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Determination of the H^+/ATP ratio of the H^+ transport-coupled reversible chloroplast ATPase reaction by equilibrium studies

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Fluorescence quenching of 9-aminoacridine was followed to ascertain the state of light-induced transthylakoidal $\Delta\tilde{\mu}H^+$ at energetic equilibrium with a subsequently added mixture of ATP, ADP and phosphate. In the measured range, the logarithm of relative fluorescence quenching at equilibrium is a linear function of the imposed phosphate potential. The slope of the line is shown by mathematical deduction to be equal to the ATP/H^+ stoichiometry of the H^+-coupled reversible ATPase reaction. Determination of the stoichiometry by this method neither relies on the standard phosphate potential nor on the internal thylakoid volume. The results confirm that during ATP synthesis or hydrolysis 3 H^+ per ATP are translocated through the CF_0-CF_1 complex.

ATPase; Chloroplast; H^+/ATP ratio; Phosphate potential; Protonmotive force

1. INTRODUCTION

The formation of ATP from ADP and inorganic phosphate in chloroplasts is coupled to the translocation of H^+ from the intrathylakoidal space through CF_0-CF_1 to the stroma side. Conversely, hydrolytic cleavage of ATP by the enzyme complex is related to a flux of H^+ in the opposite direction. The overall reaction is described by

$$n\,H^+_{in} + ADP + P_i \xrightleftharpoons{H^+\text{-ATPase}} n\,H^+_{out} + ATP + H_2O$$

The change of free enthalpy of the two coupled reactions is

$$\Delta G = -n\cdot\Delta\tilde{\mu}H^+ + \Delta G_p \qquad (1)$$

The electrochemical potential difference of protons, $\Delta\tilde{\mu}H^+$, is composed of the transmembrane differences of pH and the electrical potential difference $\Delta\psi$ [1]

$$\Delta\tilde{\mu}H^+ = 2.303\cdot RT\cdot\Delta pH + F\cdot\Delta\psi \qquad (2)$$

The phosphate potential ΔG_p is defined as

$$\Delta G_p = \Delta G_p^0 + 2.303\cdot RT\cdot\log\frac{[ATP]}{[ADP]\cdot[P_i]} \qquad (3)$$

The factor n in eq 1 signifies the number of H^+ translocated through the CF_0-CF_1 complex per one ATP synthesized or hydrolyzed. The H^+/ATP ratio is an important parameter, both from the energetic and mechanistic points of view. Accordingly manifold determinations by different experimental techniques are reported in the literature. Early studies were performed by comparison of steady state rates of vectorial proton flux and ATP formation, respectively, which can be measured simultaneously by a pH electrode. Initially a ratio of 2 was obtained [2]; redetermination under well-defined conditions, however, yielded a quotient of 3 [3,4]. This value was confirmed by evaluation of results on acceleration of the 515 nm absorption change by

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Abbreviations: 9-AA, 9-aminoacridine; DTT, dithiothreitol; PMS, phenazine methosulfate

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phosphorylation [5]. In another experimental approach the H^+/ATP ratio was derived from measurements of the phosphorylation rate as a function of the transmembrane ΔpH ascertained by the distribution of radioactive amines [6]. Based on the observation that the logarithm of the rate of ATP formation is linearly related to ΔpH and the mathematical demonstration that the slope of the line is equal to the stoichiometric factor n, corresponding measurements were conducted under a variety of experimental conditions [6]. A ratio around 3 was always obtained and this value was found to hold true for ATP synthesis as well as ATP hydrolysis [7].

One recent report made use of the calculation of n from the magnitudes of $\Delta\tilde{\mu}H^+$ and ΔG_p in thermodynamic equilibrium [8]. In equilibrium (i.e. $\Delta G = 0$), the H^+/ATP stoichiometry is simply given by

$$n = \Delta G_p / \Delta\tilde{\mu}H^+ \qquad (4)$$

Equilibrium was determined by measurements of initial rates of ATP synthesis and hydrolysis as a function of ΔpH imposed by acid/base jumps at a given phosphate potential [8]. Hence the calculation of n relied solely on the accuracy of ΔG_p^0 which was taken from the literature [9]. Surprisingly a value as large as 4.5 was obtained. The authors speculated that the generally used value for ΔG_p^0 probably was overestimated by a factor of about 2 [8].

These results gave rise to re-determination of the H^+/ATP ratio under equilibrium conditions. In variance to the mentioned study, the approach employed in this work does not rely on the magnitude of ΔG_p^0. The results confirm that the H^+/ATP stoichiometry is 3.

2. MATERIALS AND METHODS

Broken chloroplasts from spinach leaves were isolated as described in [10]. All experiments were conducted in a self-constructed fluorometer containing a cylindrical cuvette of 2.5 ml volume with magnetic stirring device and temperature control (20°C). The stock medium contained 25 mM tricine buffer, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 50 nM valinomycin and 50 μM PMS. Thylakoids equivalent to 25 μg chl/ml final volume were added before the addition of 5 μM 9-aminoacridine. Fluorescence of 9-aminoacridine was measured at 494 nm; the excitation wavelength was 400 nm. Photosynthesis was excited with red light > 630 nm. The different wavelengths were adjusted by suitable combinations of filters. Light was transmitted to the cuvette and the detector, respectively, by light pipes. The reaction mix was preilluminated for 2 min in order to activate the ATPase. Further treatments and additions are described in the text and legends, respectively.

For measurement of photophosphorylation, ^{32}P-labeled inorganic phosphate was added. 15, 30 and 45 s after addition of the substrate mix (which in addition contained ADP and ATP), 0.2 ml samples were taken from the cuvette and deproteinized by $HClO_4$ (final concentration 0.5 M). Organic phosphate was separated from inorganic phosphate by precipitation as described [11]. Correspondingly, ATP hydrolysis was measured by employing γ-^{32}P-labeled ATP which was prepared as described [12,13]. Separation of released $[^{32}P]P_i$ was carried out as described [13,14].

3. RESULTS

The principle of the here employed equilibrium measurements is based on the concept that the steady state proton motive force of illuminated thylakoids should not be changed by the addition of ATP + ADP + P_i when the phosphate potential of the substrate mix is in energetic equilibrium with $\Delta\tilde{\mu}H^+$. ΔpH changes can be conveniently followed by the quenching of 9-aminoacridine fluorescence [15]. In spite of the criticism concerning the quantitative calculation of ΔpH values from 9-aminoacridine signals [16,17], there is no doubt that in a certain ΔpH range under standardized experimental conditions fluorescence quenching yields a reliable relative measure of the proton gradient [17,18]. The results presented below also confirm this assumption.

Fig.1 illustrates the procedure of measurement. In order to induce reversible H^+-ATPase activity, isolated thylakoids were illuminated for 2 min at maximum light intensity in a medium containing DTT. Then light intensity was decreased. After achievement of a new constant fluorescence signal (1 min), a substrate mix consisting of ATP, ADP and P_i was injected. The final concentrations of ATP and ADP were 0.25 mM and that of P_i 1.1 mM. Depending on the light intensity employed, the addition of the substrate mix either caused a decrease or an increase of the 9-aminoacridine signal. The values must be corrected for the fluorescence quench caused by the nucleotides themselves [19]. This contribution was determined by following the signal change upon addition of the mix to a non-illuminated thylakoid suspension.

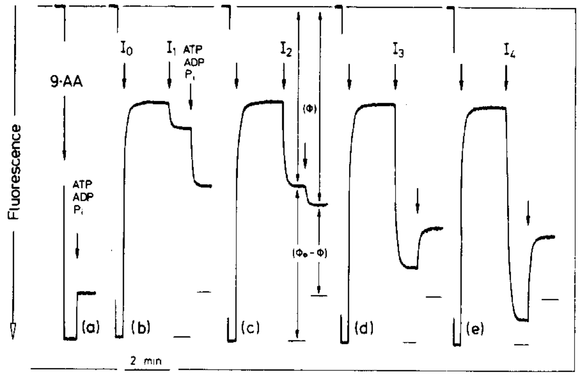

Fig.1. Effect of attenuation of light intensity and subsequent addition of ATP/ADP/P_i on the light-induced 9-aminoacridine fluorescence signal. Trace (a) shows the change of fluorescence by addition of the substrate mix to a non-illuminated sample. In traces (b) to (e) after 2 min illumination with 250 W/m^2, light intensity was decreased to different values I_1 to I_4, before the substrate mix was added after 1 min. In trace (c) the distances ϕ and $\phi_0 - \phi$ are indicated which were used for the quantitative evaluation of the signals obtained before and after addition of the substrates. Addition of the substrate mix yielded concentrations of 0.25 mM ATP, 0.25 mM ADP and 1.1 mM P_i, respectively.

The original theory of calculation of ΔpH by 9-aminoacridine fluorescence [15] led to the relationship

$$\Delta \mathrm{pH} = \log \frac{\phi_0 - \phi}{\phi} + \log \frac{V_o}{V_i} \qquad (5)$$

(ϕ_0, fluorescence obtained upon addition of 9-aminoacridine; ϕ, fluorescence at energization of the thylakoids; V_o, outer volume; V_i, internal thylakoid volume).

It has been shown that the internal thylakoid volume is not significantly changed by different light intensities [20]. Hence the variable term of eqn 5 is $\log(\phi_0 - \phi)/\phi$. In fig.2 corrected positive or negative changes of the fluorescence signal, $\Delta(\log(\phi_0 - \phi)/\phi)$, caused by addition of the substrate mix are plotted versus the fluorescence signal $\log(\phi_0 - \phi)/\phi$ observed before the addition. The value of $\log(\phi_0 - \phi)/\phi$ where addition of the substrates did not change fluorescence at all is designated $(\log(\phi_0 - \phi)/\phi)_{eq}$). The conditions and performance of the experiment were the same as in fig.1, but in one set of measurements P_i was labeled by ^{32}P and in another set ATP was ^{32}P-labeled in the γ-position. By the formation of $[^{32}P]$ATP and the release of $[^{32}P]P_i$, respectively, initial rates of phosphorylation and ATP hydrolysis were followed while 9-aminoacridine fluorescence was recorded. In the lower part of fig.2 the obtained rates as a function of $\log(\phi_0 - \phi)/\phi$ are plotted. The results show that the rate of ATP synthesis equals the rate of ATP

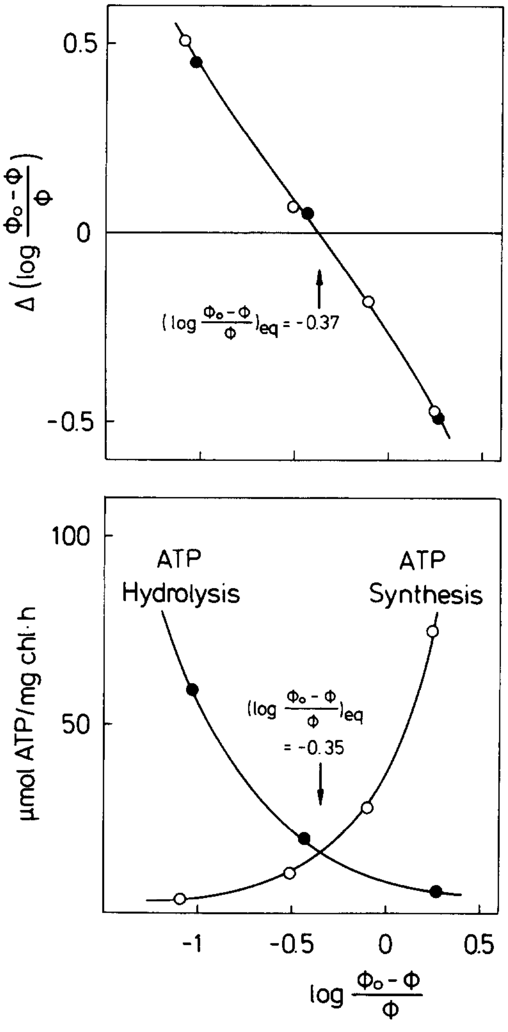

Fig.2. Change of the 9-aminoacridine signal by the addition of ATP/ADP/P_i (upper part) and rates of ATP synthesis and hydrolysis (lower part) as function of the fluorescence signal $\log(\phi_0 - \phi)/\phi$ adjusted by different light intensities. The substrate mix (see fig.1) contained either ^{32}P-labeled P_i or ^{32}P-labeled ATP. Initial rates of $[^{32}P]$ATP synthesis and hydrolysis were measured as described in section 2.

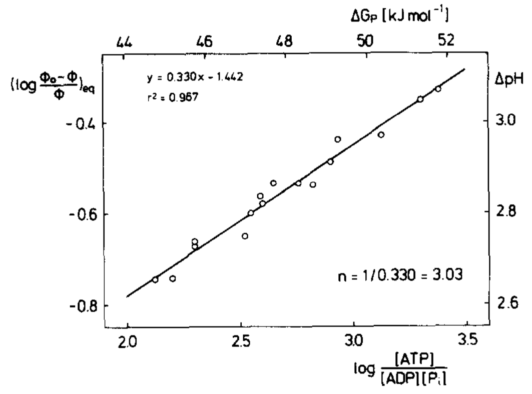

Fig.3. 9-Aminoacridine fluorescence signals at equilibrium (expressed as $(\log(\phi_0 - \phi)/\phi)_{eq}$) as a function of the imposed phosphate potential (expressed as $\log[\mathrm{ATP}]/[\mathrm{ADP}] \cdot [\mathrm{P_i}]$). The different phosphate potentials were adjusted by changing the $\mathrm{P_i}$ concentration from 0.4 to 7.5 mM at constant concentrations of ATP and ADP (0.5 mM each). The line was drawn according to the indicated equation which results from linear regression analysis. The validity of the linear fit is demonstrated by the coefficient of determination, r^2, which is close to 1.

hydrolysis at nearly the same value $(\log(\phi_0 - \phi)/\phi)_{eq}$ where the fluorescence change upon substrate addition is zero. This result proves that our approach indeed can be used for the determination of equilibrium states.

In a series of experiments equilibrium $(\log(\phi_0 - \phi)/\phi)_{eq}$ for a variety of different imposed phosphate potentials was ascertained. They are plotted against $\log[\mathrm{ATP}]/[\mathrm{ADP}] \cdot [\mathrm{P_i}]$ in fig.3. The concentrations of ATP and ADP were kept constant (both 0.5 mM) whereas the concentration of phosphate was varied between 0.4 and 7.5 mM. In spite of some scattering of the data, a linear relationship is quite evident. The drawn line results from linear regression analysis. The slope of the line is 1/3.03.

4. DISCUSSION

When the electrical potential term $\Delta\psi$ is cancelled – in the here reported experiments by addition of valinomycin/$\mathrm{K^+}$ – the equilibrium state as presented in eqn 4 can be expressed by

$$\Delta\mathrm{pH} = \frac{1}{n} \cdot \log\frac{[\mathrm{ATP}]}{[\mathrm{ADP}] \cdot [\mathrm{P_i}]} + \frac{\Delta G_\mathrm{p}^0}{2.303 \cdot n \cdot RT} \tag{6}$$

or, since the second term on the right side of the equation consists of constants only:

$$\Delta\mathrm{pH} = \frac{1}{n} \cdot \log\frac{[\mathrm{ATP}]}{[\mathrm{ADP}] \cdot [\mathrm{P_i}]} + a \tag{7}$$

In equilibrium the context between fluorescence and the phosphate potential can be defined by combining eqns 5 and 6:

$$(\log\frac{\phi_0 - \phi}{\phi})_{eq} = \frac{1}{n} \cdot \log\frac{[\mathrm{ATP}]}{[\mathrm{ADP}] \cdot [\mathrm{P_i}]} + \frac{G_\mathrm{p}^0}{2.303 \cdot n \cdot RT} - \log\frac{V_\mathrm{o}}{V_\mathrm{i}} \tag{8}$$

If the constant volume term is set b, eqn 8 yields

$$(\log\frac{\phi_0 - \phi}{\phi})_{eq} = \frac{1}{n} \cdot \log\frac{[\mathrm{ATP}]}{[\mathrm{ADP}] \cdot [\mathrm{P_i}]} + (a - b) \tag{9}$$

This equation predicts a linear relationship between the fluorescence term and the concentration term of the phosphate potential in thermodynamic equilibrium with a slope of the line being equal to $1/n$. In fact linearity is demonstrated experimentally by fig.3, at least for the indicated range of phosphate potentials from 45 to 52 kJ/mol. Calculation of these phosphate potentials is based on $\Delta G_\mathrm{p}^0 = 32.8$ kJ/mol which was computed for our conditions (~0.1 M ionic strength, 5 mM $\mathrm{Mg^{2+}}$, pH 8.0) by using the data of Rosing and Slater [9]. Accordingly n can be taken from experiments as shown in fig.3 without knowing the precise value of the standard phosphate potential and the internal thylakoid volume. The number for n is 3.03, thus confirming the results obtained by non-equilibrium studies [3–6]. The validity of the 9-aminoacridine technique for the quantitative determination of ΔpH was repeatedly challenged [16,17,19]. It was shown that fluorescence quenching is not solely caused by uptake of the amine into the thylakoid lumen following the pH gradient but also by binding to the membrane surfaces [16,22]. Binding can be largely abolished by cations such as $\mathrm{Mg^{2+}}$ [21,22]. From the data reported by Hope and Matthews [22] we may conclude that under our conditions (5 mM $\mathrm{Mg^{2+}}$) binding of 9-aminoacridine is rather low. Moreover, binding of the amine would not affect the determination of n significantly, provided that its contribution to fluorescence quenching is relatively constant because linearity between ΔpH and $\log(\phi_0 - \phi)/\phi$ is the only prerequisite. Approximate linearity between the two parameters in a certain ΔpH range was demonstrated by Van et al. [18] using the technique of additional H^+ uptake by imidazole for ΔpH calibration. Finally our own results prove the linear relationship between fluorescence quenching and ΔpH because of the theoretical proportionality between ΔpH and ΔG_p in equilibrium.

The method of determination of fluorescence quenching in thermodynamic equilibrium could be used for a precise assignment of the 9-aminoacridine signals to ΔpH if either the thylakoid volume or the standard phosphate potential is known. On the other hand, one of these two parameters can be calculated from our results by eqn 8 if the other one is known. Assuming that ΔG_p^0 for our conditions is 32.8 kJ/mol, the data of fig.3 yield an internal thylakoid volume of 16 μl/mg chl (chl concentration in the cuvette: 25 μg/ml). This value is in good agreement with biochemical volume determinations (10–20 μl/mg chl) [17]. The ΔpH and ΔG_p scales in fig.3 were calculated on the basis of $\Delta G_p^0 = 32.8$ kJ/mol.

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