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H^+/ATP coupling ratio at the unmodulated CF_0CF_1-ATP synthase determined by proton flux measurements

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Abstract

The H^+/ATP coupling ratio is determined kinetically by comparing ATP synthesis and proton flow across the ATP synthase in spinach thylakoids under conditions of continuous illumination. Net proton flow at steady-state is zero, therefore the initial efflux in the dark is taken. A new method of kinetic analysis of the relaxation of the transmembrane proton gradient is presented, allowing direct determination of phosphorylating and basal proton fluxes at the same time. Special care is taken for the influence of dark phosphorylation and the H^+ diffusion potential. The investigations give evidence of a H^+/ATP coupling ratio of four for the oxidized or unmodulated state of the ATP synthase being at par with the coupling ratio for the reduced or modulated state of the enzyme.

Keywords: F_0F_1-ATP synthase; H^+/ATP coupling ratio; Chloroplast

1. Introduction

ATP synthase of the F_0F_1 type is the source of ATP in chloroplasts, mitochondria and bacteria. This enzyme is membrane-embedded and uses a H^+ gradient, generated across the membrane by electron flow, to drive the synthesis of ATP from ADP and inorganic phosphate (details in Refs. [1–6]). The H^+/ATP coupling ratio is defined as the number of H^+ ions to be translocated across the ATP synthase for each ATP molecule being synthesized. This number is of crucial importance with respect to (1) the mechanism of the coupling process, (2) the energy requirement of maintaining a high ATP/ADP ratio and (3) the overall ATP/e ratio. In the literature up to now a number of 3 H^+ per ATP is preferred, though a number of 4 H^+ per ATP has been put forward in recent times.

Surveying the literature with reference to the H^+/ATP coupling ratio at the ATP synthase from chloroplasts of green plants, a distinction between the oxidized and the reduced states of the enzyme seems to be important. In the older literature, where consistently a H^+/ATP value of 3 has been reported, the ATP synthase was in the oxidized or unmodulated state throughout [7–14]. A modulation of the enzyme takes place if it is reduced under energizing conditions by use of thioredoxin or dithiothreitol. Thereby the transmembrane ΔpH needed for enzyme activation is lowered [15]. As a consequence the hydrolysis of ATP is no longer hindered and unequivocal energy balance measurements are possible from which a H^+/ATP value of 4 emerges [16].

Here, we carry out a thorough re-examination of the H^+/ATP coupling ratio at the unmodulated ATP synthase in order to solve the discrepancies caused by the divergent data reported earlier. The coupling ratio is determined kinetically by comparing both the rates of proton backflow across the ATP synthase and coupled ATP synthesis under continuous illumination. Because net H^+ flow across the thylakoid membrane in the steady-state is zero, the flow rate is obtained from the initial H^+ efflux in the dark, indicated by a transient external pH decrease.

2. Materials and methods

The experiments were performed with suspensions of spinach thylakoids in an optical cuvette (cross-section 15×15 mm) at 20°C and pH 8.00 ± 0.05. The basic reaction medium contained in a volume of 4 ml 50 mM KCl, 3 mM free $MgCl_2$, 0.4 mM imidazole, 1 mM P_i, 1 mM ADP, 15 μM pyocyanine and thylakoids equivalent to 10 μM chlorophyll. 20 μM phenol red was added for

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optical detection of external pH changes and 4 μM *N*-(1-naphthyl)ethylendiamine (NED) for optical detection of internal pH. Red actinic light was fed into the cuvette from two opposite sides.

External pH changes were obtained from the difference of absorbance at 575 and 557 nm, which guarantees elimination of contributions from light scattering changes. The monitoring light was produced by two groups of LEDs pulsed alternately at a frequency of 8 kHz and the signal sequence was fed into a lock-in amplifier. The observed pH changes never exceeded 0.05 units, which makes safe a linear relationship of both changes of pH and absorbance to H^+ turnover. Calibration with respect to H^+ turnover was achieved by injecting 3 times 10 μl of 3 mM HCl into the stirred suspension at the end of each experiment. Internal pH was obtained from the fluorescence quenching of NED as described in Ref. [17] using light of 353 nm for excitation and 463 nm for detection of fluorescence, which was guided by quartz fibres dipping into the suspension from above. Transmembrane electric field changes were obtained from the electrochromic absorbance difference measured between 520 and 535 nm.

The rate of ATP production was read out from the coupled external pH changes taking into account the net consumption of 0.94 H^+ per each ATP formed at the experimental conditions of pH 8.0, pMg 2.52, 0.05 M ionic strength and 20°C used here [18,19]. Proton efflux out of the thylakoid lumen was obtained from the dark decay of the proton gradient as described in the text.

3. Results

3.1. Improvement of H^+ flux measurement by addition of imidazole

The outer pH changes caused by light-induced proton uptake into the lumenal phase of the thylakoid vesicles are shown in Fig. 1. In the presence of 400 μM imidazole the extent of proton uptake is considerably enhanced due to a corresponding enhancement of the internal buffer capacity. This is caused by lumenal trapping of the protonated form of imidazole, which is charged and hardly permeable, in contrast to the unprotonated form, which is uncharged and highly permeable [20]. The enlargement of the pH signal in the presence of imidazole is accompanied by an expanded time scale during its rise and decay, without any detectable retardation of the initial rates. Both of these effects contribute to a considerable improvement of the conditions for the execution of efflux measurements. The degree of improvement depends on the internal proton concentration. In detail this is shown in Fig. 2. The results may be described empirically as follows:

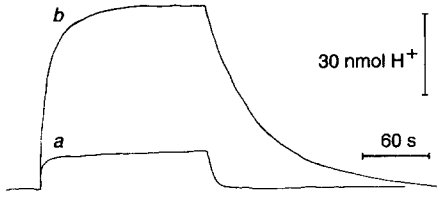

Fig. 1. Time course of light-induced external pH changes, caused by the transient proton uptake into the thylakoid lumen, (*a*) in the absence and (*b*) in the presence of 400 μM imidazole. In this experiment phosphate has been replaced by 0.3 mM Tricine buffer and 4 μM nonactin has been added for suppression of transmembrane potential changes due to uncompensated proton transport. Equivalent internal proton concentrations of 4 μM during steady-state conditions have been realized by addition of 6 nM nigericin in the absence of imidazole, mimicking the slight uncoupling activity of this agent. Additionally the signals have been normalized for equivalent external buffer capacity.

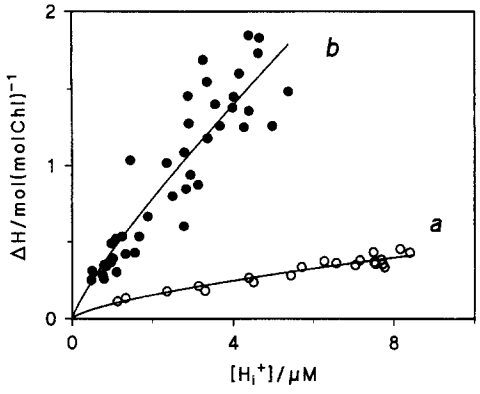

Fig. 2. Extent of proton uptake in dependence on the internal proton concentration, (*a*) in the absence and (*b*) in the presence of 400 μM imidazole. Variation of $[H_i^+]$ was realized by different light intensities. Other experimental conditions as in Fig. 1. The plotted curves are the basis of Eqs. 1 and 2, respectively.

$$\Delta H_{intr}/\mathrm{mol(molChl)}^{-1} = 0.095\left([H_i^+]/\mu M\right)^{0.689} \quad (1)$$

$$\Delta H_{imid}/\mathrm{mol(molChl)}^{-1} = 0.437\left([H_i^+]/\mu M\right)^{0.838} \quad (2)$$

ΔH_{intr} signifies the intrinsic proton uptake and ΔH_{imid} the apparent one in the presence of 400 μM imidazole (see below). Eq. (1) and Eq. (2) are valid in the region $[H_i^+] \gg [H_a^+] = 10^{-8}$ M.

The uptake of uncharged imidazole is coupled to the additional uptake of H^+ in a 1:1 ratio. Unfortunately, the corresponding loss of H^+ in the outer phase is partially masked by the dissociation of charged imidazole ($ImH^+ \rightleftharpoons Im + H^+$) due to the loss of the uncharged species. Therefore, the difference between the true proton uptake, ΔH^*_{imid}, and the amount which is indicated in the outer phase, ΔH_{imid}, is given by

$$\Delta H^*_{imid} = \Delta H_{imid} + (\Delta H_{imid} - \Delta H_{intr})[H_a^+]/K \quad (3)$$

where K is the acidity constant of imidazole which amounts to 6.6 10^{-8} M as determined from titration at the experimental conditions of 20°C and 0.05 M ionic strength used here. With respect to the corresponding proton fluxes, J, a

conversion factor, f, emerges which according to Eq. (3) is given by

$$f = \frac{J^*_{imid}}{J_{imid}} = \frac{d\Delta H^*_{imid}/dt}{d\Delta H_{imid}/dt} = 1 + \left[1 - \frac{d\Delta H_{intr}}{d\Delta H_{imid}}\right]\frac{[H^+_a]}{K} \quad (4)$$

By combination of Eq. (1) and Eq. (2) we find (restricted to $[H^+_i] \gg [H^+_a] = 10^{-8}$ M)

$$\frac{d\Delta H_{intr}}{d\Delta H_{imid}} = 0.179\left([H^+_i]/\mu M\right)^{-0.149} \quad (5)$$

For the measurements in this paper the value of $[H^+_i]$ was 4 μM, which gives according to Eq. (4) and Eq. (5) a correction factor of $f = 1.13$.

3.2. Determination of H^+/ATP by use of the tangent method

The pH changes during continuous illumination and the subsequent dark period, which are observed during ongoing ATP synthesis, are shown in Fig. 3*a*. Continuous pH increase in the light due to ATP production is followed by a transient pH decrease in the dark due to H^+ efflux. The initial proton efflux is determined from the tangent just at the point of light off. The slope of the continuous pH increase in the light has to be used as a base line because phosphorylation goes on in the dark as long as the H^+ gradient is sufficiently large to drive ATP synthesis. This

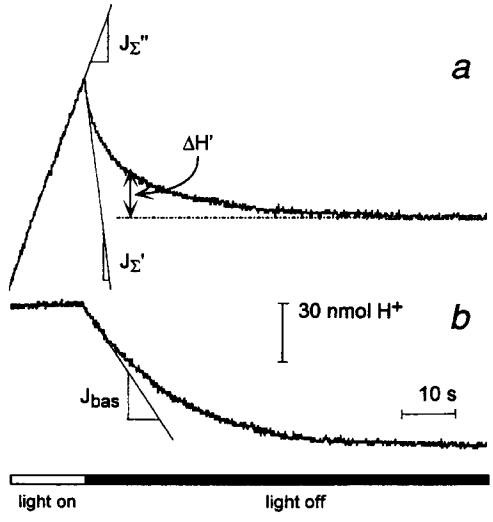

Fig. 3. Time course of external pH changes during continuous illumination and the subsequent dark period. (*a*) Complete reaction system. The pH increase during the illumination period indicates the stationary ATP production due to net consumption of exact 0.94 H^+ per each ATP formed at the experimental conditions used here. The transient pH decrease in the dark is caused by the efflux of protons out of the thylakoid lumen. 4 μM nonactin has been added for suppression of the transmembrane diffusion potential arising in the dark. (*b*) Phosphorylation is totally blocked by addition of 1 μM venturicidin. Internal pH has been adjusted to the same value as in *a* by decrease of light intensity.

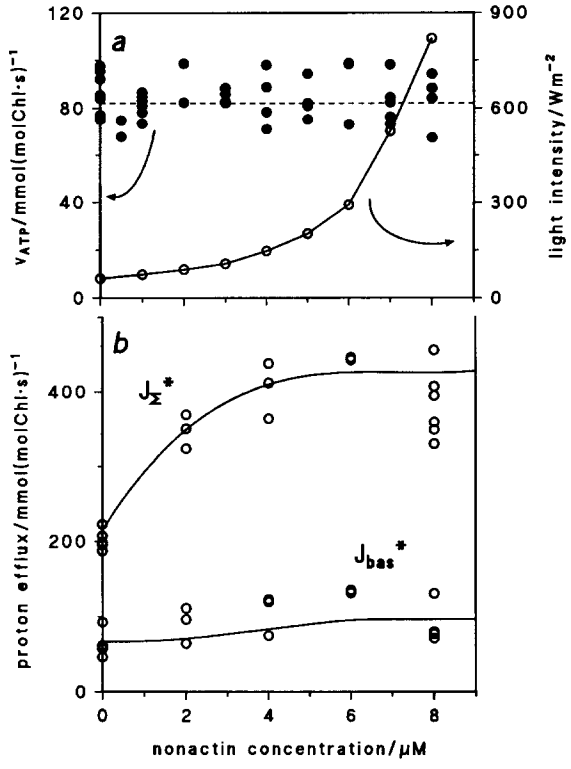

Fig. 4. (*a*) Rate of light-induced ATP synthesis, v_{ATP}, and (*b*) proton effluxes at $t = 0$ in dependence on the nonactin concentration. The rate of ATP synthesis has been obtained from the continuous pH increase in the light and the effluxes from the pH decrease in the dark by application of the tangent method as described above. The light intensity has been adjusted as shown in order to maintain a constant internal pH of 5.4. Suppression of the diffusion potential is indicated by the plateau regions of the proton fluxes above 4 μM nonactin.

is the case for several seconds of dark time as can be seen from Fig. 6. Therefore, the total efflux, J_Σ, is given by $J_\Sigma = J'_\Sigma + J''_\Sigma$ (see Fig. 3*a*). In order to obtain the phosphorylating proton flow, J_p, the basal flow, J_{bas}, which stems from the intrinsic leakiness of the membrane, has to be determined independently (see Fig. 3*b*) and subtracted from the total one. Altogether we obtain:

$$J^*_p = f\left(J'_\Sigma + J''_\Sigma - J_{bas}\right) \quad (6)$$

Correct H^+ flux measurements require suppression of the transmembrane diffusion potential, which is caused by the uncompensated H^+ efflux in the dark, and which gives rise to its retardation. This is achieved by the addition of nonactin, which enhances the permeability of the counterion K^+ [21]. Unfortunately this agent additionally inhibits the electron transport system and reduces phosphorylation this way. This effect was overcome by adjusting the light intensity for constant internal pH, giving rise to a constant rate of ATP synthesis independent of the nonactin concentration (Fig. 4*a*). The results of corresponding flux measurements are shown in Fig. 4*b*. As expected, both J^*_Σ and J^*_{bas} are stimulated by addition of nonactin up to a plateau

in the region above 4 μM nonactin. The plateau indicates the complete suppression of the diffusion potential and for that reason corresponding flux values only equal the true H^+ flow at steady-state. Additionally the diffusion potential has been measured directly by means of the electrochromic absorbance difference between 520 and 535 nm. The value of 73 mV in the absence of nonactin is continually reduced by increasing nonactin concentrations and reaches 1.5 mV at 4 μM nonactin. These results give independent evidence for the total quenching of the diffusion potential above 4 μM nonactin.

According to the foregoing results the H^+/ATP coupling ratio should be given by the J_p^*/v_{ATP} ratio at the limit $\Delta\Psi_{Diff} = 0$ ($v_{ATP} = J''_\Sigma/0.94$ being the rate of ATP synthesis). The J_p^*/v_{ATP} ratio, analyzed in dependence on the nonactin concentration, starts at a value around 2 and levels off at a value around 4 in the region above 4 μM nonactin (Fig. 5). The exact plateau value is 4.0 ± 0.5 ($n = 13$). It indicates the true H^+/ATP coupling ratio.

3.3. Determination of H^+/ATP by kinetic analysis of proton efflux

The procedure of tangent plotting may be replaced by a detailed kinetic analysis of the dark relaxation of the H^+ gradient. By this approach a well founded extrapolation procedure is established, which uncovers the superposition of the basal and phosphorylating proton fluxes and enables their simultaneous determination.

A first order course of the relaxation process during basal conditions and a biphasic one during phosphorylating conditions are becoming evident if a logarithmic amplitude scale is used (see Fig. 6). Taking this into account the following rate law for the relaxation process during phosphorylating conditions is put to the test (for definition of $\Delta H'$ see Fig. 3*a*):

$$-d\Delta H'/dt = k_1\Delta H' + k_2\Delta H'^n \quad (7)$$

Integration yields the following implicit time law:

$$\ln\frac{\Delta H'}{\Delta H'_0} - \frac{1}{n-1}\ln\frac{k_1 + k_2\Delta H'^{n-1}}{k_1 + k_2\Delta H'^{n-1}_0} = -k_1 t \quad (8)$$

By adjustment of k_1, k_2 and n excellent fitting to the experimental data is obtained (see Fig. 6). The first term on the right of Eq. (7), which is of first reaction order with respect to $\Delta H'$, takes into account the basal portion of the H^+ efflux, and the second one, which is of the reaction order $n = 3.15$, takes into account the phosphorylating portion. Therefore, at $t = 0$ the different contributions to the total efflux are given by

$$J'_{bas} = k_1\Delta H'_0 \quad (9a)$$

$$J'_p = k_2\Delta H'^n_0 \quad (9b)$$

Two corrective factors have to be applied, the first one with regard to the imidazole uptake according to Eq. (4), and the second one with regard to the pH change induced by dark phosphorylation. We thus obtain

$$J_i^* = f(1 + J''_\Sigma/J'_\Sigma)J'_i \quad (10)$$

where J_i^* is the true efflux and the suffix i denotes either the basal or the phosphorylating portions.

It should be emphasized that Eq. (7) is an empirical one without a direct mechanistical basis. The reason is that $[H_i^+]$ rather than $\Delta H'$ represents the driving force for proton efflux. $\Delta H'$ is related to $[H_i^+]$ through the buffer capacity inside the thylakoids, which depends on the amount of added imidazole. Therefore, the reaction orders in Eq. (7) emerging here are valid only for the imidazole concentration of 0.4 mM used here. However, the reaction order with respect to $\Delta H'$ can be transformed to the

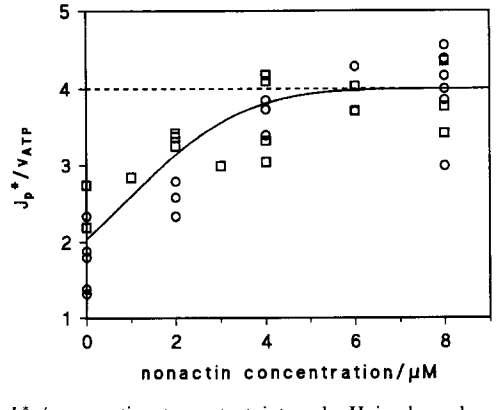

Fig. 5. J_p^*/v_{ATP} ratio at constant internal pH in dependence on the nonactin concentration. The proton efflux at $t = 0$ has been evaluated by the tangent method. Circles correspond to the data from Fig. 4 and squares to data for a different thylakoid preparation. The plateau value of 4 above 4 μM nonactin corresponds to the H^+/ATP coupling ratio at the ATP synthase.

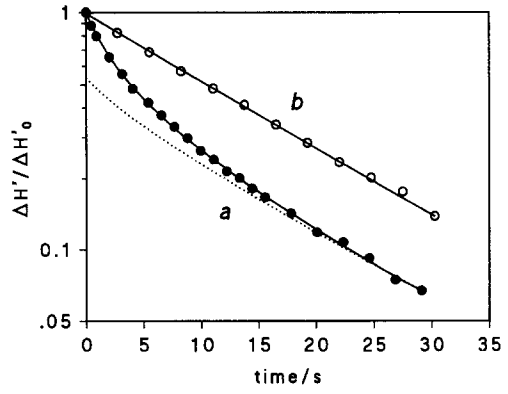

Fig. 6. Experimental data from Fig. 3 (meaning of *a*, *b* as above) fitted by curves according to Eq. 7, taking into account $k_2 = 0$ in the case of basal conditions. The dotted curve indicates the basal portion of the transported protons in the case of the complete reaction system. $\Delta H'_0$ corresponds to 1.75 mol (mol Chl)$^{-1}$. For this procedure 4 single measurements have been sampled and digitized.

corresponding reaction order with respect to $[H_i^+]$ if Eq. (2) is taken into account. By this procedure reaction orders of 0.84 and 2.64 with respect to $[H_i^+]$ are calculated for the basal and phosphorylating proton fluxes, respectively, in the region $[H_i^+] \gg [H_a^+]$. This is sufficiently close to the direct results of 0.80 and 2.60 which have been obtained during steady state-conditions where $[H_i^+]$ has been changed by change of the light intensity (Fig. 7). Hence, the analysis of the steady-state data in Fig. 7 verifies the correctness of Eq. (7) independently of the relaxation analysis of the H^+ gradient in Fig. 6, in particular the splitting of the total H^+ flux into the independent basal and phosphorylating portions.

The J_p^*/v_{ATP} ratio measured by application of the method of kinetic analysis as described above has been analysed in the dependence on the nonactin concentration as in the case of the tangent method. The results are shown in Fig. 8. The J_p^*/v_{ATP} ratio starts at a value around 2 and levels off at a value around 4 in the region above 4 μM nonactin. The exact plateau value is 4.0 ± 0.4 ($n = 29$). The results of both methods are identical within experimental error and indicate a true H^+/ATP coupling ratio of four.

4. Discussion

The H^+/ATP coupling ratio at the ATP synthase of chloroplasts has been a matter of controversy over three

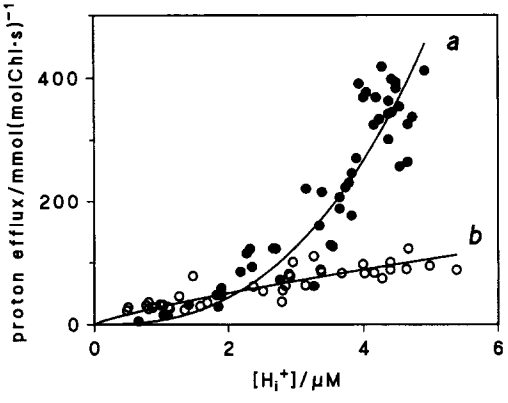

Fig. 7. Steady-state rates of (*a*) the phosphorylating proton flux, J_p, and (*b*) the basal proton flux, J_{bas}, in dependence on the internal proton concentration, which was regulated by the light intensity. J_p was obtained from the steady-state rate of ATP synthesis taking into account the H^+/ATP ratio of 4. J_{bas} has been obtained from the dark decay of the proton gradient as described in Section 3.2. For this last measurement phosphate has been replaced by 0.3 mM Tricine buffer and 4 μM nonactin was present. The plotted curves are based on powers of 2.6 and 0.8 with respect to $[H_i^+]$ as described in the text. The observed $[H_i^+]$ profile of ATP synthesis stems essentially from the need for $[H_i^+]$ to put the unmodulated enzyme in the active state [15]. This is in contrast to the modulated (reduced) enzyme, where the activation barrier is shifted substantially downwards, exposing the $[H_i^+]$ profile of the catalytic reaction cycle, which is located between the activation profiles of the modulated and unmodulated enzyme.

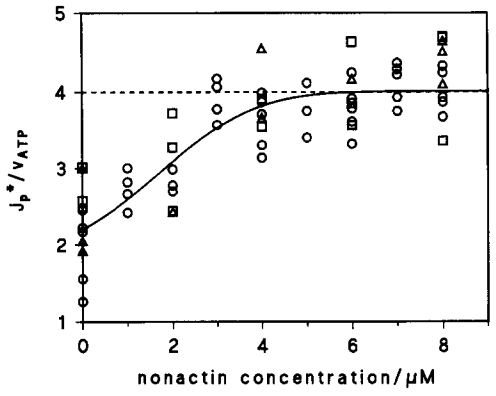

Fig. 8. J_p^*/v_{ATP} ratio at constant internal pH in dependence on the nonactin concentration. The proton efflux at $t = 0$ has been evaluated by the analytical extrapolation method. Circles, squares and triangles correspond to different thylakoid preparations. The plateau value of 4 above 4 μM nonactin corresponds to the H^+/ATP coupling ratio at the ATP synthase.

decades. Initially a number of 2 has been put forward by Mitchell [22]. Over the years a value of 3 seemed to be more realistic [7–14]. However, some of these measurements (Refs. [9,12,14]) have been based on ATP/e determinations taking into account a H^+/e ratio of 2 and ignoring the Q-cycle activity which shifts the H^+/e ratio towards 3 and accordingly the H^+/ATP ratio to a value higher than 3. In fact, in 1990 our laboratory gave clear evidence of the real H^+/ATP coupling ratio to be 4 [17]. This has been derived mainly from energy balance measurements and was supported by others [16]. Nevertheless the number of 3 continued to prevail in the literature. The results presented here produce further evidence that the real H^+/ATP is 4 indeed. These results are based on H^+ flux measurements, where the H^+ flux is obtained from the initial efflux in the dark, monitored by the external pH decrease. This method yields correct results only, if (1) the influence of the pH change due to dark phosphorylation is taken into account, (2) the total flow is corrected for the nonphosphorylating basal flow and (3) the transmembrane diffusion potential produced by the uncompensated H^+ efflux in the dark is suppressed.

The following comments are worth to be added: (1) Contrary to the former investigations [16,17], where ATP synthases were reduced by DTT or thioredoxin, no such modification has been applied here. Therefore, it can be stated that the H^+/ATP coupling ratio is 4, independent of the reduction state of the ATP synthase. (2) Kinetic modelling of the ATP synthase as put forward recently also suggests a H^+/ATP coupling ratio of 4 [23]. (3) An interesting hypothesis assumes the H^+/ATP coupling ratio to be equal to the stoichiometric ratio of the H^+ binding subunits III in CF_0 (corresponding to subunit c in the *E. coli* enzyme) and the nucleotide binding subunits β in CF_1 [24]. A value of 4 for the H^+/ATP ratio would suggest a number of 12 c-subunits, which falls into the span of 9–12

c-subunits determined so far [25]. (4) Focussing on the overall process of plant photosynthesis where an ATP/NADPH ratio of 1.5 is needed to accomplish CO_2 reduction, a number of 3 H^+ to be translocated across the thylakoid membrane per each electron transported emerges on the basis of $H^+/ATP = 4$. Taking into account the additional contribution of approx. 20% basal H^+ translocation, this number has to be raised to 3.6. If compared with the maximal H^+/e ratio of 3.0 during linear electron transport [26], a deficit emerges which has to be covered by an additional cyclic electron transport route.

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