van Wezel [3]). The factor n corresponds to the number of protons translocated by the F₁F₀ per molecule ATP synthesised or hydrolysed (the H⁺/ATP ratio). This ratio is a very important parameter in the functional mechanism of the ATP synthase (see e.g. Gräber [4]). Determinations of this ratio of ATP synthases from various sources have been performed for several decades. In the first experiments performed with a glass electrode in isolated chloroplasts [5] a ratio of 2 H⁺/ATP was found. This was in harmony with

the value predicted by Mitchell [1]. Since then the H⁺/ATP of chloroplast F₁F₀ (CF₁F₀) has been determined by different methods using different approaches; the values tend to increase over the years from 2 H⁺/ATP [6] to ca. 3 or sometimes more H⁺/ATP [7–16]. A number of 3 H⁺/ATP is presently more or less generally accepted.

In this paper the H⁺/ATP is re-evaluated. With different techniques based on independent approaches it appears that this ratio is 4 in two membrane systems: thiol-modulated (reduced) broken spinach chloroplasts and membrane vesicles from two cyanobacterial strains, the thermophile *Synechococcus* 6716 and the transformable *Synechocystis* 6803. The F₁F₀ from those cyanobacteria appears to be very closely related to CF₁F₀ in sequence homology of the subunits [17,18] and functionally corresponds to the reduced form of the latter [19,20].

2. H⁺/ATP determinations

2.1. From equilibrium between $\Delta\bar{\mu}_{H^+}$ and phosphate potential

The total driving force of reactions 1 and 2 equals:

$$\Delta G = \Delta G_p - n \cdot \Delta\bar{\mu}_{H^+} \quad (n = H^+/ATP) \quad (3)$$

ATP synthesis requires that $\Delta G < 0$ whereas ATP hydrolysis occurs at $\Delta G > 0$. When $\Delta G = 0$ no net ATP synthesis or hydrolysis occurs and under this condition it is possible to determine the H⁺/ATP from $\Delta G_p/\Delta\bar{\mu}_{H^+}$.

Apart from being an energetic intermediate for the catalytic reactions of the ATP synthase, a proton gradient is also required for activation of the enzyme. In the case of chloroplasts the influence of the redox state of the enzyme has to be accounted for [21]. CF₁F₀ from isolated chloroplasts is present in the oxidised inactive state. The $\Delta\bar{\mu}_{H^+}$ needed for activation is much higher than needed for actual ATP synthesis at most values of ΔG_p so that ATP hydrolysis is practically excluded. For this reason oxidised chloroplast F₁F₀ is not suitable for thermodynamic studies and should first be reduced. Upon reduction the $\Delta\bar{\mu}_{H^+}$ for activation is well under the $\Delta\bar{\mu}_{H^+}$ needed for ATP synthesis, and ATP hydrolysis of the active enzyme may occur. The presence of the region in the F₁ γ -subunit where reduction takes place and as a probable consequence the presence of an oxidised form is, as far as we know, unique for CF₁F₀ (see Van Walraven et al. [17]). Therefore, cyanobacterial vesicles from the strains studied here can be used for thermodynamic studies without the need for thiol modulation (see Krab et al. [20]). In the following studies, CF₁F₀ of broken

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Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical potential difference; ΔG_p , phosphate potential; ΔG° , standard free energy; F₁, hydrophilic part of the ATP synthase (prefix C refers to chloroplasts); F₀, membrane-bound part of the ATP synthase; NED, *N*-(1-naphthyl)ethylenediamine.

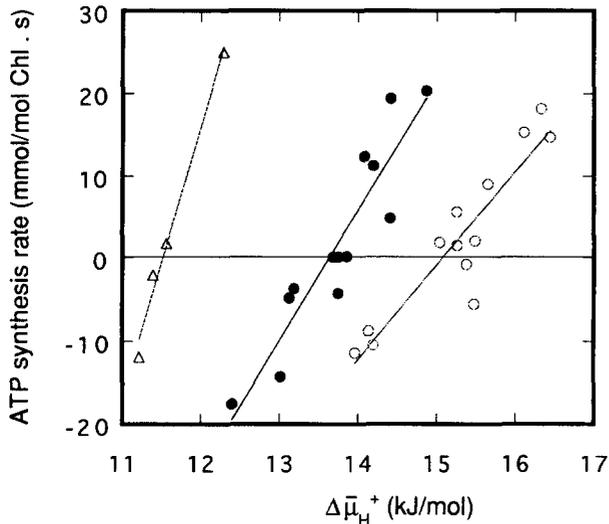


Fig. 1. Dependence of the ATP synthesis and hydrolysis activities on the size of light-induced $\Delta\bar{\mu}_{H^+}$ (ΔpH) in reduced chloroplasts for three different mixes of ATP, ADP and P_i . The H^+/ATP was calculated from $\Delta G'_0/\Delta\bar{\mu}_{H^+}$ at equilibrium and was 4.24 (Δ , 48.84/11.52); 4.19 (\bullet , 57.11/13.64) and 4.10 (\circ , 61.83/15.08). The Figure was modified from Rumberg et al. [23].

spinach chloroplasts was thiol-modulated by cyclic electron transport during pre-illumination in the presence of DTT and the experiments were carried out at pH 8.

In the studies of Rumberg and Becher [22] and Rumberg et al. [23] $\Delta\bar{\mu}_{H^+}$ (ΔpH) was varied by light intensity and the size was measured with the use of imidazol [22] as well as fluorescence quenching of *N*-(1-naphthyl) ethylenediamine (NED) [23]. ATP synthesis and hydrolysis were determined by the uptake resp. release of scalar protons (see reaction 1). The $\Delta\bar{\mu}_{H^+}$ at which no net reaction occurred was determined at different mixtures of ATP, ADP and P_i . The result is given in Fig. 1 and a H^+/ATP of at least 4 can be calculated. Rather than assuming a value of H^+/ATP higher than 3, Rumberg and Becher [22] suggested at first that the value of $\Delta G'_0$ was overestimated. However, the slope of $\lg [ATP]/[ADP] \cdot [P_i]$ as a function of $\Delta\bar{\mu}_{H^+}$ (ΔpH) (see [23]) also gave a ratio of 4; and at the same time a normal value for $\Delta G'_0$ of 31.2 kJ/mol, in full agreement with Rosing and Slater [24].

Strotmann and Lohse [16] plotted the \lg of 9-aminoacridine fluorescence signals at equilibrium (no change in fluorescence after addition of a mix of ATP, ADP and P_i) as a function of $\lg [ATP]/[ADP] \cdot [P_i]$ in a similar type of experiment as shown in Fig. 1. In this way they not only circumvented uncertainties in $\Delta G'_0$ but also in the size of $\Delta\bar{\mu}_{H^+}$ measured by the use of fluorescent amines. Initially a H^+/ATP of about 3 was found for reduced chloroplasts [16], but in those experiments the background ΔpH due to the fluorescence excitation light was disregarded. Accordingly the maximal 9-aminoacridine fluorescence Φ_0 and $\lg (\Phi_0 - \Phi)/\Phi$, respectively, were underestimated. Since the relative effect of the measuring light increases with decreasing ΔpH , the $\lg (\Phi_0 - \Phi)/\Phi$ over $\lg [ATP]/[ADP] \cdot [P_i]$

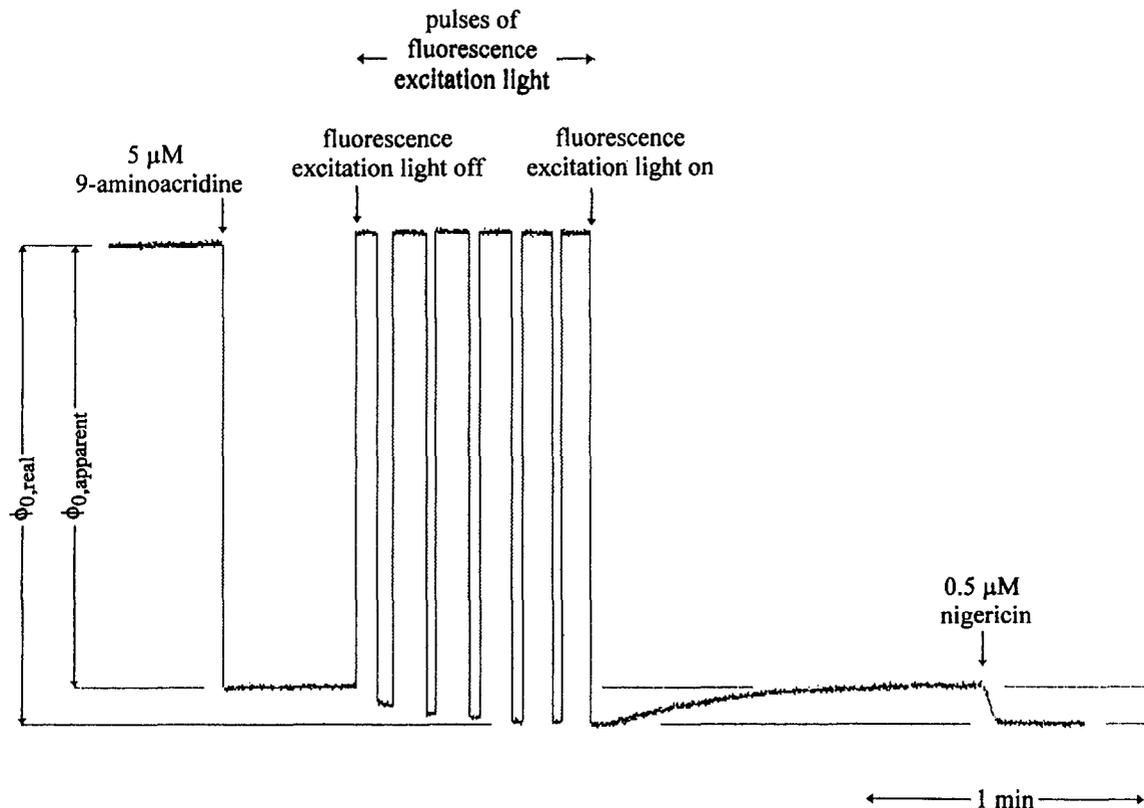


Fig. 2. Generation of ΔpH by the 9-aminoacridine fluorescence excitation beam. Initially continuous fluorescence excitation light was given. After addition of 9-aminoacridine the excitation beam was pulsed. The increase of fluorescence following the intercalated dark phases indicates the relaxation of ΔpH . The finally obtained fluorescence level therefore records the real maximal fluorescence Φ_0 . Subsequent application of continuous excitation light shows the new generation of ΔpH as demonstrated by the effect of the uncoupler nigericin.

relationship would be steeper and the calculated H^+/ATP smaller than its real value. Fig. 2 shows a technique which allows the correct determination of Φ_0 by employing short pulses of the measuring beam separated by long dark periods. Re-determination of H^+/ATP by this technique yielded a value close to 4 (Fig. 3).

Determination of H^+/ATP with light-driven $\Delta\bar{\mu}_{H^+}$ relies heavily on the methods used for measuring $\Delta\bar{\mu}_{H^+}$. Furthermore there is still the theoretical possibility that the relevant coupling $\Delta\bar{\mu}_{H^+}$ is not the one between the bulk-phases separated by the membrane but has a more local character. This will lead to an overestimation of H^+/ATP . By an imposed gradient by acid-base transition, a valinomycin-induced K^+ diffusion potential or a combination of both one can be sure that possible local gradients are not higher than the $\Delta\bar{\mu}_{H^+}$ between the bulk phases. This method is used by Gräber et al. [25] to determine H^+/ATP in reduced chloroplasts. A stoichiometry of 4.25 was found at the point where the sum of the ATP synthesis and hydrolysis rate is zero (Fig. 4).

In membrane vesicles from two cyanobacterial strains *Synechococcus* 6716 and *Synechocystis* 6803 a H^+/ATP of ca. 4 was either deduced from the threshold value of $\Delta\bar{\mu}_{H^+}$ for ATP synthesis as determined by acid-base transition [19,26] (Fig. 5A) as well as from the value of $\Delta\bar{\mu}_{H^+}$ imposed by a valinomycin-induced K^+ diffusion potential where ATP hydrolysis was just inhibited (Fig. 5B) [19,27]. ATP synthesis in *Synechocystis* 6803 vesicles induced by acid-base transition essentially yields the same graph as Fig. 4A with also a H^+/ATP ratio of ca. 4 (Scholts, M.J.C., Aardewijn, P., Krenn, B.E. and Van Walraven, H.S. unpublished results).

We conclude that thermodynamic studies at equilibrium between proton gradient and phosphate potential in reduced chloroplast- and cyanobacterial ATP synthase points to a H^+/ATP value of 4.

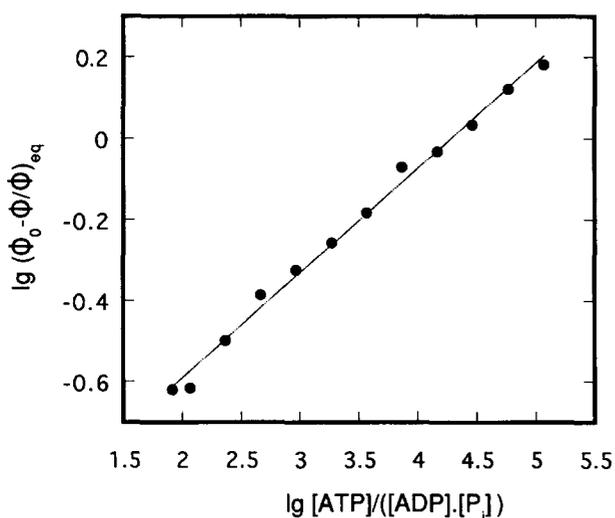


Fig. 3. 9-Aminoacridine fluorescence signals at equilibrium (expressed as $\lg(\Phi_0 - \Phi)/(\Phi)_{eq}$) as a function of $\lg[ATP]/([ADP] \cdot [P_i])$ in an experiment with reduced chloroplasts in which equilibrium was obtained between light-induced $\Delta\bar{\mu}_{H^+}$ (ΔpH followed with 9-aminoacridine) and ΔG_p at different mixes of ATP, ADP and P_i . From the slope of the line a H^+/ATP of 3.87 can be calculated. The method was described in Strotmann and Lohse [16] but here the effect of the excitation light for the 9-aminoacridine fluorescence on ΔpH was taken into account. Φ_0 , fluorescence upon addition of 9-aminoacridine; Φ , fluorescence upon illumination.

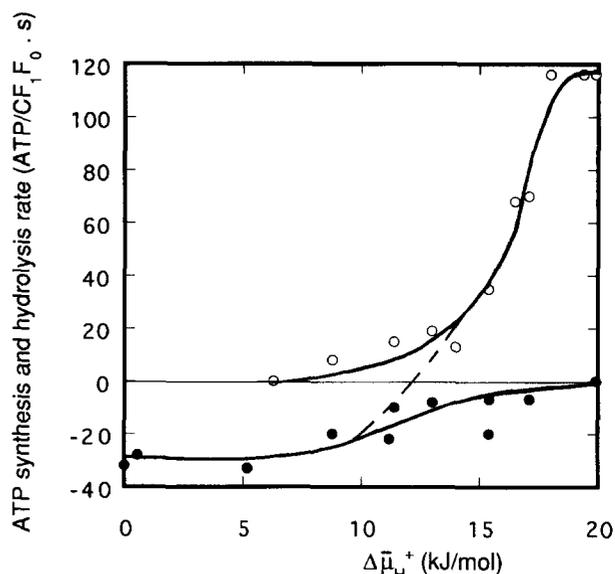


Fig. 4. Rate of ATP synthesis (\circ) and hydrolysis (\bullet) of reduced chloroplasts as a function of $\Delta\bar{\mu}_{H^+}$ (ΔpH) by acid-base transition imposed with a rapid-mixing quenched flow apparatus. ATP synthesis and hydrolysis were determined separately by the incorporation of ^{32}P - P_i and the breakdown of $[\gamma\text{-}^{32}P]ATP$, respectively. The H^+/ATP of 4.25 was calculated from the equilibrium values of ΔG_p (51 kJ/mol) and $\Delta\bar{\mu}_{H^+}$ (at 12 kJ/mol the rate of ATP synthesis plus hydrolysis is zero). The Figure was modified from Gräber et al. [25].

2.2. From flux measurements

Determination of H^+/ATP by flux measurements is possible both in oxidised and reduced chloroplast ATP synthase as long as the enzyme is in the active form. For reduced activated chloroplasts Rumberg et al. [23] found a H^+/ATP of 3.94 from the rate of ATP hydrolysis on the one hand and electron (e) flow and H^+/e on the other under conditions of identical $\Delta\bar{\mu}_{H^+}$ (ΔpH) as determined by NED fluorescence.

3. Discussion

Comparison with other literature

In the literature there are many discrepancies between published values of the H^+/ATP ratio for CF_1F_0 . Here we show that most of those are due to differences in experimental conditions. Much of the older work has been done with oxidised chloroplasts. As already stated in the previous section, ATP synthesis in these chloroplasts is not limited by $\Delta\bar{\mu}_{H^+}$ as a driving force for the reaction but by a much higher $\Delta\bar{\mu}_{H^+}$ needed for activation. In the equilibrium studies on oxidised CF_1F_0 of Avron [8], Portis and McCarty [9], Dewey and Hammes [11], Hangarter and Good [14] and Mills and Mitchell [15] this was not accounted for. However, the latter authors found a H^+/ATP of 4.6 in reduced pea chloroplasts, when they studied the effect of thiol modulation on ATP synthesis driven by acid-base transition. This value agrees well with values shown in this paper.

In the case of flux measurements in which an activation constraint as compared to thermodynamic measurements is not present also a H^+/ATP of 3 dominates for oxidised chloroplasts

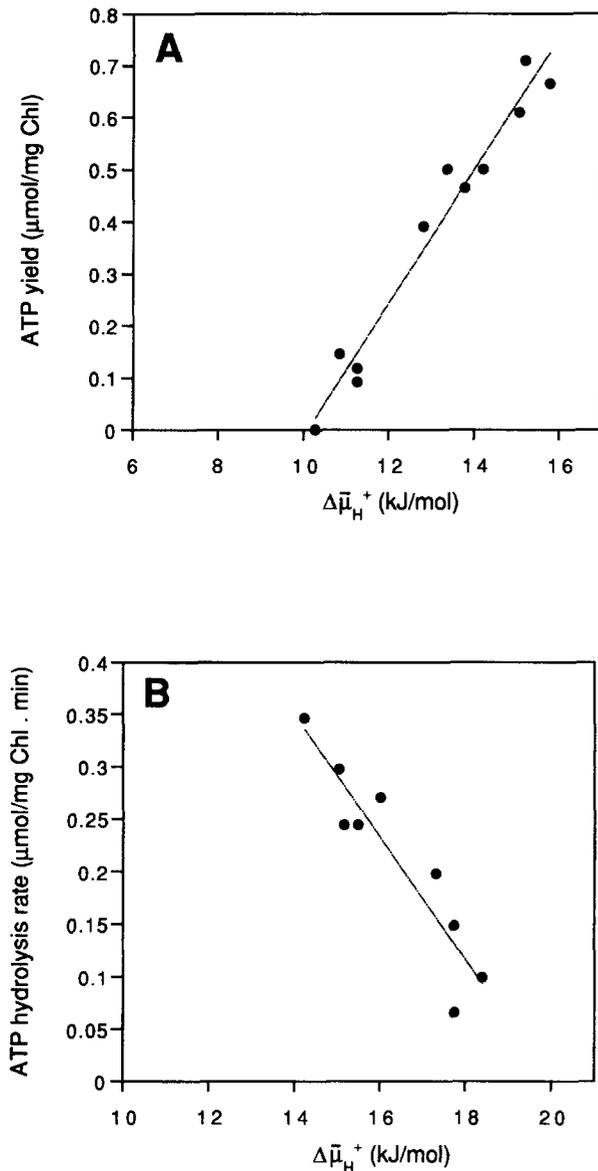


Fig. 5. (A) ATP yield as a function of $\Delta\bar{\mu}_{H^+}$ (Δ pH) by acid-base transition in membrane vesicles from *Synechococcus* 6716. The H^+/ATP of 3.96 was calculated from the equilibrium values of ΔG_p (39.95 kJ/mol) and $\Delta\bar{\mu}_{H^+}$ (10.10 kJ/mol). Data from Bakels et al. [19] and Krenn et al. [26] were used and ΔG_p was calculated according to Krab and Van Wezel [3]. (B) ATP hydrolysis rate as a function of a valinomycin-induced K^+ -diffusion potential in membrane vesicles from *Synechococcus* 6716. The H^+/ATP of 4.25 was calculated from the equilibrium values of ΔG_p (85 kJ/mol) and $\Delta\bar{\mu}_{H^+}$ (20 kJ/mol). Data from Van Walraven et al. [27] were used. A similar experiment is also given in Bakels et al. [19].

[7,10,13]. However, in one case [7], where flash groups are used for stimulation of ATP synthesis, the reported lower limit for H^+/ATP of 3.2 is easily corrected to 4 if the proton flux portions which take place between the flashes of each flash group are additionally taken into account. In the other cases [10,13] a too low value of 2 H^+/e has been used for calculation of H^+/ATP from P/e measurements, not taking into account the additional proton which is translocated due to the Q-cycle activity at the

cytochrome b_6f complex. In measurements of ATP hydrolysis induced by illumination in the presence of DTT (thus in reduced chloroplasts), linked to inward proton translocation a H^+/ATP higher than 3 (3.4 ± 0.3) was found [12]. Obviously, there are many possible pitfalls in these types of measurements and the group of Rumberg will present convincing evidence elsewhere that also in the case of oxidised chloroplasts a H^+/ATP of 4 is found by kinetic analysis of ATP synthesis and proton flow across the ATP synthase under illumination (Berry, S. and Rumberg, B., unpublished data).

We conclude that the H^+/ATP ratio of thiol-modulated chloroplasts is at least 4, values varying between 4 and 4.25 H^+/ATP . Cyanobacterial vesicles from the thermophile *Synechococcus* 6716 and the transformable *Synechocystis* 6803, of which the F_1F_0 functionally corresponds to reduced CF_1F_0 , have the same H^+/ATP . Since the majority of recent studies on CF_1F_0 is performed with the thiol-modulated form which is physiological relevant for catalytical activity, at least 4 H^+/ATP must be taken into account.

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