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Studies on well-coupled Photosystem I-enriched subchloroplast vesicles – energy-dependent switching between two different active states of the proton-translocation adenosine triphosphatase

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Single-turnover flash-induced ATP synthesis coupled to natural cyclic electron flow in Photosystem I-enriched subchloroplast vesicles (from spinach) was continuously followed by the luciferin-luciferase luminescence. The ATP yield per flash was maximal (1 ATP per s per 1000 Chl) around a flash frequency of 0.5–2 Hz. It decreased both at lower and higher flash frequencies. The decrease at high flash frequency was due to limitation by the electron-transfer rate, while the decrease at low flash frequency was directly due to intrinsic properties of the ATPase itself. Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) decreased the yield at low frequency more than at high frequency. The same behaviour was observed if electron transfer was artificially mediated by pyocyanin. If the ADP concentration was increased from 40 to at least 80 μM, or if the vesicles were preincubated with 5 mM dithiothreitol (DTT), the decrease of the yield at flash frequencies below 0.5 Hz was no longer observed. Incubation with DTT increased the rates of ATP hydrolysis and synthesis at any flash frequency. The decrease of the yield could be elicited again by addition of 50 nM FCCP. It is concluded that at low levels of the protonmotive force ($\Delta\tilde{\mu}_{H^+}$), the ATPase is converted into an active ATP-hydrolyzing state in which ATP synthesis activity is decreased due to a decreased affinity towards ADP and/or to a decreased release of newly synthesized ATP, that can be cancelled by increasing the ADP concentration or by addition of DTT in the absence of uncoupler.

Introduction

By mild digitonin treatment of broken spinach chloroplasts, PS I-enriched membrane vesicles can be prepared that are devoid of PS II activity and are capable of cyclic electron transfer around PS I and of cyclic phosphorylation under single-turnover as well as under steady-state conditions [1–6].

In these vesicles, the ATPase is already active [4,5] before reduction (modulation) with thiols

Abbreviations: Chl, Chlorophyll; DTT, threo-2,3-dihydroxy-1,4-dithiol butane; ΔG_P, Gibbs free-energy change for ATP hydrolysis (phosphate potential); $\Delta\tilde{\mu}_{H^+}$, electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$ inside minus outside); FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; PS I, Photosystem I; PS II, Photosystem II; pyocyanine, 1-hydroxy-5-methylphenazinium hydroxide inner salt; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

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(c.f. Refs. 7–12), possibly as a result of the digitonin treatment during preparation of the vesicles [13,14], or due to the presence of the thioredoxin system [9,15–20] allowing reduction of the ATPase directly by PS I (in the light) or by NADPH.

Single-turnover experiments, in which the rate and number of turnovers (each induced by one light flash) can be easily controlled, are valuable for kinetic and quantitative studies on cyclic electron flow and the resulting ATP synthesis. In chloroplasts [21–25], chromatophores [25–27] and PS I vesicles [5], the ATP yield per single-turnover flash varies with the flash frequency. Usually, the yield is maximal around 1 Hz and drops at lower and at higher flash frequency.

At high frequency, electron transfer and the associated proton translocation have been proposed to be rate-limiting [5,22,27,28]. The decrease of the yield at low frequency has been attributed by different authors to different effects: (1) to the decay of the $\Delta\tilde{\mu}_{H^+}$ in the interval between the flashes, resulting in a lower level of $\Delta\tilde{\mu}_{H^+}$ at the moment of flashing [21,25,27]; (2) to the decay of a specific redox state of some membrane component [22]; (3) to a decrease of the intrinsic turnover rate of the ATPase below a certain level of $\Delta\tilde{\mu}_{H^+}$ [5]; or (4) to an increased chance that ATP, synthesized on a previous flash and still bound to the ATPase, becomes hydrolyzed again, before its release is induced by a subsequent flash [23].

The possibility that the decrease of the ATP yield at low flash frequency is caused by a decrease of the intrinsic turnover rate of the ATPase below a certain threshold level of the $\Delta\tilde{\mu}_{H^+}$ (third possibility mentioned above) was suggested by us on the basis of recent experiments, carried out with the above-mentioned PS I vesicles and involving flash frequency modulation and mild uncoupling [5]. The implications of such a $\Delta\tilde{\mu}_{H^+}$-dependent transition of the intrinsic ATPase turnover rate were detailed in a previously published kinetic model [5]. With the model we were able to simulate our experimental data.

Although the decrease of the yield per flash was ascribed solely to the behaviour of the ATPase and not to changes in the efficiency of electron transfer or of the electron transfer-associated proton translocation [5], this was not rigorously tested experimentally. In addition, the results in Ref. 5 did not reveal the physiological mechanism responsible for the proposed decrease of the intrinsic ATPase turnover rate at low $\Delta\tilde{\mu}_{H^+}$.

In our vesicles, a true ATPase deactivation (leading to inhibition of ATP hydrolysis) occurred only after several minutes in the dark, in agreement with what was observed by others in chloroplasts [29–31]. The ATPase hydrolysis rate in the dark immediately after flashing was independent of the flash frequency [5], provided the dark times did not exceed a few minutes, whereas the ATP yield changed instantaneously (and in a completely reversible way) in response to variations of the flash frequency [5]. Apparently, the transition of the yield at low flash frequency did not reflect a transition of the ATPase to a true deactivated state [32–42].

Thus, the low-turnover state of the ATPase as proposed in our previously published model [5] seems to be also an active state, although it is characterized by intrinsically lower ATP synthesis rates.

Junesch and Gräber [30,31] have shown that two different ATP-synthetically active states of the ATPase can occur, viz. oxidized or reduced by dithiothreitol (DTT). The reduced state readily catalyzed ATP synthesis as well as ATP hydrolysis. The oxidized state (in the absence of DTT) became initially activated only at a higher ΔpH than was necessary to drive ATP synthesis [31]. If the deactivation which occurs in response to a decrease of the ΔpH would be slow enough, it might allow some ATP hydrolysis. ATP hydrolysis by the oxidized ATPase was actually demonstrated in Refs. 32 and 33.

Recent observations by Komatsu-Takaki suggested the existence of even three different ATP hydrolytically active states of the ATPase in chloroplasts [32,33], characterized by different hydrolase activities, different reduction levels, different pH profiles and a different susceptibility to uncoupler stimulation. Only in one of these states (generated by illumination in the presence of thiol), ATP hydrolysis could be stimulated by uncoupler. The other two states were not susceptible to uncoupler stimulation, which could point to intrinsic uncoupling of the ATPase itself (c.f. Refs. 24 and 43). The uncoupler-insensitive states displayed relatively low ATP hydrolase activity. Of these two

states, one (reduced) state evolved spontaneously from the uncoupler-sensitive (reduced) state after some time in the dark or could be generated after illumination in the absence of thiol by subsequent addition of thiol in the dark, before true inactivation occurred [33]. The other (oxidized) uncoupler-insensitive state was generated by illumination in the absence of thiol, ADP and phosphate [32,33]. Although capable of ATP hydrolysis, this latter state was not immediately able to synthesize ATP [43]. (By illumination in the absence of thiol, but in the presence of ADP and phosphate, conversely, an ATP hydrolytically inactive but ATP synthetically active state was generated.)

In addition to these observations, it is noteworthy that several papers [44–47] have pointed to the possibility that the unidirectional reactions of ATP synthesis and hydrolysis could be regulated independently from each other. It has been proposed that the unidirectional forward (ATP synthesis) reaction could be specifically increased at higher levels of $\Delta\tilde{\mu}_{H^+}$ [44,45].

The present experiments with PS I vesicles involve the use of artificial electron-transfer mediator, thiol reduction, and variation of the nucleotide concentration.

Materials and Methods

The experimental procedures were as in Ref. 5, unless indicated otherwise. By varying the intensity of the light flashes, it was checked that the flashes, which decayed to half maximal amplitude in only a few microseconds, were saturating. It was also checked (with the use of a transient recorder) that the shape and intensity of the flashes remained unchanged at high flash frequency. The ATP level was monitored continuously in the reaction cuvette by the ATP-specific luciferin-luciferase assay. The constant ATP hydrolysis rate in the dark remained unchanged after flashing, provided the dark times did not exceed a few minutes [4,5]. In trains of single-turnover flashes, the ATP level in the cuvette changed at a constant rate, displaying either net ATP synthesis or decreased ATP hydrolysis with respect to the dark situation. This flash-induced effect was always corrected [5,23,48] for ATP hydrolysis occurring in the dark. Adenylate kinase activity [49] was apparently very low or absent in these PS I vesicle preparations. By way of precaution, diadenosyl pentaphosphate (purchased from Sigma, St. Louis, MO, U.S.A.) was added to a final concentration of 5 μM. Thiol modulation was carried out by addition of dithiothreitol (DTT, also from Sigma) to 5 mM, followed by a dark incubation of 10 min. Thereafter, a short train of flashes sufficed to induce a lasting ATP hydrolysis in the dark, with a rate 2–3 times higher than in unmodulated vesicles.

In some experiments, the ATP level was artificially increased. For this purpose, only 'standard' ATP was used (purchased from LKB-Wallac, Turku, Finland, for the calibration of the luciferase assay). ADP (purchased from Boehringer, Mannheim, F.R.G.) was always treated with glucose and hexokinase and subsequently heated to destroy the enzyme. Solutions of ATP and ADP were stored frozen. Small aliquots were thawed, subsequently kept on ice and used within a couple of hours. At the moment of experimentation, the ATP solution contained no detectable amounts of contaminating nucleotides and in the ADP solutions, the contaminating fraction of ATP was less than 1% (mol/mol), as determined by means of high-performance liquid chromatography (HPLC). In some experiments, ADP was used that had been further purified by HPLC, and contained less than 0.01% contaminating ATP. This will be denoted below as 'purified ADP'. The purity of both types of ADP was confirmed by the luciferase test.

Results

Flash frequency-dependent variation of the ATP yield – the high flash-frequency range

From Fig. 1, it appears that the ATP yield per flash not only decreases at low flash frequency, but also at high flash frequency (above 3 Hz in the native system and above 1.3 Hz in the pyocyanin-mediated system). We will examine the decrease of the yield at high flash frequency separately from the decrease at low flash frequency.

As far as the behaviour of ATP synthesis at high flash frequency (above 2–4 Hz) is concerned, the following points are noteworthy. (1) In con-

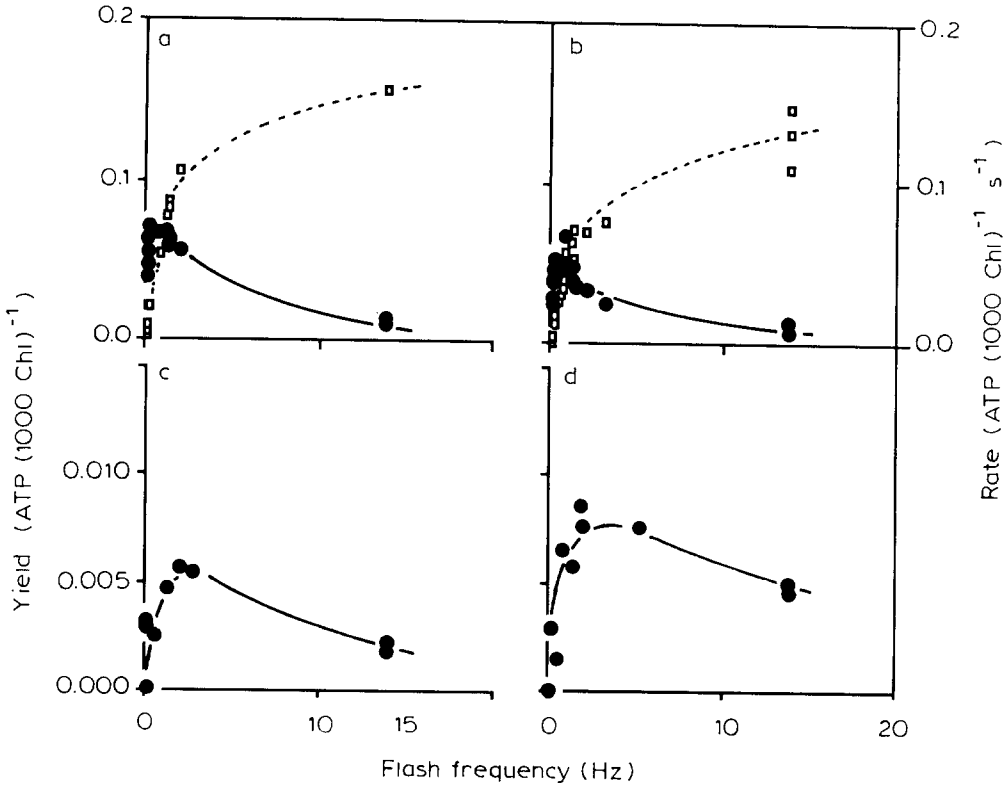

Fig. 1. The ATP yield per flash and the ATP synthesis rate as a function of flash frequency. Note the different scalings. Solid symbols: yield per flash; open symbols: corrected synthesis rate (see Materials and Methods). Ferredoxin was present at 5 μM in a and c; pyocyanin at 10 μM in b and d. FCCP was added to 500 nM in c, and to 250 nM in d. The reaction mixtures further contained: 10 mM $KHCO_3$, 2 mM K_2HPO_4, 38 μM ADP, 5 μM diadenosyl pentaphosphate, 1.75 mM NADPH, 40 mM Tes-KOH buffer (pH 8.0) and 350 μM O_2. The temperature was 10 ° C.

trast to the ATP yield per flash, the actual ATP synthesis rate continued to increase at increasing flash frequency, at least until 14 Hz, which was the highest flash frequency tested in the present experiments (Fig. 1a and b). (2) At 14 Hz, the net synthesis rate varied from 0.2 to only 0.9 ATP per s per 1000 Chl (mol/mol). This cannot be the limiting rate of ATP synthesis in our system: the ATPase is able to drive ATP synthesis at steady-state rates varying from 2.3 to 12.4 ATP per s per 1000 Chl, i.e., from 2.7 to 14 nmol ATP per s per mg Chl (not shown) in the same reaction mixture under continuous illumination. (3) In general, the presence of uncouplers will strongly decrease the effective level of $\Delta\tilde{\mu}_{H^+}$. In the presence of uncouplers, the same level of $\Delta\tilde{\mu}_{H^+}$ and the same ATP synthesis rate (also the limiting turnover rate!) will thus be reached at much higher flash frequencies than in the control situation. However, the decrease of the yield at high flash frequency was not much changed in the presence of uncoupler (Fig. 1). (4) If the decrease of the yield at high flash frequency would be due to rate limitation in the ATPase, it would be expected that the yield always started to decrease more or less at the same ATP synthesis rate, even in the presence of uncouplers. It is clear from Fig. 1 that this was not the case, since the maximal yield was much lower in the presence of FCCP or valinomycin, while the onset of the decrease remained more or less in the same flash frequency range. (5) At flash frequencies above 0.5–1.0 Hz, the flash-induced extra transfer of electrons in the cytochrome *bf* complex (and the flash-induced increase of membrane potential, not shown) is clearly diminished (Fig. 2).

From (1)–(5), it appears that the decrease of the ATP yield at high flash frequency (above 2–4

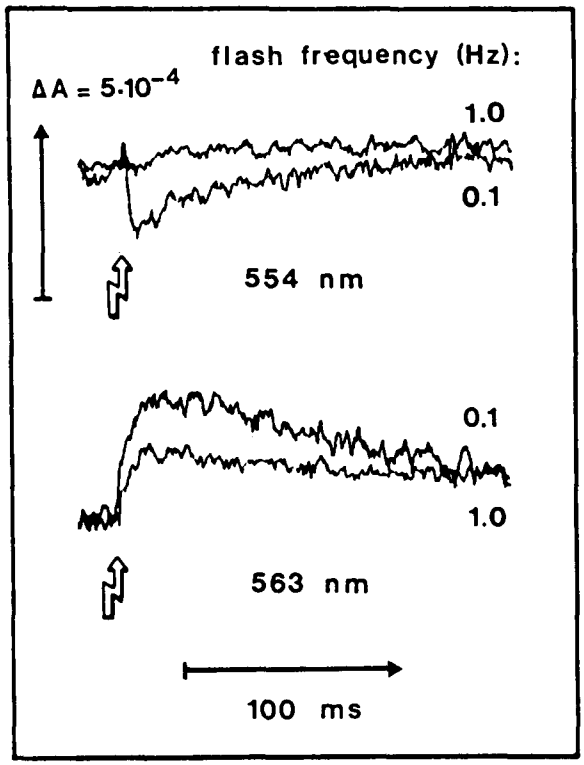

Fig. 2. Flash-induced electron-transfer-related absorption transients. ADP and ATP were absent. The arrows indicate the moment of flashing. Further conditions as in Fig. 1a.

Hz) is not the result of rate limitation at the level of the ATPase, but rather at the level of electron transfer-driven proton translocation, in agreement with earlier conclusions about chloroplast and bacterial systems [5,22,27,28].

Flash frequency-dependent variation of the ATP yield – the low flash frequency range

We compared the native (ferredoxin-mediated) system to a system in which electron transfer was artificially mediated by pyocyanin (Fig. 3). Pyocyanin-mediated electron transfer by-passes the quinone pool and the cytochrome *bf* complex [50].

Fig. 3 shows that the behaviour of the yield was indeed comparable in both systems in the low flash frequency range (below 2 Hz). The behaviour is similar to the behaviour observed in previous batches of PS I vesicles (Fig. 2 in Ref. 5). Under mild uncoupling conditions (Fig. 3c and d), the maximal ATP yield that could be reached was smaller and the frequency-dependent yield transition was less sharp than in the absence of uncoupler. (This effect of mild uncoupling is also predicted by our kinetic model of ATP synthesis, described in Ref. 5.)

The similarity between the results obtained with the native and with the pyocyanin-mediated system indicates that the frequency-dependent behaviour of ATP synthesis below 2 Hz is not related to special properties of electron transfer through the quinone pool and the cytochrome *bf* complex. This is corroborated by the observation in the native (ferredoxin-mediated) system, that flash-induced electron transfer (and the associated membrane potential generation, not shown) decreases (Fig. 2) but that the ATP yield increases (Figs. 1 and 3) at increasing flash frequency in the 0.1–1.0 Hz range. Consequently, the transition must be ascribed to the behaviour of the ATPase itself. This is further substantiated by the specific effect of ADP on the transition, which will be discussed below.

Characterization of the ATPase-associated transition of ATP synthesis in the 0–1 Hz range – variation of nucleotide concentration

So as to further characterize the ATPase-associated rate transition, we examined the effect of changed nucleotide concentration on the frequency-dependent behaviour of the ATP yield per flash.

According to the kinetic model in Ref. 5, the frequency at which the transition of the ATPase occurs is expected to shift in response to the ambient phosphate potential, ΔG_P. This because the effective proton flux through the ATPase was assumed to be proportional to $\Delta\tilde{\mu}_{H^+} + (\Delta G_P/n)$ in our system, where n is the intrinsic H^+/ATP ratio of the ATPase.

It was difficult to determine the exact levels of ADP and ATP, and thus of ΔG_P, such as they occurred in the course of the experiment. ATP was continuously hydrolyzed in the dark and synthesized in the light. Hydrolysis occurred already during the mixing of the compounds, just after addition of ATP to the reaction mixture. An estimate was calculated on the basis of (1) the initially added amounts of ADP and ATP (see Materials and Methods) and of (2) the overall

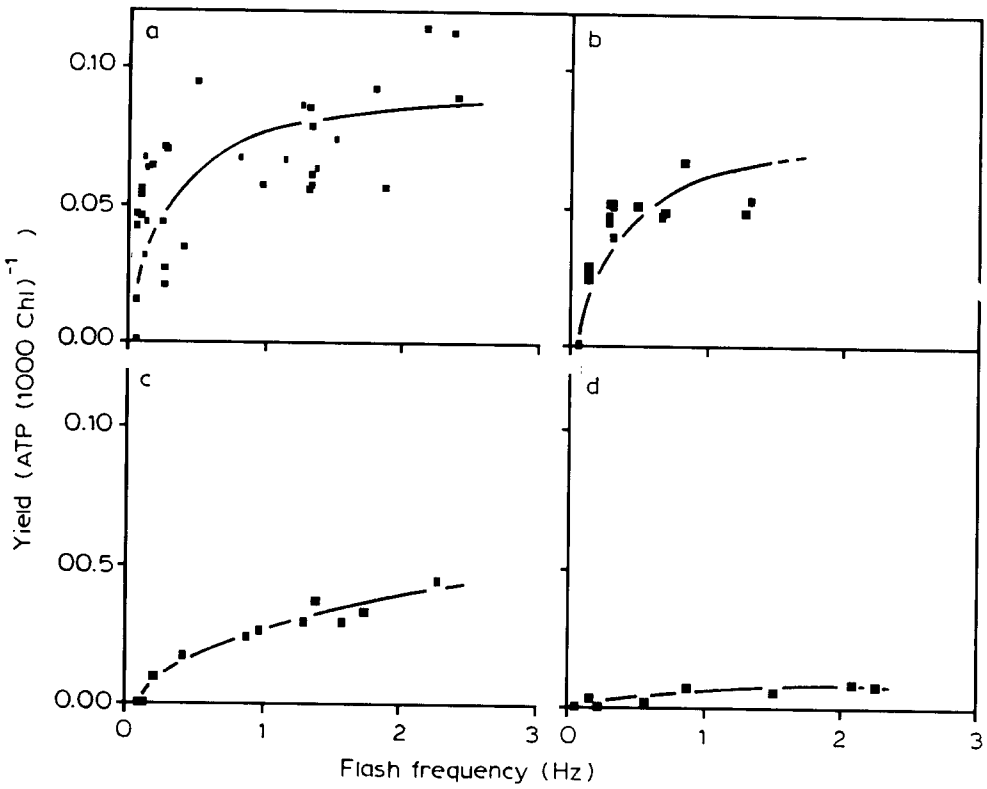

Fig. 3. The ATP yield per flash as a function of flash frequency. Either 5 μM ferredoxin (a,c), or 10 μM pyocyanin (b,d); was present. ADP (containing less than 1% ATP, mol/mol) was present at 39 μM. In c and d, FCCP was added to 250 nM. Further conditions as in Fig. 1a and b. The results in a represent a compilation of several experiments.

balance of (luciferase-monitored) ATP hydrolysis and synthesis, taken between the moment of the initial addition of nucleotides and the moment at which the actual recording of the flash frequency-dependent ATP-synthesis was started. In various experiments, the values of ΔG_P at the moment the of the actual data acquisition varied from about -56 to -28 kJ $\cdot$ mol^{-1}. Finally, during the actual recording of a set of data, the application of flash trains was manipulated in such a way as to keep the average nucleotide levels more or less constant.

As it appears from Table I, there was no consistent relation between the estimated ΔG_P and the occurrence of the ATP yield-transition in the flash frequency range of 0.1–0.8 Hz. The occurrence of the yield transition was neither related to the ATP concentration, in the range of about 0–1 μM (Table I). However, the ADP concentration appeared to have a marked effect on the occurrence of the transition: the transition was only observed at a relatively low ADP concentration (40 μM or lower).

In general, deactivation of the ATPase is associated with tight binding of ADP and a concurrent decrease of the K_M for ADP [8,36–38,55–57]. Indeed, we observed such a deactivation at higher ADP concentrations (Fig. 4). If the transition of the yield at low flash frequency would reflect ATPase deactivation, ADP would be expected to induce even a more pronounced transition, due to enhanced deactivation at low $\Delta\tilde{\mu}_{H^+}$ (low flash frequency). This was not observed. The transition was, in contrast, apparently obscured at 80 μM ADP (Table I, Fig. 4), where the ATPase was not significantly deactivated (compare the absolute

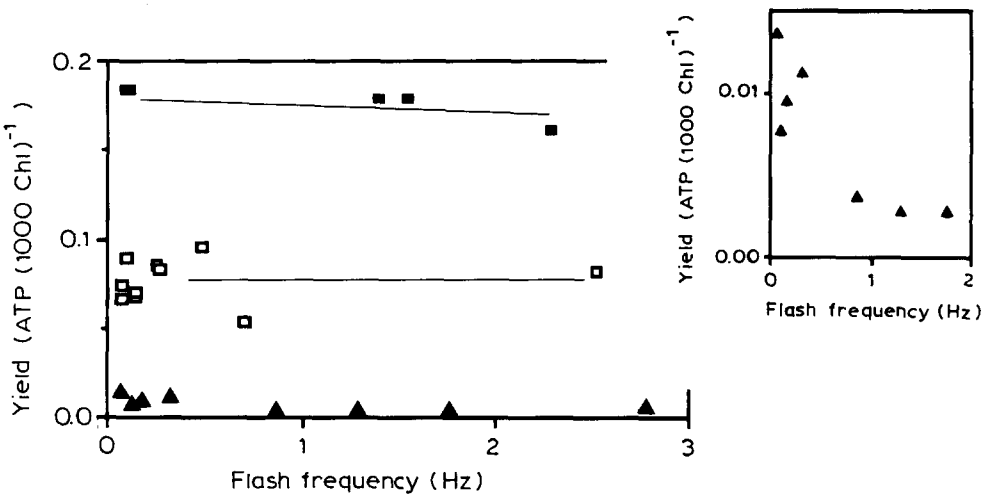

Fig. 4. The frequency-dependent behaviour of the ATP yield per flash, at increased levels of ADP (compare with Figs. 1a and 3a). ADP was present at 80 (■), 156 (□) or 830 (▲) μM. The panel on the right shows the data at 830 μM ADP in more detail. At 80 and 830 μM, the ADP contained less than 1% ATP (mol/mol); at 156 μM, purified ADP was used, containing no ATP. Further conditions as in Fig. 1a.

TABLE I

EFFECT OF NUCLEOTIDE CONCENTRATION ON THE OCCURRENCE OF THE ATP YIELD TRANSITION AT LOW FLASH FREQUENCY

The nucleotide concentrations at the moment of data aquisition were calculated from the initially added amounts, after correction for ATP synthesis and hydrolysis that occurred between the initial addition of nucleotides and the moment of data aquisition. The flash frequency was varied from 0.07 to 2 Hz. The ATP yield per flash at 0.07–0.2 Hz was compared to the ATP yield at 0.8–2 Hz. If the average yields in these two frequency ranges differed by at least a factor 2 (c.f. Figs. 1 and 3), the occurrence of a yield transition is indicted in this table. If the transition was indicated to be absent, the yields in these frequency ranges did not significantly differ (c.f. Fig. 4). Apart from the indicated concentrations of phosphate, ADP and ATP, conditions were as in Fig. 1a.

Concentration of:			ΔG_p (kJ·mol^{-1})	Transition: (present = +; absent = −)
phosphate (mM)	ADP (μM)	ATP (μM)		
2.0	0.001	0.05	−54 to −56	+
2.0	0.3	0.5 to 1.0	−46 to −48	+
2.0	0.5	1	−47	+
2.0	39	0.5 to 0.8	−35 to −36	+
6.9	39	0.07 to 0.08	−30 to −31	+
2.0	80	1 to 1.5	−35 to −36	−
21.6	156	0.1 to 0.3	−28 to −30	−
2.0	830	8	−34 to −35	−

yields in Figs. 1 and 4). Thus, the transition of the ATPase seems not to be due to changes of the overall ATPase activity.

The effect of thiol reduction

Treatment of the ATPase with DTT enhanced both the dark ATP hydrolysis (not shown) and the flash-induced ATP synthesis (Fig. 5) by a factor

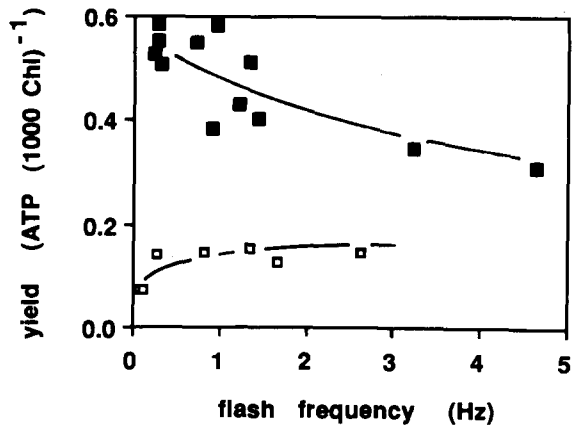

Fig. 5. The effect of DTT on the frequency-dependent behaviour of the ATP yield per flash, under standard conditions (at 39 μM ADP). □, control; ■, after subsequent thiol modulation. Further conditions as in Fig. 1a.

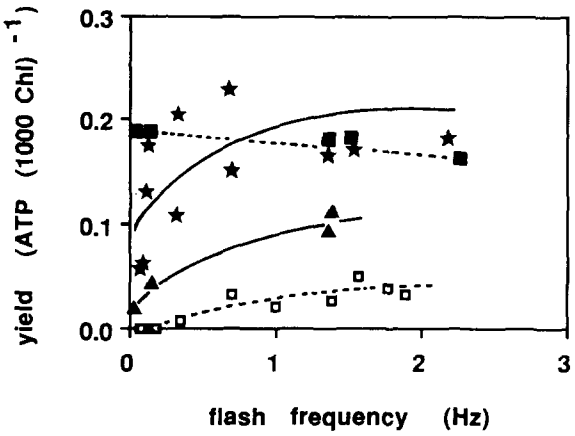

Fig. 6. The effect of uncoupling and addition of DTT on the frequency-dependent behaviour of the ATP yield per flash, at a somewhat elevated ADP concentration (80 μM). The ADP contained less than 1% ATP (mol/mol). ■, control (start of the experiment); □, after addition of 50 nM FCCP (DTT and nigericin were not yet present); *, after subsequent thiol reduction by DTT; ▲, finally, after addition of 100 nM nigericin. Further conditions as in Fig. 1a.

3–4 throughout the flash frequency range. (The hydrolysis rates in the dark, in the presence of DTT, varied from 0.03 to 0.12 ATP per 5 per 10^3 Chl). Thus, DTT appeared to elicit extra ATPase activation. In the presence of DTT, like in its absence, the observed ATP hydrolysis rate in the dark, immediately after flashing, was independent of flash frequency and was in most cases equal before and after flashing, in agreement with [4,5].

The ATP yield-transition at flash frequencies below 0.5 Hz and at relatively low ADP concentration (Figs. 1 and 3) is apparently obscured also in the presence of DTT (Fig. 5).

The combined effect of enhanced ADP concentration, thiol reduction and uncoupling

Although at 80 μM ADP, the yield transition (see Fig. 4) was obscured, it could be elicited again by addition of a relatively low amount (c.f. Refs. 4,5) of the uncoupler FCCP (Fig. 6), be it that the absolute yield was small in the presence of uncoupler. The transition is also clearly seen after subsequent thiol reduction with DTT (Fig. 6). This contrasts with the experiment shown in Fig. 5. The lower ADP concentration in the experiment of Fig. 5 would only tend to enhance the yield transition! Thus, it seems that the presence of uncoupler is responsible for the occurrence of he transition in Fig. 6.

Just like in the experiment of Fig. 5, thiol reduction increased the absolute level of the yield (Fig. 6). A stronger uncoupling, by subsequent addition of nigericin, caused again a decrease of the yield. The relative effects of uncoupler and DTT will be discussed below.

A relation between the ATPase transition and the flash-induced membrane potential?

If part of the decay of the flash-induced membrane potential reflects the proton efflux through the ATPase associated with ATP synthesis [23,29,34,51,52], it might be possible to monitor the transition in the ATPase directly with the decay kinetics of the electrochromic absorption changes at 518 nm so as to allow a better characterization of the ATPase transition (see, however, Ref. 53 for a contrasting opinion).

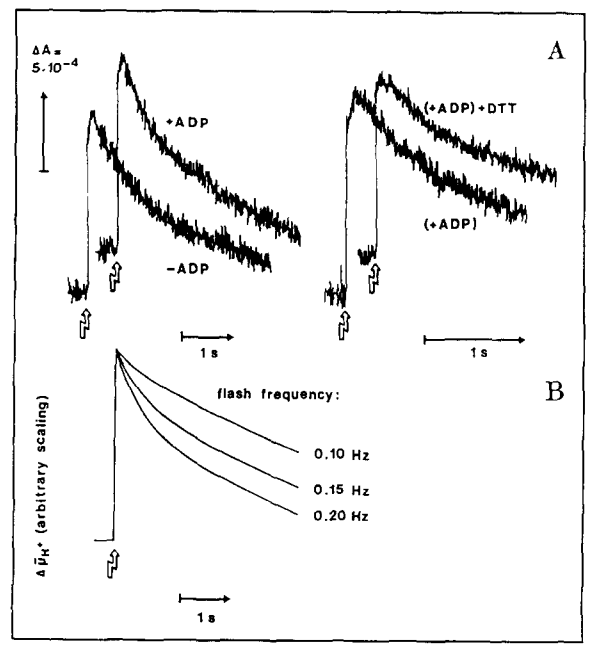

Fig. 7. (A) The flash-induced electrochromic signals recorded at 518 nm. Note the different time scales. The absorption changes were corrected for non-electrochromic changes occurring in the presence of 10 μM gramicidin D. Where indicated, ADP was present at 40 μM and DTT at 5 mM. The flash frequency was 0.1 Hz. The arrows indicate the moment of flashing. Further conditions as in Fig. 2. (B) Simulated behaviour of the flash-induced changes of $\Delta\tilde{\mu}_{H^+}$. The trace was calculated with the same model [5] and parameter values that were used to fit the data of Fig. 2 in Ref. 5.

Fig. 7A shows the flash-induced electrochromic absorption changes occurring in our experimental system under phosphorylating conditions (+ADP) and non-phosphorylating conditions (−ADP), as well as under conditions in which ATP synthesis was enhanced by DTT reduction of the ATPase.

A sharp transition is not apparent. Also at higher and lower flash frequencies, a sharp transition was not observed. However, simulations according to the above-mentioned kinetic model [5] show that the stimulated decay of the flash-induced $\Delta\tilde{\mu}_{H^+}$ will not reveal a sharp transition (Fig. 7B), even at flash frequencies where the simulated transition of the ATPase is most prominent. If such a sharp transition is not apparent in the behaviour of $\Delta\tilde{\mu}_{H^+}$, it is not reasonable to expect a sharp transition in the decay rate of the flash-induced membrane potential, even though the behaviour of the membrane potential is not necessarily similar to that of $\Delta\tilde{\mu}_{H^+}$. (The model described in Ref. 5 does not discern the contributions of membrane potential and ΔpH to the overall $\Delta\tilde{\mu}_{H^+}$, but the decay of the simulated $\Delta\tilde{\mu}_{H^+}$ traces is similar to that of the measured electrochemic signal at 518 nm).

Unfortunately, it remains doubtfull whether there is indeed a direct relation between the proton flux through the ATPase and the decay of the absorption changes at 518 nm in PS I vesicles (c.f. Ref. 53), since we observed that ADP and DTT had apparently no effect on the 518 nm signal. (The lack of effect of ADP could possibly be explained by an increased intrinsic proton leakage in the absence of ADP.) In any case, the 518 nm signal appeared to provide no detailed information on the transition of the ATPase.

Discussion

It is generally accepted that the reduction level of the ATPase can be modulated by PS I via the thioredoxin system [15–20]. We have not found ferredoxin-thioredoxin oxidoreductase in our vesicles, but thioredoxin seems to be present (Galmiche, J.M., Jacquot, J.-P. and De Wolf, F.A., unpublished data) and we cannot exclude that the reduction level of the ATPase is varied in another way, in response to the varying flash frequency (i.e., the varying turnover speed of PS I, c.f. Ref. 54).

Our previously-published model [5] did not discern (thiol)-reduced and oxidized states of the ATPase, because at that time, we did not have any experimental information in this respect [5]. On the other hand, the reduction level of the ATPase cannot be the most important factor that governs the observed transition of the ATPase, since experiments with uncouplers (Figs. 1, 6 and Ref. 5) show that the appearance of the transition can be manipulated by varying only the level of $\Delta\tilde{\mu}_{H^+}$. Moreover, the transition not only occurs under standard conditions (in the absence of DTT), but, in the presence of uncoupler, also in the presence of DTT, even at increased ADP concentration (which only tends to suppress the transition). If the transition would be only due to changes in the reduction level of the ATPase, DTT should abolish the transition under all conditions.

Since in the PS I vesicles, DTT appears to enhance both ATP hydrolysis in the dark and flash-induced ATP synthesis, the phosphorylation-associated proton flux through the ATPase will also be enhanced. As a result (1) the average level of $\Delta\tilde{\mu}_{H^+}$ in the dark will be increased, due to enhanced ATP hydrolysis; and (2) the relative importance of proton leakage and uncoupling will decrease, since an increased fraction of all translocated protons will then flow through the ATPase. (This is directly due to the enhanced proton flux through the ATPase.) Thus, the effect of DTT on the frequency-dependent transition of the ATPase is probably due to its effect on the ambient level of $\Delta\tilde{\mu}_{H^+}$. In agreement, the effects of DTT and uncoupler in Fig. 6 seems to be antagonistic. According to our previously proposed model [5], an increase of the dark $\Delta\tilde{\mu}_{H^+}$ level would in itself be sufficient to abolish the transition of the ATPase: the threshold level of $\Delta\tilde{\mu}_{H^+}$, where the transition was assumed [5] to occur, would then be exceeded already at very low frequencies, and may-be even in the absence of light flashes.

Since, in the absence of uncouplers and DTT, the transition is only observed at relatively low ADP concentration (Table I, Fig. 4), the decrease of the yield at low frequency (low $\Delta\tilde{\mu}_{H^+}$) could reflect a decrease of the affinity towards ADP.

This would agree with the observation by others [8,58] that the K_M for ADP is apparently increased in chloroplasts at low concentrations of uncoupler.

The significance of those K_M values [8,58] has been contested [57] in view of the fact that the $\Delta\tilde{\mu}_{H^+}$ does not remain constant if the ADP and uncoupler concentrations are varied [59] during the determination of the K_M. We observed that the decrease of the ATP yield at low flash frequency was abolished by increasing the ADP level, in the absence of uncoupler (Figs. 5 and 6), but enhanced (if anything) at low $\Delta\tilde{\mu}_{H^+}$ (under mild uncoupling). Addition of extra ADP will tend to decrease the level $\Delta\tilde{\mu}_{H^+}$ (by inhibition of ATP hydrolysis, and possibly also by stimulating ATP synthesis). Therefore, the effect of ADP in our vesicles was not indirectly due to changes of the $\Delta\tilde{\mu}_{H^+}$, since in that case, the transition would be only clearer at increased levels of ADP.

In a previous study on chloroplasts [23], it was concluded that the decrease of the yield at low flash frequency is possibly due to a decreased release of bound, newly synthesized ATP from the ATPase. This was ascribed to an increased change of the bound ATP to become hydrolyzed before its release on the next flash [23]. Another interpretation might be that the release itself [44,45,60–67], rather than the synthesis of bound ATP [68,69], would be energy-dependent. At low flash frequency, the average level of $\Delta\tilde{\mu}_{H^+}$ will be lower. Thus, the chance of bound ATP to be released upon a flash would possibly decrease.

If the decrease of the yield would indeed be related to a decreased release of ATP, our findings would suggest that, to some extent, ADP could displace the ATP from the ATPase [60,62,64,70, 71]. The dispacement would not occur if the level of $\Delta\tilde{\mu}_{H^+}$ is too low, such as in the presence of uncoupler: in the presence of uncoupler (i.e., at much decreased $\Delta\tilde{\mu}_{H^+}$), the $\Delta\tilde{\mu}_{H^+}$-dependent transition of the ATPase turnover rate is even observed at increased ADP concentration (Fig. 6).

In conclusion, there are two possible interpretations of our results. (1) At low $\Delta\tilde{\mu}_{H^+}$, the affinity towards ADP is largely decreased. (2) At low $\Delta\tilde{\mu}_{H^+}$, the release of ATP is hampered, but can be stimulated to some extent by ADP (due to displacement of ATP by ADP). Both interpretations refer specifically to a decrease of the ATPase-catalyzed forward reaction (unidirectional ATP synthesis). Such an exclusive effect on the forward reaction agrees well with the finding that the ATP hydrolysis immediately following the flashes is not influenced by the flash frequency.

Boork and Wennerström, [44] have shown that such a decrease of the forward reaction around a certain value of $\Delta\tilde{\mu}_{H^+}$ will theoretically result in a sharp ($\Delta\tilde{\mu}_{H^+}$-dependent) decrease of the apparent proportionality constant, which relates the overall phosphorylation rate (the net effect of ATP synthesis and hydrolysis) to the level of $\Delta\tilde{\mu}_{H^+}$. At very low levels of $\Delta\tilde{\mu}_{H^+}$, even unidirectional ATP hydrolysis could theoretically occur. Being a non-equilibrium reaction, this would no longer be dependent on $\Delta\tilde{\mu}_{H^+}$, as long as $\Delta\tilde{\mu}_{H^+}$ stayed low enough. In appearance, such behaviour of the proportionality (rate) constant bears resemblance to the behaviour which was assumed in our previously described model to simulate our data [5], although, for simulation-related mathematical reasons, the model described in Ref. 5 did not discern the forward and backward reactions catalyzed by the ATPase. Nevertheless, those stimulations appeared satisfactory.

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