REGULATION OF THE MEMBRANE PERMEABILITY OF SPINACH CHLOROPLASTS BY BINDING OF ADENINE NUCLEOTIDES

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1. Introduction

It is well known that the proton transport at isolated chloroplast membranes is influenced by the addition of adenine nucleotides (ADP, ATP) [1–11]. The main results are as follows:

1. Under continuous illumination, the proton efflux after illumination was increased under phosphorylating conditions (+P_i, +ADP) as compared to non-phosphorylating conditions [1]. Accordingly, the steady state proton uptake (and correspondingly the ΔpH) was decreased under phosphorylating conditions [1–3].

2. Under continuous illumination, after addition of ATP or ADP under non-phosphorylating conditions (i.e., in the absence of P_i), the proton efflux was decreased and, correspondingly, the proton uptake was increased [2,4].

3. After flash excitation, the decay of the transmembrane potential difference measured by the absorption change at 515 nm [5] is accelerated under phosphorylating conditions as compared to non-phosphorylating conditions [6–9]. Thus, the effect of phosphorylation on the membrane permeability is similar in flash light and in continuous light.

4. On addition of ATP, an accelerated decay of the electrochromic absorption change as compared to that without ATP has been observed [10,11]. This effect has been interpreted by an increased proton conductivity of the membrane due to the bound ATP. Consequently, the acceleration under phosphorylating conditions was interpreted to be due to the newly synthesized ATP [10,11], in contrast to the interpretation given above. Furthermore, as mentioned above, addition of ATP, however, decreases the proton efflux under continuous illumination.

In order to clarify the effect of ADP and ATP on the membrane permeability, we investigate the influence of ATP and ADP under phosphorylating and non-phosphorylating conditions on the decay of the flash-induced electrochromic absorption changes. Since it is known that the nucleotides are bound almost exclusively to the coupling factor, CF_1 [12], the observed effects directly show the influence of nucleotide binding to CF_1 on the membrane permeability under different conditions. It is found that under non-phosphorylating conditions low concentrations (μM range) of ADP and ATP decrease the membrane permeability in accordance with the measurements in continuous light. Also, in the presence of P_i, i.e., under phosphorylating conditions, low concentrations of ADP (1–2 μM) decrease the membrane permeability. Higher concentrations (2 μM–1 mM) increase the membrane permeability.

2. Materials and methods

The spinach (*Spinacea oleceria*, var. 'Matador') was grown in a phytochamber (conditions: day, 8 h, 15°C, light intensity 2 mW/cm²; night, 16 h, 10°C, 80% humidity). After harvesting, the leaves were kept in the dark on ice for at least 60 min, homogenized with a Waring blender for 3 bursts of 10 s in a medium containing 0.3 M NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 50 mM tricine (pH 7.4) and 10 mM Na-ascorbate, squeezed through 3 layers of a nylon net (2 layers N45, 1 layer N53, H. Simon, England) and

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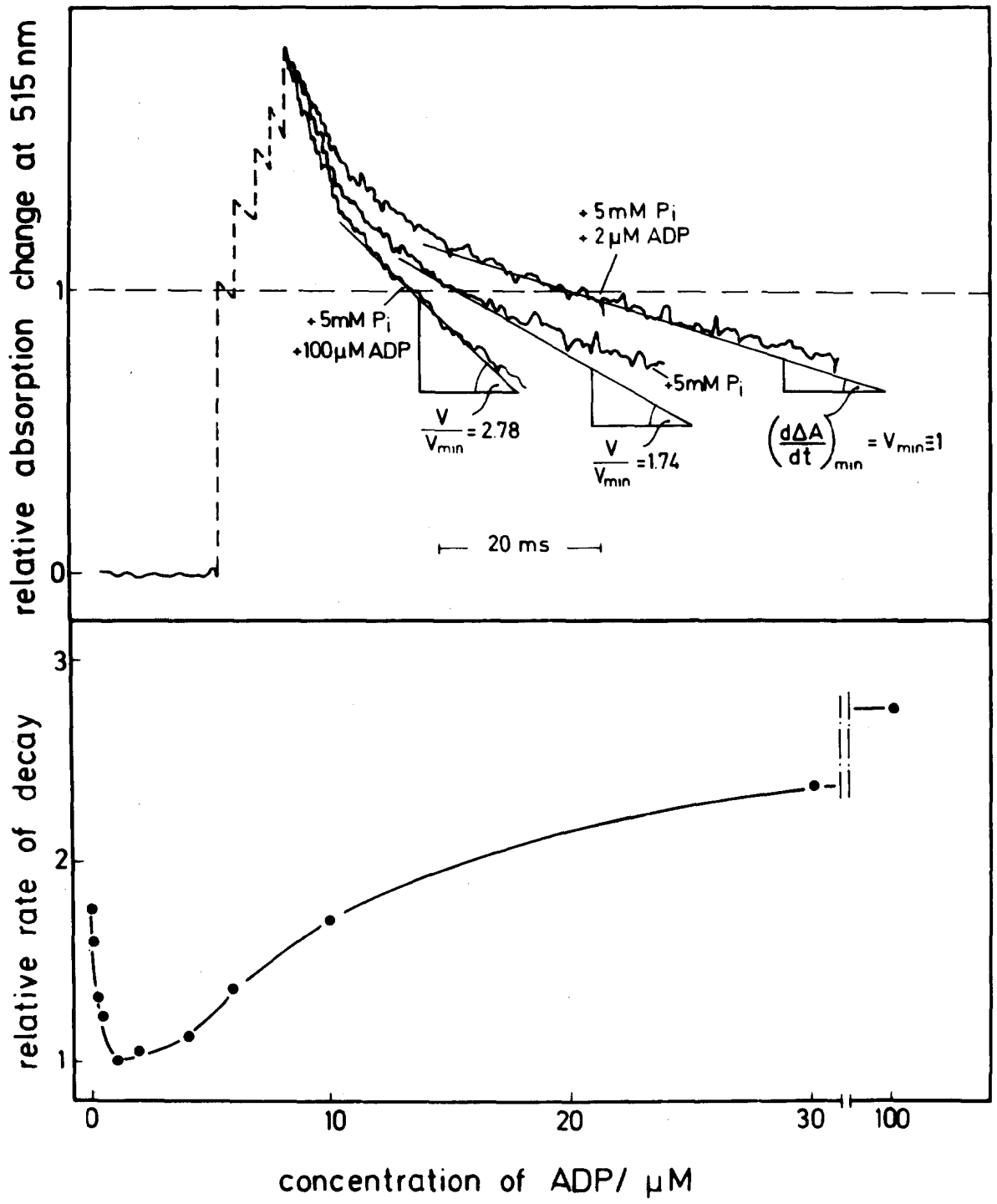

Fig.1. (Top) Relative absorption change at 515 nm elicited by a flash group in the presence of 5 mM P_i and either no further addition or addition of 2 μM ADP or 100 μM ADP. The rate of decay at the relative absorption change 1 in the presence of 2 μM ADP is arbitrarily set to 1. The relative rates of the additional traces are indicated. (Bottom) Relative rate of the decay of the absorption change as a function of the ADP concentration. Data from fig.1 (top) and additional measurements. Details are in the text.

centrifuged (7 min, 1000 × *g*). The sediment was washed twice (7 min, 1000 × *g*) in a medium containing 0.4 M sucrose, 10 mM NaCl, 2 mM $MgCl_2$, 20 mM tricine (pH 7.4) and finally resuspended in the same medium at ~4 mM chl. The chloroplasts were kept on ice and used within 3 h after preparation.

The reaction medium contained 20 mM tricine adjusted to pH 8 with NaOH, 5 mM $MgCl_2$, 20 mM sucrose, 0.1 mM benzylviologen and chloroplasts giving 10 μM chl final conc. Thus, endogeneous nucleotides are at $\sim 10^{-8}$ M, if the amount of tightly bound nucleotides is AdN/chl $\approx 10^{-3}$ [12,13]. Addition of nucleotides and P_i as indicated in the figures. ATP, ADP, and AMP were obtained from Boehringer with highest purity available.

Illumination of 5 ml of this suspension was carried out in an optical cell (optical pathlength 20 mm) by flash groups (wavelength > 610 nm). Each group consists of 5 single turnover flashes spaced 2 ms; the dark time between groups was 10 s. The absorption change at 515 nm (measuring light intensity 180 μW/cm², optical bandwidth $\Delta\lambda = 8$ nm) was measured in a flash spectrophotometer [14] and the signals were sampled in an averager (NIC 1072) in order to increase the signal-to-noise ratio. To avoid induction phenomena, the signals from the last 16 groups out of a train of 18 flash groups were sampled.

3. Results

3.1. *Effect of ADP on the potential decay in the presence of P_i*

Fig.1 (top) shows the decay of the transmembrane potential difference ($\Delta\psi$) after excitation with a flash group under 3 different conditions. It can be seen that in the presence of P_i without any added ADP (endogeneous nucleotides are estimated to be at <0.1 μM; section 2) the rate of the decay is fast; addition of ADP in low concentrations ([ADP] = 2 μM) decreases the rate (deceleration effect); addition of higher concentrations ([ADP] = 30 μM) increases the rate to higher values than without ADP (acceleration effect). Obviously, in the presence of P_i, the addition of ADP creates two effects:

(i) At low concentrations a decrease of the membrane permeability;
(ii) At higher concentrations an increase of the membrane permeability.

This is shown in more detail in fig.1 (bottom). Here, the relative rate of the $\Delta\varphi$ decay is plotted as a function of the ADP concentration. The relative slope was obtained as shown in fig.1 (top) at the relative absorption change 1. This corresponds to the potential generated in a single turnover flash ($\Delta\psi = 50$ mV). The slope in the presence of 2 μM ADP has been set arbitrarily to 1. The decrease of the membrane permeability occurs over 1–2 μM, the increase over 10–20 μM. No increase was observed in the absence of P_i even at up to 100 μM ADP.

In order to exclude a possible superposition of electrochromic absorption changes and changes in light scattering, measurements have been carried out at 515 nm and 482 nm where the electrochromic absorption has an opposite sign and the proportion of light scattering changes is different from that at 515 nm.

Fig.2 (left) shows that the rate of decay is increased at both wavelengths if, in the presence of 5 mM P_i, 100 μM ADP is added. Fig.2 (right) shows that the rate of decay is decreased at both wavelengths if, in the absence of P_i, 20 μM ADP is added. P_i has been omitted in the latter case for the sake of simplicity. Without P_i (i.e., under non-phosphorylating conditions) the increase in the rate of decay at higher ADP concentrations (fig.1) does not take place. These results imply that the ADP binding influences the membrane permeability and that the observed effects are not due to a superposition of electrochromic absorption changes and light scattering changes.

3.2. *Regulating effect of nucleotide binding under non-phosphorylating conditions*

To obtain more quantitative results, the following procedure was used. The rate of the potential decay ($-d\Delta\psi/dt$) which is proportional to the rate of the absorption change, ($-d\Delta A/dt \equiv v$) can be described as follows:

$$v = kf(\Delta\psi) - k'f'(\Delta\psi)\,[E \cdot ADP] \qquad (1)$$

where the first term on the right side describes the rate, if no ADP is present, v_o, i.e., if $[E \cdot ADP] = 0$. The dependence of the rate on $\Delta\psi$ is described as $f(\Delta\psi)$, i.e., it is not specified, since in the following the rates are compared at a constant $\Delta\psi$. (In the simplest case, the rate is proportional to $\Delta\psi$; however, a logarithmic plot of the data shows that the decay is at least triphasic under these conditions.) The second term describes the dependence of the rate on the ADP concentration, and it is assumed that the measured rate

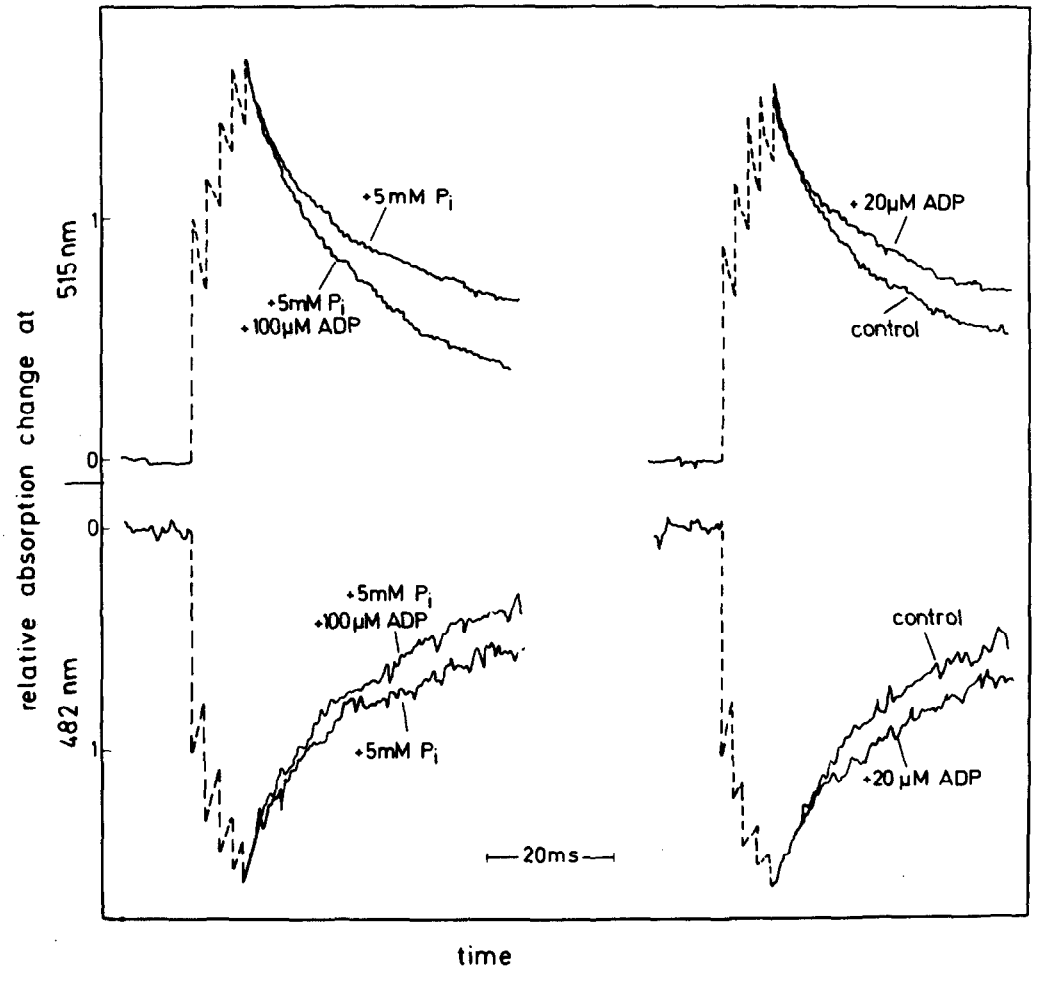

Fig.2. Kinetics of the relative absorption change at 515 nm and 482 nm. (Left) Acceleration of the decay by addition of 100 μM ADP in the presence of 5 mM P_i. (Right) Deceleration of the decay by addition of 20 μM ADP in the absence of P_i.

v is smaller if ADP is bound at the ATPase, E. It follows that the rate, with high ADP concentration (v_∞) is given by:

$$v_\infty = kf(\Delta\psi) - k'f'(\Delta\psi)\,[E_t] \qquad (2)$$

where E_t is the total amount of ATPases present, because all ATPases are saturated with ADP under these conditions. The maximum rate regulated by ADP binding is then $v_m = v_o - v_\infty$ (at $\Delta\psi$ = const.). A simple association/dissociation equilibrium is assumed under energized conditions, that is:

$$\text{E (high permeability)} + \text{ADP} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{E} \cdot \text{ADP (low permeability)}$$

With the dissociation constant, $K_d = k_2/k_1$, it results in:

$$[E \cdot ADP] = \frac{[E_t]\,[ADP]}{K_d + [ADP]} \qquad (3)$$

From eq. (1) and (3) results:

$$v_o - v = k'f'(\Delta\psi)\,[E_t]\,\frac{[ADP]}{K_d + [ADP]} \qquad (4)$$

Thus, a plot of $1/(v_o - v)$ versus $1/[ADP]$ gives the dissociation constant, K_d.

Fig.3 shows the rate of potential decay ($v_o - v$) as

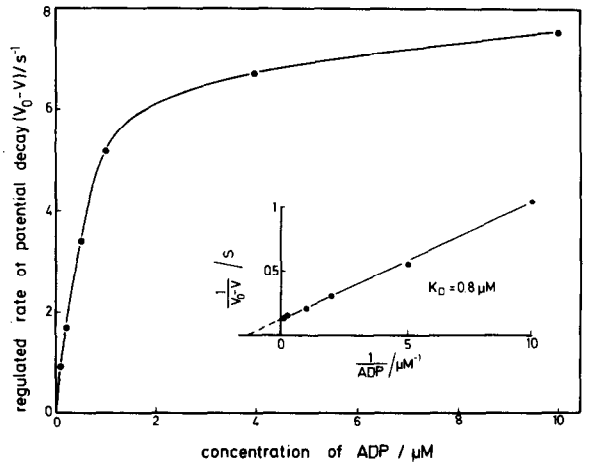

Fig.3. Regulated rate of potential decay ($v_o - v$) as a function of the ADP concentration. (Inset) Determination of the dissociation constant. Details see text.

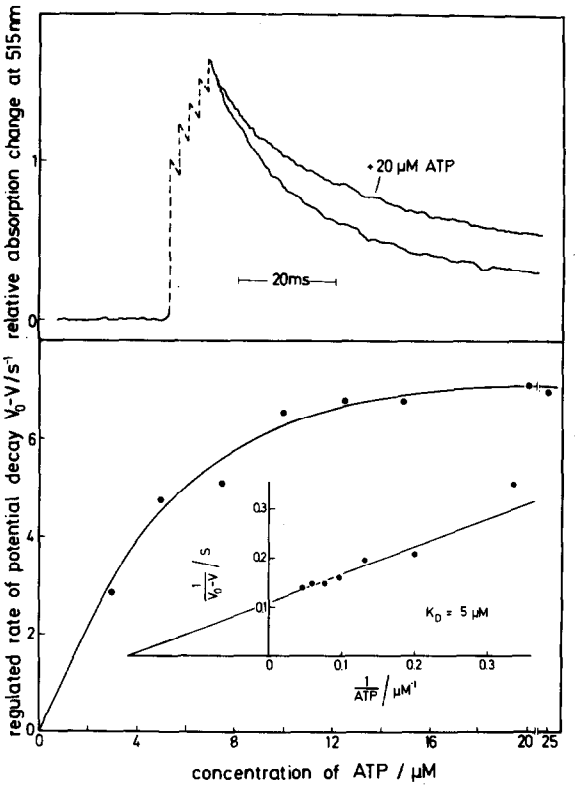

Fig.4. (Top) Effect of ATP on the kinetics of potential decay. (Bottom) Regulated rate of potential decay ($v_o - v$) as a function of ATP concentration. (Inset) Determination of the dissociation constant. Details see text.

a function of the ADP concentration in the absence of P_i. Under these conditions, a monotonic decrease of the rate with increasing ADP concentration is observed. Obviously, the increase of the rate at higher ADP concentrations occurs only in the presence of P_i or, more generally, only under phosphorylating conditions. The insert in fig.3 shows a double reciprocal plot of the data, giving a value of K_d = 0.8 μM. The mean value of different chloroplast preparations and of measurements carried out at 515 nm and 482 nm is $K_{d,ADP}$ = 1.5 ± 1 μM. Omission of Mg^{2+} from the reaction medium does not influence the deceleration effect caused by ADP. ([Mg^{2+}] in the experiment without added Mg^{2+} was actually 8 μM due to the suspension medium of the added chloroplasts.)

Fig.4 (top) shows the effect of ATP on the rate of potential decay. Also in this case, a decrease of the rate is observed. Fig.4 (bottom) shows the rate of potential decay ($v_o - v$) as a function of the ATP concentration in the absence of P_i. A decrease of the rate is observed at least up to [ATP] = 25 μM. A quantitative evaluation of the data (see fig.4, inset) gives a dissociation constant of $K_{d,ATP}$ = 5 μM. The mean value of different chloroplast preparations is $K_{d,ATP}$ = 6 ± 2 μM.

3.3. *The influence of phosphorylating conditions and internal pH*

The acceleration effect was also measured as a function of P_i and ADP concentration. (For the acceleration effect the presence of Mg^{2+} is absolutely required.) For a quantitative evaluation a similar procedure as described above was used. It results in a Michaelis-Menten constant (K_m) for ADP in the presence of 2.5 mM P_i of $K_{m,ADP}$ = 25 ± 8 μM and a K_m for P_i in the presence of 100 μM ADP, K_{m,P_i} = 220 ± 50 μM.

In order to check which type of ion flux is regulated by the ADP and ATP binding, a background illumination (or an increased measuring light intensity) has been used to increase the internal proton concentration. At the top of fig.5, the absorption change of a single turnover flash is depicted indicating that at the highest light intensity used ~10% of the reaction centers are saturated. Fig.5 (bottom) shows the effect of the increased [H^+_{in}] (corresponding to the light intensity) on the rate of potential decay under the different conditions. On increasing the light intensity the rate in the presence of ADP does not increase up

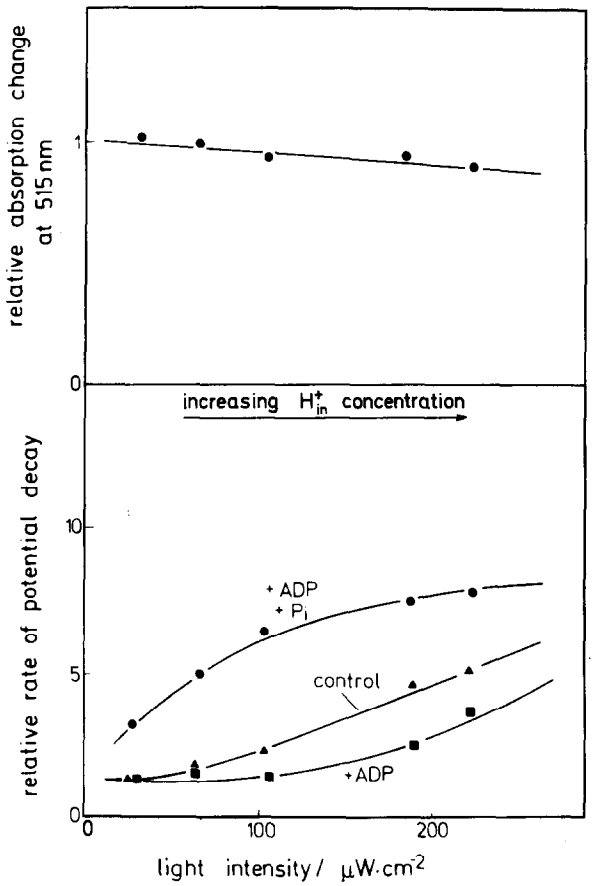

Fig.5. (Top) Relative absorption change of a saturating single turnover flash as a function of light intensity of the measuring light. (Bottom) Relative rate of potential decay at the relative absorption change 1 as a function of the light intensity in the absence of P_i and of ADP, in the presence of 20 μM ADP and in the presence of 100 μM ADP and of 5 mM P_i. At the top, the direction of increasing $[H^+_{in}]$ is indicated. Details see text.

to 100 μW/cm², whereas, in the absence of ADP, the increase of the rate occurs at lower light intensities. Under phosphorylating conditions the rate is always higher at all light intensities.

4. Discussion

Usually, under single turnover conditions, the basal decay of $\Delta\psi$ is not due to protons but to other ions, e.g., K^+, Cl^-, Mg^{2+} as can be seen by comparing the proton efflux after a single turnover flash ($\tau \approx 3$ s) with the rate of potential decay ($\tau \approx 0.1$ s). However, on increasing the internal $[H^+]$ (and the transmembrane ΔpH) the proportion of H^+ in the basal flux increases. Thus, the dependency of the decay rate of $\Delta\psi$ on light intensity or $[H^+_{in}]$ (see fig.5) might be interpreted as follows: in the absence of ADP the decay rate increases with increasing light intensity from 20 μW/cm², indicating that the $[H^+_{in}]$ is high enough that the $\Delta\psi$ decay is partly due to a proton flux. In the presence of ADP an increase of the decay rate with light intensity is seen only above 100 μW/cm², indicating that under these conditions a higher $[H^+_{in}]$ must be obtained before the $\Delta\varphi$ decay is partly due to a proton flux. Thus, μM levels of ADP ($K_{d,ADP} \approx 1.5$ μM) or ATP ($K_{d,ATP} \approx 6$ μM) decrease the proton permeability of the membrane. This finding is in accordance with earlier observations obtained by measurements of the proton uptake and proton efflux in continuous light [4].

The deceleration effect of nucleotides on the $\Delta\psi$ decay had not been reported [7,9,10]. According to fig.5, this is expected at low measuring light intensities (as used in [7,9,10]): the nucleotide binding obviously regulates only the proton flux (and not that of other ions responsible for the $\Delta\psi$ decay) and the proton flux at low $[H^+_{in}]$ is too small to influence the basal $\Delta\psi$ decay significantly. However, at high measuring light intensities the deceleration effect has been observed also by others (U. Siggel, unpublished). The acceleration effect by ATP (see section 1) has been observed under different conditions: low measuring light intensity and mM ATP levels [10,11]. In this case the internal $[H^+]$ is increased by proton pumping due to ATP hydrolysis and consequently after flash excitation an increased efflux is observed [10,11].

Also, in the presence of P_i, i.e., under phosphorylating conditions, the addition of low concentrations of ADP decreases the proton permeability. The dissociation constant under these conditions can only be estimated because of the superposition of the acceleration effect. It is practically identical with the one under non-phosphorylating conditions ($K_{d,ADP} \approx$ 1.5 μM). Addition of ADP in higher concentrations increases the proton permeability. The acceleration effect is assumed to be due to the phosphorylation-coupled proton efflux [6,7,9] and in accordance with this assumption, the K_m value found here for the acceleration by ADP, $K_{m,ADP} = 25$ μM (in presence of 2.5 mM P_i) is similar to that found for phosphorylation under comparable conditions [15,16]. Also, the

K_m value for the acceleration by P_i, K_{m,P_i} = 220 μM (in the presence of 300 μM ADP) is similar to that found for phosphorylation under comparable conditions (K_{m,P_i} = 350 μM [17]).

Obviously, addition of ADP in the presence of P_i produces contrary effects on the proton permeability of the membrane: at low concentrations a decrease of the permeability, at high concentrations an increase of the permeability. Whereas the latter effect depends on the simultaneous presence of P_i and Mg^{2+}, the former occurs also in the absence of P_i and Mg^{2+}.

The relevance of these observations with regard to the mechanism of phosphorylation is not completely clear. As discussed in [18] the dissociation constant of ADP for the site which decreases the proton flux is practically identical with the dissociation constant of the tightly bound ADP [19]. As shown here, the K_m values for the site which increases the potential decay (for ADP as well as for P_i) are similar to the corresponding constants for phosphorylation. Therefore, it might be assumed that the different permeability regulating sites are identical with the 'tight site' and the catalytic site.

The release of tightly bound AdN may be connected with the activation of the ATPase [13,20]. It was shown, in particular, that the fast phase of the AdN release reflects the number of active ATPases [13]. The rate of ATP synthesis was directly proportional to the number of 'active' ATPases, i.e., to the number of those ATPases which have rapidly released the tightly bound AdN [13,21]. Because of the microscopic reversibility, the ATPases which have released their tightly bound nucleotides, must also be active in ATP hydrolysis. The correlation between the release of tightly bound nucleotides and ATP hydrolysis has been investigated by different authors, and it was found that the ATPases which have released their tightly bound AdN are able to catalyze ATP hydrolysis [22–24]. Thus, it seems to be clear that the binding of ADP to the tight binding site inhibits not only the proton flux through the ATPase but also ATP synthesis [13] and ATP hydrolysis [22–24].

The increase of the proton permeability of the ATPase after activation draws protons from the inner aqueous phase preferentially through the ATPase. This might be of some advantage in a subsequent utilization of protons in ATP synthesis at these active ATPases.

Acknowledgements

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