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Studies on well-coupled Photosystem-I-enriched subchloroplast vesicles. Kinetic aspects of flash-induced energy transduction

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Flash-induced ATP synthesis, coupled to cyclic electron flow in Photosystem-I-enriched subchloroplast vesicles (from spinach) was studied by continuous registration of luciferin-luciferase luminescence. In flash trains, the ATP yield per flash was in any case constant after 2–3 flashes and remained so throughout the train. However, valinomycin (not nigericin) induced a lag phase which lasted about 10 flashes at 0.1–0.5 Hz and about 50 flashes at 5 Hz. Valinomycin (10–100 nM) as well as nigericin (35–100 nM) or the uncoupler FCCP (50–250 nM) partly inhibited phosphorylation throughout the flash train; a combination of 50 nM of each inhibited phosphorylation completely. In the absence of ionophores, the flash-induced energization increase seems to be mainly dependent on electric-potential changes, while a pH gradient (ΔpH) only contributes to the steady-state energization level in the dark. In trains of single flashes, the ATP yield increased with flash frequency, but only between about 0.1 and 0.8 Hz. A model is presented, which explains this behaviour assuming a sharp, non-linear increase of the turnover rate of the ATPase molecules above a certain level of the proton-motive force ($\Delta\tilde{\mu}_{H^+}$). In groups containing two or three flashes at variable intervals, the ATP yield per flash was sharply decreased at intervals within the group shorter than 40–50 ms, especially in the presence of nigericin or the uncoupler FCCP. The decrease seemed to be less pronounced in the presence of electron-transfer inhibitors. It is probably due to kinetic limitation of electron transfer and proton translocation. Nigericin and FCCP have a larger effect on single flashes than on flash groups. Their effect supports the assumption that the ATPase turnover rate depends non-linearly on $\Delta\tilde{\mu}_{H^+}$.

Introduction

Photosystem-I-enriched vesicles, derived from spinach chloroplasts by mild digitonin treatment contain all the natural components necessary for cyclic electron transfer [1,2,3] and photophosphorylation [4], except ferrodoxin. After addition of ferredoxin and adjustment of its redox poise by means of NADPH [4,5], these vesicles show a relative large redox-associated 'slow component' of electrical potential formation [6–9] and are

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Abbreviations: Ch, Chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzo-quinone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; ΔG_P, phosphate potential corresponding to ATP hydrolysis; $\Delta\mu_{H^+}$, electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$ (positive when driving protons from the vesicle lumen to the outside); ΔpH, pH difference across the membrane ($pH_{in} - pH_{out}$); FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; PS I or II, Photosystem I or II, Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino} ethanesulphonic acid.

capable of pronounced ATP synthesis under steady state [4] and single-turnover [5] conditions. Under non-phosphorylating conditions, a clear light-induced steady-state proton uptake was observed in these vesicles (Krab, K. Hotting, E.J. and De Wolf, F.A., unpublished data). However, single-turnover proton uptake could not be demonstrated [10].

Single-turnover light flashes and short light pulses are valuable tools for the study of photophosphorylation kinetics. In this paper, we will evaluate the contribution (extent and kinetics) of proton translocation, viz. the contribution of membrane potential and ΔpH, to single-turnover flash-induced phosphorylation in these PS I vesicles. Therefore, we will consider the effects of ionophores on the phosphorylation. We will also vary the intervals between the flashes in order to study the kinetics of the formation and dissipation of the high-energy state which drives phosphorylation.

It has been observed in chromatophores from photosynthetic bacteria and chloroplasts [11–21] that at the first few flashes or during the first period of illumination, ATP synthesis is mainly driven by membrane potential, while pH gradients (ΔpH) contribute only in a later stage [21–29].

In the absence of ionophores, a lag in the ATP synthesis has sometimes been observed during the first period of flashing or illumination [11,12,14, 18–22,30] (not in [15–17,31,32]). These lag phases have been attributed to the time needed for (1) the dissociation of bound ATP [14], (2) ATPase activation [20,30], (3) the generation of a sufficient electrochemical proton gradient ($\Delta\mu_{H^+}$) to overcome the counteracting effect of the phosphate potential [12,17], or (4) the saturation of localized proton pools in or on the membrane [19,21]. In PS I vesicles, such a lag was not observed [5]. A considerable ATPase activity was already present before flashing and in agreement with [33], this activity was apparently not changed by flashing [5].

In several studies on chloroplasts and chromatophores, the amount of ATP synthesized per single-turnover flash varied with the dark time between the successive flashes [12,13,18,22–24,30]. Maximal ATP yields were observed at flash intervals between 100 and 1000 ms. The observed decrease of the yield at flash intervals shorter than 100–200 ms has been associated with the limited turn-over rate of electron transfer and/or proton translocation [22,30]. On the other hand, the decrease of the yield at flash intervals longer than 1000 ms, i.e., at flash frequencies lower than 1 Hz, has been explained by: (1) the decay of the electrochemical proton gradient during the flash interval [12,18,30]; (2) changes of the redox state of membrane components [22]; or (3) a decreased release of ATP bound to the ATPase molecules, and consequently, an increased fraction of ATP that is hydrolysed before its release from the ATPase [24].

Similar results presently obtained with PS I vesicles, can be explained by a model which assumes a sharp increase of the intrinsic ATPase turnover rate above a certain level of $\Delta\mu_{H^+}$. From the present experiments with ionophores, we conclude that in PS I vesicles, the steady state level of $\Delta\mu_{H^+}$ in the dark is mainly determined by ΔpH, while the flash-induced increase of $\Delta\mu_{H^+}$ above this level is mainly determined membrane potential.

Materials and Methods

PS I vesicles were isolated from market spinach as described previously [2,10] and stored under liquid nitrogen at 1 mg Chl per ml in a medium containing 250 mM Sorbitol, 20 mM NaCl, 20 mM KCl, 2.5 mM KH_2PO_4, 5 mM $MgCl_2$ and 5 mM Tes-KOH buffer (pH 7.8). Vesicles denoted as 'freshly prepared' were not frozen; they were prepared in the same medium and used directly after preparation. In the experiments, the vesicles were diluted to a final concentration of 50 μg Chl per ml in a medium containing 10 mM $KHCO_3$, 2 mM K_2HPO_4, 40 mM Tes-KOH buffer (pH 8.0) 38 μM ADP, 4 μM diadenosyl pentaphosphate, 5 μM ferrodexin, 1.3–2.0 mM NADPH and a small amount of dry luciferin-luciferase mixture (4–5% of the total contents of one vial of LKB 'ATP monitoring reagent' no. 1243–200). Valinomycin, nigericin, FCCP, DBMIB, DNP-INT and DCCD were solved in ethanol and added as indicated: in the reaction mixture, ethanol was always below 1–2% (v/v), which (as tested) had no effect on these vesicles.

Experiments were carried out at 10°C under continuous stirring in a (1-cm) 4-sided quartz cuvette. Saturating actinic xenon flashes (2 μs at half maximal amplitude) from two FX-6A tubes (EG & G. U.S.A.), controlled by synchronized Stroboslave 1539A units (General Radio, France) were applied simultaneously to opposite sides of the cuvette. The synchronous flashes were either fired separately (denoted as 'single flashes') or in groups consisting of 2–9 flashes. The intervals between the flashes in one such group could be varied between 3 and 400 ms as required. The frequency at which either the single flashes or the flash groups were fired was variable (usually 0.1–10 Hz). The flash light was passed through a Calflex X2 (Balzers, Liechtenstein) and a RG630 (Schott, F.R.G.) filter. Luminescence was detected with a cooled 9558QB photomultiplier (EMI, U.K.), screened from the actinic light by a Specivex DH485b (MTO, France) filter. Changes of the luminescence level were continuously recorded and frequently calibrated with small amounts of ATP.

The rate of ATP synthesis was always corrected for the dark ATP hydrolysis observed immediately after the flash train (c.f. Ref. 32). The rate of this hydrolysis was usually equal to that observed just before the flash train, provided the dark period preceding the flash train was not longer than 1 or 2 min. This shows that dark ATPase activity was not changed if the dark periods between the flash trains were not too long [5]. The ATP yield per flash was calculated from the ratio between the corrected ATP synthesis rate and the flash frequency.

Luciferin-luciferase mixture and ATP (standard) were purchased from LKB-Wallac (Turku, Finland), FCCP was purchased from Du Pont De Nemours, Inc. (Wilmington, DE, U.S.A.) and Valinomycin from Sigma (St. Louis, MO, U.S.A.). DNP-INT and DBMIB were a gift from Prof. A. Trebst (Ruhr-Universität, Bochum, F.R.G.) and nigericin from the Ely Lilly Laboratories (Indianapolis, IN, U.S.A.). All other chemicals were purchased from Merck (Darmstadt, F.R.G.) and Prolabo (Paris, France).

Results

Some general characteristics of flash-induced ATP synthesis in PS I vesicles

Trains of single flashes as well as trains of flash groups (consisting of three flashes) induced a marked ATP synthesis in the PS I vesicles, as shown in Fig. 1 (typical experiment). The maximal ATP yield per single flash varied from 0.05 to 0.08 molecules ATP per 1000 molecules of chlorophyll in ordinary vesicles and from 0.07 to 0.16 ATP per 1000 Chl in freshly prepared vesicles. In groups of two or three flashes, the average yield per flash was maximally a factor 1.3–1.4 higher. These yields are significantly lower than those observed in chloroplasts (0.4–1.3 ATP/(1000 Chl)) [19,24,32, 33] or chromatophores from photosynthetic bacteria (0.5–2.5 ATP/(1000 Chl)) [12,30]. Also in steady-state experiments (not shown), the yield was (10–20)-times lower than in chloroplasts [33] and (5–50)-times lower than in bacterial chromatophores [12,44]. This may be due to the smaller steady-state light-induced proton uptake observed in these vesicles, as compared to chloroplasts (Krab, K. Hotting, E.J. and De Wolf, F.A., unpublished data).

In the absence of ionophores, the envelope of the noise showed a linear increase from the first flash on, even if the flashes were preceded by several minutes of darkness (Fig. 1, traces labeled 'control'; see also Ref. 5). The ATP synthesis rate did apparently not depend on the number of flashes fired. Only in some experiments, a slight decrease of the rate was observed after about 10 flashes. The small amount of ATP synthesized after every single flash could not be discerned due to the noise, which varied from 5 to 20 pmol ATP (peak to peak) in different experiments. However, the yield per flash should have reached more than 90% of its final value within 1–3 flashes.

In the following sections we will study (1) the contribution of membrane potential and ΔpH to single-turnover phosphorylation and (2) the formation and dissipation characteristics of the flash-induced increase of $\Delta\mu_{H^+}$.

Effects of ionophores: contribution of membrane potential and ΔpH

Valinomycin induced a lag in the onset of ATP

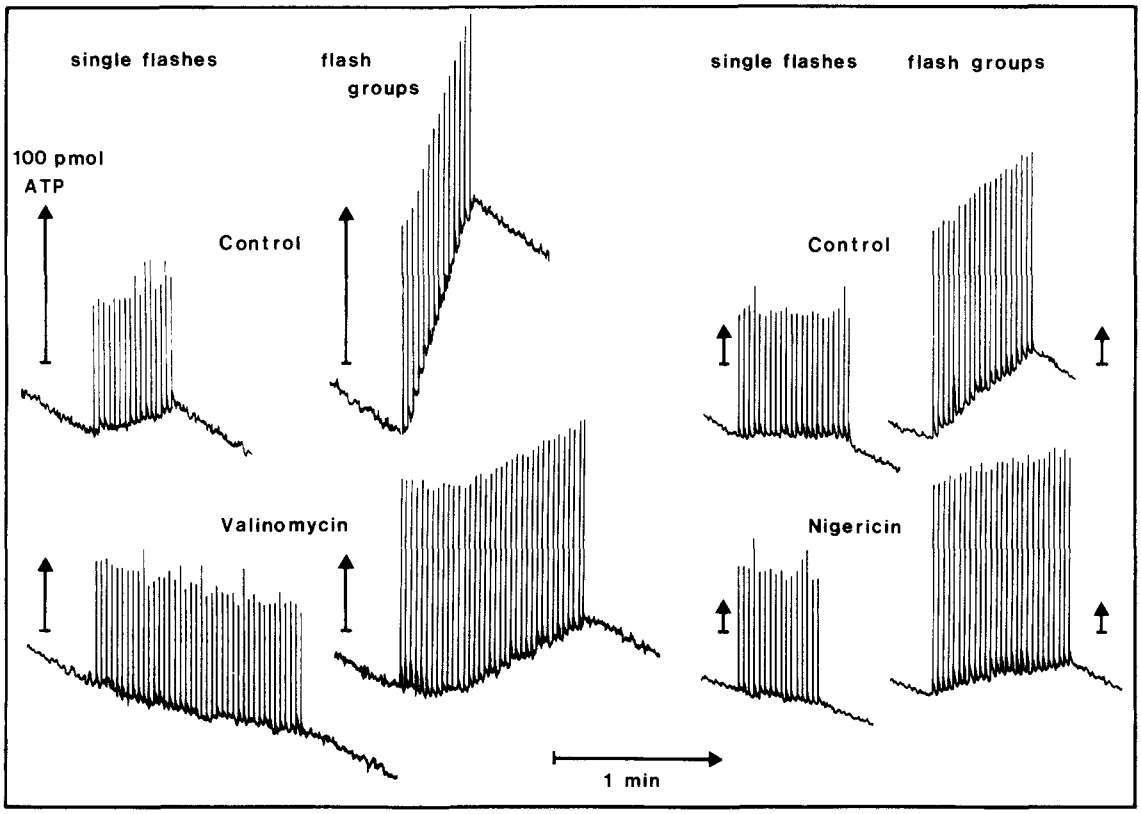

Fig. 1. The effect of nigericin and valinomycin on flash-induced ATP synthesis in PS I vesicles. ATP was monitored continuously by luciferin luciferase luminescence. Each spike represents an artifact caused by a single flash or a flash group (containing three flashes spaced at 50 ms). The vertical arrows all correspond to 100 pmol ATP. Nigericin was present at 35 nM and valinomycin at 50 nM, as indicated. Further conditions as in Materials and Methods.

synthesis (in agreement with [11,12,14–20]): it took 7–10 flashes at 0.1–0.6 Hz flash frequency (Fig. 1, traces labeled 'valinomycin'), and 35–65 flashes at 5 Hz (not shown), before a constant ATP yield per flash was established. In steady-state experiments (not shown), the normally observed lag of about 0.7 s in these vesicles, was extended to 2.0–2.5 s by addition of valinomycin. In agreement with [11,18], nigericin did not induce a lag phase under single-turnover conditions (Fig. 1), nor under steady-state conditions (not shown).

Nigericin (35–100 nM), FCCP (50–100 nM) and valinomycin (50–100 nM) partly inhibited flash-induced ATP synthesis throughout the flash train. This is shown in Table I in which the values for valinomycin correspond to the yields after the lag phase. Freshly prepared (not frozen) vesicles, which have a better membrane integrity [5], were much less sensitive to nigericin (Table I, valinomycin not tested). A combination of 50 nM valinomycin and 50 nM nigericin inhibited ATP synthesis completely (c.f., Ref. 34).

The results obtained with nigericin point to a contribution of ΔpH to the vesicle energization. The valinomycin-induced lag shows that ΔpH does not contribute significantly to the flash-induced increase of $\Delta\mu_{H^+}$, at least during the first few flashes. It probably contributes to the steady-state level of $\Delta\mu_{H^+}$ (c.f. Ref. 35), which is maintained in the dark by ATP hydrolysis (see Discussion).

High concentrations (500–1000 nM) of valinomycin or nigericin inhibited ATP synthesis completely (Table I). This may be due to side effects. Nigericin, which affects the ΔpH by electro-neutral H^+/K^+ exchange at low concentrations, may induce electrogenic ion transport at

TABLE I

EFFECT OF IONOPHORES AND UNCOUPLER ON FLASH-INDUCED ATP SYNTHESIS

Flashes were applied as trains of single flashes or as trains of flash groups. The frequency at which the single flashes or flash groups were fired was 0.56–0.63 Hz. Each flash group consisted of three flashes, 50 ms apart. The data in parenthesis were obtained with freshly prepared (not frozen) vesicles. The 100% yields varied between 30 and 65 pmol ATP per mg Chl per flash.

Concentration (nM)	Relative ATP yield per flash (%) in the presence of					
	valinomycin		nigericin		FCCP	
	single flashes	flash groups	single flashes	flash groups	single flashes	flash groups
0	100	100	100 (100)	100 (100)	100	100
10	54	61	77	100	90	100
20	–	–	65	100	–	–
35	–	–	59	93	–	–
50	38	51	25 (100)	50 (87)	73	86
75	–	–	– (100)	– (83)	–	–
100	32	33	9 (60)	50 (83)	49	83
250	–	–	– –	– –	0	24
500	6	6	0 (51)	35 (60)	–	–
1000	0	0	0 (41)	9 (57)	0	0

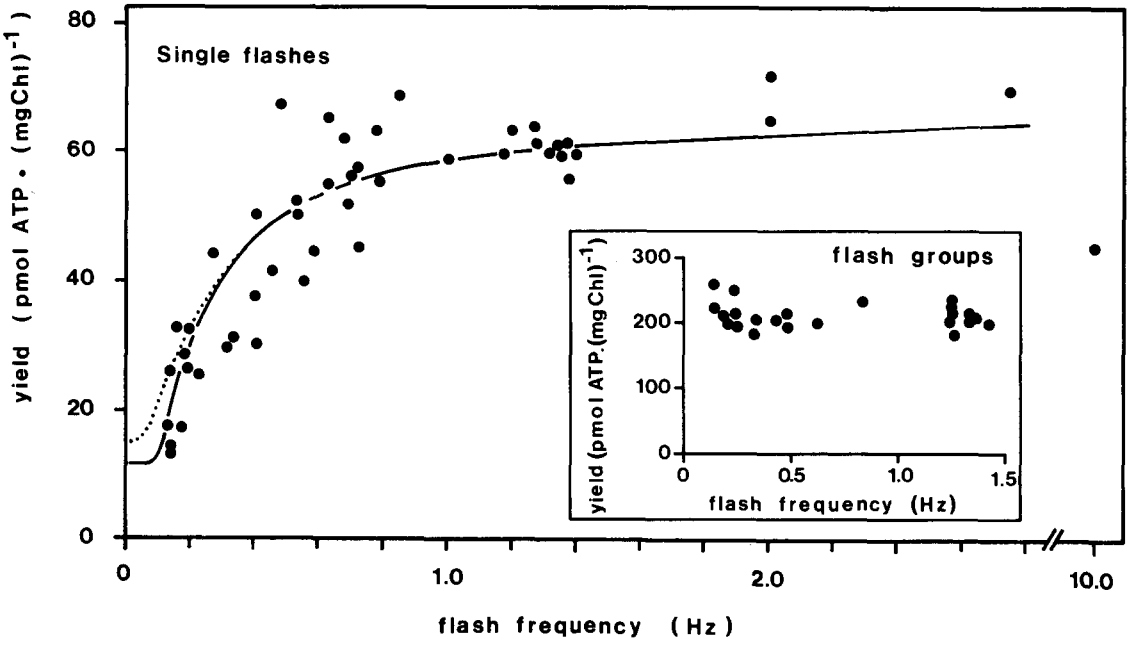

Fig. 2. The ATP yield per flash as a function of flash frequency in trains of single flashes. The curves are calculated according to the model described in the Appendix. The model parameters were (solid line): $T = -\Delta G_P/n = 17.5$ kJ·mol^{-1}; $H^+/e^- = 2$; $n = 2$; $k_1 = 0.10$ s^{-1}; $k_2 = 0.02$ s^{-1}; $k_3 = 1.00$ s^{-1}; $P = 11.58$ kJ·mol^{-1}; and $U = 6.41$ pmol ATP per mg Chl per flash per (kJ/mol); or (dashed line): $T = -\Delta G_P/n = 11.67$ kJ·mol^{-1}; $H^+/e^- = 2$; $n = 3$; $k_1 = 0.15$ s^{-1}; $k_2 = 0.03$ s^{-1}; $k_3 = 1.00$ s^{-1}; $P = 11.58$ kJ·mol^{-1} and $U = 6.70$ pmol ATP per mg Chl power flash per (kJ/mol). The inset shows the yield of flash groups as a function of flash frequency. The groups contained six flashes spaced at 3 ms. Further conditions as in Fig. 1.

these high concentrations [34] and may thus also affect the membrane potential. Valinomycin, above 100 nM, inhibits electron transfer as well as phosphorylation in chloroplasts [36,37]; it may act as a surfactant [38].

If the ATP yield of single flashes and of flash groups consisting of three flashes, 50 ms apart, is set to 100% in the absence of ionophores, nigericin (50–100 nM) or FCCP (50–250 nM) affected the relative ATP yield of single flashes to a larger extent than that of the flash groups. This was hardly or not the case with valinomycin (Table I). The origin of this difference will be considered in the discussion, but see also below under 'ATP synthesis in flash groups' (Figs. 3B vs. Figs. 4C, 4D and 5).

Effects of flash frequency: kinetics of $\Delta\mu_{H^+}$ dissipation

We will first consider the effects of flash frequency observed in trains of single flashes. Above 0.8 Hz, the yield per flash in PS I vesicles is practically independent of flash frequency (Fig. 2). Consequently, the average rate of ATP synthesis increases linearly with flash frequency (not shown). The curves in Fig. 2 (solid line and dashed line) represent simulations according to the model described in the Appendix, which will be discussed below. In agreement with observations on chromatophores and chloroplasts [12,13,18,22–24,30], the ATP yield per flash in these vesicles decreases with decreasing flash frequency between about 0.8 and 0.05 Hz (Fig. 2). The observed frequency-dependent variation of the yield between 0.1 and 0.8 Hz may be shifted to higher or lower flash frequencies if the proton permeability of the membrane is increased or decreased, respectively, which indicates that $\Delta\mu_{H^+}$ plays a role: (1) the uncoupler FCCP (1 μM) completely inhibited phosphorylation at 0.6 Hz flash frequency, but hardly at 10 Hz, where even a yield of 55 pmol ATP per mg Chl per flash was measured (not shown); (2) in freshly prepared vesicles with a higher membrane integrity, the yield of single flashes (75 pmol ATP per mg Chl per flash) was independent of a flash frequency down to 0.05 Hz in the abscence of uncoupler (Table I).

In order to reveal possible non-linearities of the system, the effect of flash frequency has also been studied with flash groups, because such flash groups likely induce a larger increase of $\Delta\mu_{H^+}$ than single flashes. When the group frequency is varied, the resulting variation of the ATP yield appears to be relatively smaller than when the flash frequency is varied in the case of single flashes. The yield of single flashes at 0.14 Hz was only 30% of the yield at 0.78 Hz (55 pmol ATP per mg Chl per flash, Fig. 2). However, at 0.14 Hz the yield of flash groups containing three flashes spaced at 50 ms was still 55% of the yield at 0.78 Hz (145 pmol ATP per mg Chl per group, not shown). The yield of groups consisting of six flashes, 3 ms apart (210 pmol ATP per mg Chl per group) was even independent of a group frequency down to 0.1 Hz: this is shown in the inset of Fig. 2. The decrease of the yield observed with single flashes below 1 Hz (main plot of Fig. 2) may reflect a decrease of $\Delta\mu_{H^+}$ below a critical level. Above this level, the yield per flash is constant. This would then be the case (1) in freshly prepared vesicles, (2) in ordinary vesicles, using groups of six flashes or (3) in ordinary vesicles, using single flashes above 1 Hz.

ATP synthesis in flash groups – variation of the number of flashes in the group and of the flash interval: kinetics of $\Delta\mu_{H^+}$ generation

At flash intervals shorter than 100–200 ms, the yield per flash may start to decrease due to kinetic limitation of electron transfer and proton translocation [30]. We studied this phenomenon using flash groups of a few flashes at constant group frequency. The intervals between the flashes in the groups were varied between 10 and 400 ms. The occurrence of multiple proton-translocating electron-transfer turnovers may thus be reduced at short flash intervals in the group, due to kinetic limitation [30]. This type of experiment does not reveal the decay of the flash-induced energization between the flashes, but rather the kinetics of its formation (during 10–400 ms following each flash). (Note that in the absence of ionophores, the $\Delta\mu_{H^+}$ decay appears to occur only in the course of several seconds, for the ATP yield per flash decreases only at flash frequencies below 1 Hz, see Fig. 2).

Before studying the effect of the flash interval in the flash groups, we first studied the effect of

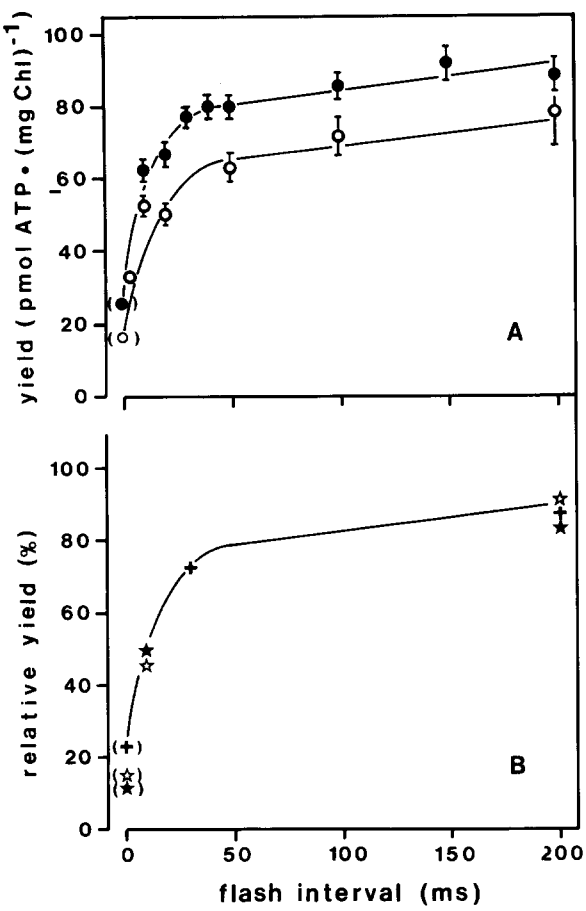

Fig. 3. The ATP yield as a function of the flash interval within a group. The groups were fired at 0.4–0.6 Hz. The results are independent of this variation. The limiting values at 'flash interval 0 ms' (in parenthesis) are derived from the yields of single flashes instead of flash groups (about 50 pmol ATP per mg Chl; see Fig. 2). (A) Absolute yields per flash in groups of 2 (●) or 3 (○) flashes (mean ± S.E., $N = 5-15$, but $N = 1$ for dark time 3 ms). (B) Yields relative to the values at dark time 400 ms (100%, not shown) in the presence of 40 μM DCCD (+), or of 50 nM (★) or 100 nM (☆) valinomycin. The 100% yields were (in pmol ATP per mg Chl per flash): 46.6 (DCCD), 32.9 (50 nM valinomycin) and 23.5 (100 nM valinomycin). Further conditions as in Fig. 1.

the number of flashes (spaced at 10 ms) in the groups. The ATP yield per group varied with the number of flashes (spaced at 10 ms) in the group. Large groups were relatively less efficient than small groups. If the yield of single flashes (occurring at the same frequency as otherwise the flash groups) is set at 100%, the yield of a group of two flashes was 280%, that of a group of 4 was 480%, that of a group of 6 was 360–480%, and that of a group of 9 was 540%. Accordingly, the average yields per flash were 140% in a group of two, 120% in a group of four, 60–80% in a group of six, and 60% in a group of nine flashes. Thus, the first flashes of a group seem to be more efficient than the following flashes. These effects were independent of flash-group frequency between 0.13 and 0.55 Hz in PS I vesicles. On the basis of these results we decided to study the effect of the flash interval (10–400 ms) in groups containing only two or three flashes.

As expected, the average efficiency in such a group was sharply reduced if the flashes were closer than about 50 ms (Fig. 3A), in agreement with observations on chloroplasts and chromatophores [22–24,30]. The yield of a group of very closely spaced (< 10 ms) flashes approached that of a single flash (Fig. 3, yield per single flash indicated in parenthesis).

If the flash-interval-dependent variation of the ATP yield (10–400 ms intervals) is indeed due to the characteristics of electron transfer, an effect of electron transfer inhibitors, but not of other types of inhibitor may be expected. The behaviour of the ATP yield was indeed not changed by DCCD, as shown in Fig. 3B. Accordingly, DCCD inhibited ATP synthesis in solitary flashes and in flash groups both to the same extent (Table II). The electron-transfer inhibitors DNP-INT and DBMIB induced a larger percentage reduction of the ATP yield in flash groups (consisting of three flashes spaced at 50 ms) than in single flashes (Table II). Accordingly, the relative decrease of the ATP yield at short flash intervals seemed to be less pronounced in the presence of DNP-INT (Fig. 4A) and DBMIB (Fig. 4B), although it is difficult to say for certain due to the large scatter on the data. Thus these inhibitors would reduce the yield of flash groups in which the flashes were not too closely spaced (intervals of at least 50 ms), rather than of groups with very closely (up to 50 or 40 ms) spaced flashes. This may be expected, because several electron-transfer turnovers will mainly occur in the groups with relatively widely spaced flashes, thereby offering more opportunity for an effect of inhibitors.

We also tested the effect of ionophores in flash groups when varying the flash intervals within the

TABLE II

EFFECT OF ELECTRON TRANSFER- AND ENERGY-TRANSFER-INHIBITORS ON FLASH-INDUCED ATP SYNTHESIS

Flash (flash group) frequency was 0.38–0.41 Hz. (Each flash group consisted of three flashes, 50 ms apart.) Further conditions were as in Table I. The 100% yields varied between 30 and 60 pmol ATP per mg Chl per flash

Concentration (μM)	Relative ATP yield (%) in the presence of					
	DBMIB		DNP-INT		DCCD	
	single flashes	flash groups	single flashes	flash groups	single flashes	flash groups
0	100	100	100	100	100	100
5	14	9	–	–	–	–
10	–	–	54	26	–	–
20	0	0	–	–	74	68
40	–	–	–	–	55	62

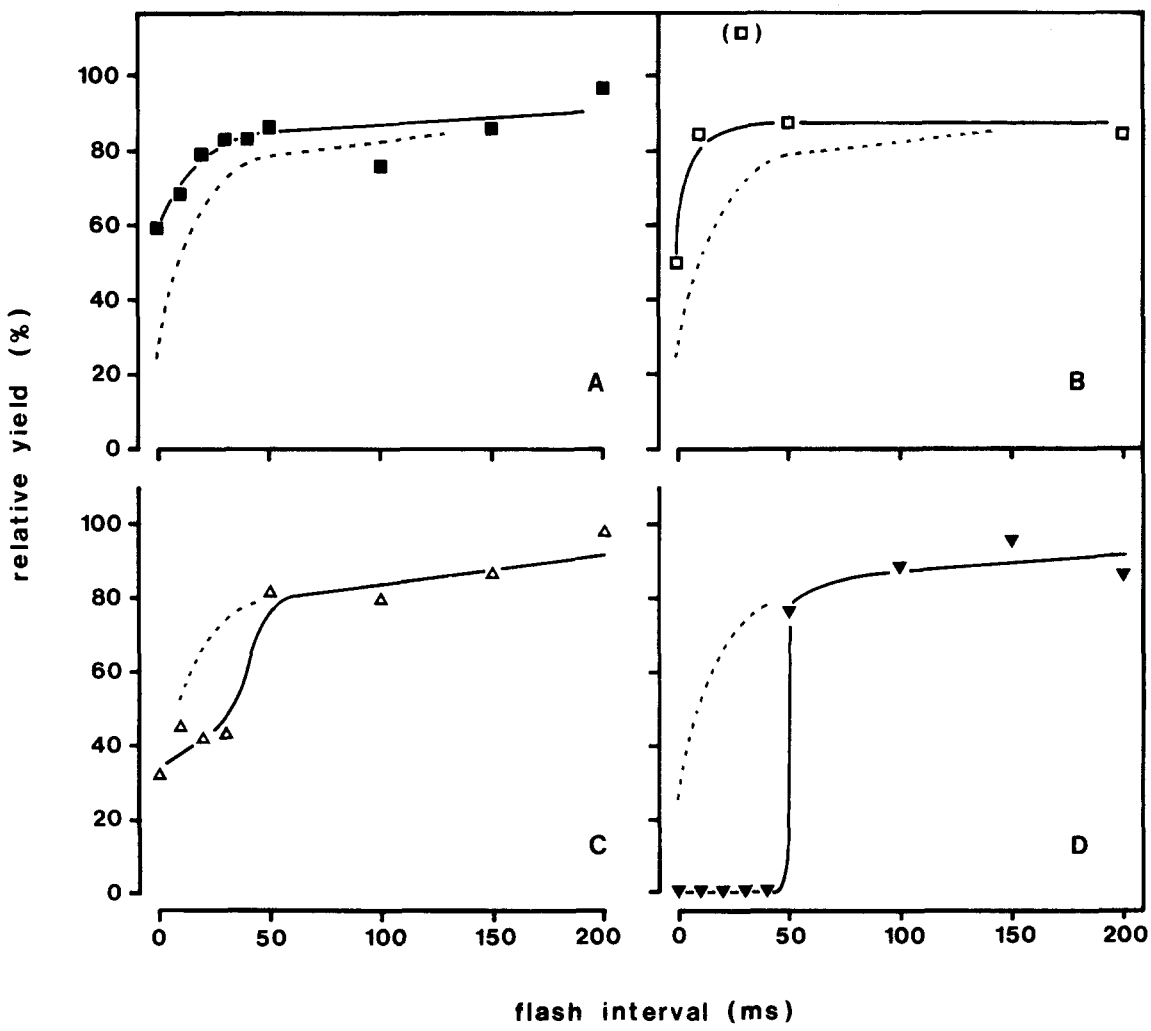

Fig. 4. The effects of inhibitors and ionophores on the relative ATP yield, as a function of the flash interval within a group. (A) 10 μM DNP-INT (■), (B) 5 μM DBMIB (□), (C) 40 nM nigericin (△), (D) 250 nM FCCP (▼). The 100% yields (at dark time 400 ms) were (in pmol ATP per mg Chl per flash): 14.0 (DNP-INT), 7.4 (DBMIB), 52.2 (nigericin) and 15.3 (FCCP). The dotted line indicates the behaviour of the relative ATP yield in control experiments. Further conditions as in Fig. 3B.

groups. Because ionophores will accelerate the dissipation rate of $\Delta\mu_{H^+}$, this dissipation could become detectable in a time span of less than 400 ms. Thus, both the generation and the dissipation of $\Delta\mu_{H^+}$ may play a role during the first 10–400 ms after each flash. Moreover, ionophores will affect the base level of $\Delta\mu_{H^+}$ in the dark (c.f. Ref. 35).

The ATP yield at short (10–50 ms) flash intervals is decreased in the presence of nigericin (Fig. 4C) and totally abolished in the presence of the uncoupler FCCP (Fig. 4D), with a sudden sharp reduction of the yield between flash intervals of 40 and 50 ms. At very short flash intervals within the groups, the groups start to behave as single flashes (see above). Therefore, the disproportionally strong effect of FCCP and nigericin on groups of closely spaced (up to 40 ms) flashes may have the same origin as the stronger effect of these agents on single flashes, compared to flash groups with 50 ms intervals in the group (Table I).

Strangely, valinomycin had no effect on the flash-interval-dependent variation of the relative ATP yield (Fig. 3B). Accordingly, it inhibited ATP synthesis in flash groups (50 ms intervals in the groups) and single flashes relatively (%) to the same extent.

Discussion

The observed decrease of the ATP yield per flash at low flash frequency (Fig. 2) agrees well with observations on chloroplasts [18,22–24] and chromatophores from photosynthetic bacteria [12,13,30]. It has been ascribed to the dissipation characteristics of the high-energy state which drives phosphorylation [12,18,30]. The experiments with ionophores indicate that coupling in these vesicles is indeed based on the formation of a $\Delta\mu_{H^+}$. In first instance, we tried to explain our results by simple first-order kinetics for the dissipation of $\Delta\mu_{H^+}$. Thus, the proton leakage was assumed to be proportional to $\Delta\mu_{H^+}$ and the proton flux through the ATPase proportional to $(\Delta\mu_{H^+} + \Delta G_P/n)$ (where $n = H^+/ATP$). The ATPase activity was assumed to be constant, in agreement with the observed constancy of the dark ATP hydrolysis rate. However, according to this model, the yield per flash would be independent of flash frequency in flash trains. This would not be in accordance with the observed frequency dependence (Fig. 2). Therefore we tentatively introduced a transition of the rate constant of the proton flux through the ATPase (see Appendix). This transition implies an non-linear increase of ATPase-associated proton conductivity above a certain level of $\Delta\mu_{H^+}$. An increase of proton conductivity [40–44] and a non-linear increase of the ATP synthesis rate [43–47] at increasing $\Delta\mu_{H^+}$ have been previously observed in photosynthetic bacteria and chloroplasts, respectively. These phenomena may be due to an increase of ATPase activity [48]. However, in our experiments we did not observe changes of dark ATPase activity during flashing at flash frequencies between 0.1 and 5 Hz (in contrast to what was observed at high flash frequency in Ref. 19). The ATP hydrolysis rates just before and just after the flash trains were identical, provided the dark times between the flash trains did not exceed a few minutes (see Fig. 1 and Ref. 5). In our model, it is essential that the establishment of the supposed rate transition only takes a time which is negligible compared to the intervals between the successive flashes of the flash train (a few ms). Thus, if the increase of the intrinsic ATPase proton conductivity at high $\Delta\mu_{H^+}$ in the model would be due to an increased fraction of active ATPase molecules, both the $\Delta\mu_{H^+}$-dependent activation and deactivation should be completed in only a few ms. ATPase activation in chloroplasts [48] and $\Delta\mu_{H^+}$-dependent increase of the proton conductivity in chromatophores [44] have indeed been shown to be completed in a few ms. Recently, the existence of an intrinsic difference between the rates of ATP hydrolysis and ATP synthesis has been proposed [49] on the basis of the theoretical effect of membrane potential on the kinetics of charge translocating reactions. Such an intrinsic difference between synthesis and hydrolysis rates would also imply a non-linear, $\Delta\mu_{H^+}$-dependent increase of the rate constant of the proton flux through the ATPase around $\Delta\mu_{H^+} = -\Delta G_P/n$ (where n is the H^+-to-ATP ratio of the ATPase).The corresponding relation between the ATP synthesis rate and $\Delta\mu_H^+$ resembles the non-linear behaviour of the proton conductivity and the ATP synthesis rate observed in Refs. 40–47.

In view of simplicity, the rate transition of the

proton flux through the ATPase was assumed to be stepwise in our model, which is already complicated because it describes the behaviour of $\Delta\mu_{H^+}$ and of the ATP synthesis during series of flashes (a series of discontinuous events). The stepwise transition can be seen as a simplification of a steep, non-linear change of the rate as in Refs. 40 and 42–46. The resulting mathematical model (see Appendix) can explain our results satisfactorily: The curves in Fig. 2 (main figure) show simulations according to the model in the Appendix. In those simulations, the yield per flash already reaches more than 90% of its 'final' value (i.e., the value reached after prolonged flashing) at the second or the third flash, irrespective of the flash frequency. This means that the yields are apparently constant from the first to the last flash, if the masking effect of the noise is taken into account. In the simulations, it was assumed that the ATP-ase associated rate transition occurs at $\Delta\mu_{H^+} = -\Delta G_P/n$ ($n = H^+/ATP$). Accordingly, ATP synthesis (at $\Delta\mu_{H^+} \geq -\Delta G_P/n$) would be intrinsically faster than ATP hydrolysis (at $\Delta\mu_{H^+} \leq -\Delta G_P/n$), in agreement with Ref. 49. Reasonable fits can be obtained with an arbitrary threshold level of $\Delta\mu_{H^+}$ anywhere between the dark steady state level of $\Delta\mu_{H^+}$ and its flash-induced peak value (not shown). However, the threshold value should preferably be around $-\Delta G_P/n$ in order to obtain a good fit (c.f. Ref. 49).

The relation between the ATP yield per flash and flash frequency (Fig. 2, main figure) bears a qualitative resemblance to the relation between the $P/2e^-$ ratio and the rate of electron transport as shown in Fig. 4 of Ref. 43, which was explained by a non-linear increase of the proton flux through the ATPase molecules. The model also agrees with the observation [50] that ADP stimulates the decay of the flash-induced membrane potential only during a short period (about 1 s) after the flash: in our simulations (see Fig. 2), fast ATP synthesis which concurs with a relatively high proton efflux through the ATPase occurs only during the first 0.5–2.0 s following the flash (at 0.1–0.6 Hz flash frequency), namely when $\Delta\mu_{H^+}$ is above the threshold level. Our model does not exclude the idea that the increase of the ATP yield at short (less than 1000 ms) flash intervals is related to an increased release of previously synthesized ATP from the ATPase as proposed in Ref. 23: the increase of the intrinsic ATPase turnover speed, assumed in our model, could cause such an increased release of previously synthesized ATP, in agreement with the predictions in Ref. 49.

In these PS I vesicles in the absence of ionophores, flash-induced pH gradients will be small, since pH gradients will be developed only slowly (c.f. Discussion in Ref. 5). Even if the buffer capacity in the vesicle lumen is not higher than in the external medium ($1.75 \cdot 10^{-2}$ M in terms of H^+ concentration, or $(5.0–62) \cdot 10^{-22}$ mol per vesicle lumen at pH 8.0), it would in theory take a factor 40–115 more translocated protons to reach a certain pH value than to reach the membrane potential which corresponds to the same proton motive force. (The diameter of these vesicles is 50–100 nm [1]. The capacitance per vesicle was calculated to be $(2.3–9.2) \cdot 10^{-17}$ C^2/J per vesicle, assuming a relative dielectric constant of 2 for their membranes [51,52]). As a consequence, the number of flashes needed to generate a considerable flash-induced pH gradient is large. This is the more so as protons will continuously flow back across the membranes, due to leakage or ATP synthesis. This agrees with the previously observed lack of pH changes in these vesicles after a single-turnover flash [10]. Such changes were only observed under steady-state conditions in PS I vesicles (Krab, K., Hotting, E.J. and De Wolf, F.A., unpublished data), in agreement with observations on chloroplasts [25–29]. After correction for (dark) steady-state rates, flash-induced phosphorylation would thus be determined by flash-induced membrane potential changes, rather than flash-induced pH changes, in the absence of ionophores. In the presence of valinomycin, the $\Delta\mu_{H^+}$ and, consequently, the back flow of protons will initially be low. In that case pH gradients could start to contribute to phosphorylation after several flashes (see Fig. 1). Otherwise, pH gradients would only contribute to the steady-state levels of energization, maintained in the dark by ATP hydrolysis. The presently observed effects of valinomycin and nigericin are in harmony with this interpretation. The decrease of the ATP yields in the presence of nigericin is likely due to a decrease of the dark steady state level of $\Delta\mu_{H^+}$

[35]. FCCP will affect both the flash-induced membrane potential and the steady state pH gradients.

These considerations, in combination with the supposed transition of proton flux through the ATPase at the threshold level of $\Delta\mu_{H^+}$, may explain the different effects of protonophores on the ATP yield of single flashes and flash groups. In the presence of the protonophoric agents nigericin and FCCP, a larger flash-induced increase of $\Delta\mu_{H^+}$ would be required in order to reach the threshold level of $\Delta\mu_{H^+}$. Flash groups consisting of flashes that are 50–400 ms apart most likely induce a larger increase of $\Delta\mu_{H^+}$ than single flashes or groups consisting of flashes that are less than 50 ms apart (see Fig. 3). If the dark steady-state level of $\Delta\mu_{H^+}$ is decreased in the presence of FCCP or nigericin [34], it is possible that the threshold level is only reached with groups in which the flashes are 50–400 ms apart but not with groups of closely spaced flashes or with single flashes fired at moderate frequencies. FCCP will also accelerate the decay of the flash-induced increase of $\Delta\mu_{H^+}$ (mainly membrane potential, see the discussion above). This would make it even more difficult to reach the threshold with single flashes or groups of closely spaced flashes at low frequency in the presence of FCCP. In contrast, in the absence of ionophores the threshold would be always reached.

It is well possible that membrane potential hardly contributes to the dark steady state level of $\Delta\mu_{H^+}$, due to the compensating movements of other ions such as Cl^- or K^+ (see below). As shown, valinomycin affects single flashes and flash groups consisting of flashes spaced at 10 to 400 ms all to the same extent. In the light of considerations above, this could mean that valinomycin does not affect the dark steady state level of $\Delta\mu_{H^+}$, supporting the idea that this level is mainly or exclusively determined by ΔpH (see also Refs. 25–29).

Appendix

A model for the observed characteristics of the ATP yield per flash in trains of single flashes

The model describes the supposed behaviour of $\Delta\mu_{H^+}$ in trains of single flashes. The model can eventually be applied to trains of flash groups, provided that each group is so short that it can be considered as a single flash. The proton flux through the ATPase is used to calculate the ATP synthesis rate. The time integral of the synthesis rate is used to calculate the total amount of ATP synthesized between two successive flashes, i.e., the yield per flash. Fig. 5 illustrates the meaning of some quantities and symbols that will be used in the model. Therefore, the most complicated model situation has been chosen: 'situation III' (see below). In this situation, the flash frequency is so high that after a few flashes, $\Delta\mu_{H^+}$ stays above the threshold level at which the rate transition of the ATPase-associated proton flux is assumed to occur.

The following quantities, symbols and units will be used in this section:

$\Delta\mu_{H^+}$, ΔG_P	as defined under abbreviations (in kJ · mol^{-1});
T	the threshold level of $\Delta\mu_{H^+}$, where the transition from k_2 to k_3 and vice versa occurs (see below). In Fig. 5, T was chosen to be equal to $-\Delta G_P/n$;
P	flash-induced increase of $\Delta\mu_{H^+}$ (kJ · mol^{-1});
n	the number of protons flowing through the ATPase molecules per molecule ATP synthesized or hydrolyzed (real H^+/ATP ratio);
k_1	rate constant of leakage-associated changes of $\Delta\mu_{H^+}$ (s^{-1});
k_2, k_3	rate constant of phosphorylation-associated changes of $\Delta\mu_{H^+}$ (s^{-1}); k_2 is only defined for $\Delta\mu_{H^+} \leqslant T$; k_3 only for $\Delta\mu_{H^+} \geqslant T$;
Y	ATP yield per flash in a train of single flashes (pmol ATP per mg Chl per flash);
Y_N	value of Y after the Nth flash;
U	unit conversion factor (positive value) which relates Y to the amount of energy (kJ · mol^{-1}) conserved as ATP (pmol ATP per mg Chl per flash per (kJ/mol);

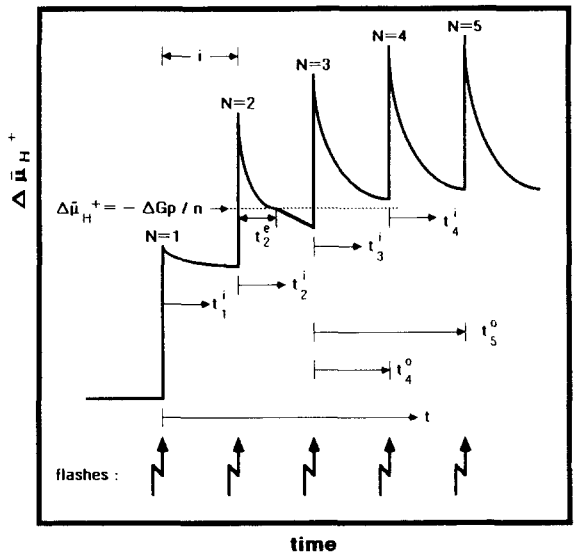

Fig. 5. Changes of $\Delta\mu_{H^+}$ during the first part of a flash train, according to the model (situation 'III') described in the Appendix. The figure illustrates some quantities and symbols defined in the appendix. 'T' was chosen to be equal to $-\Delta G_P/n$, as for the simulation of the curves in Fig. 2. The flashes are indicated by the vertical arrows. Note the discontinuity in the descending part of the trace at $\Delta\mu_{H^+} = -\Delta G_P/n$.

i	flash interval in a train of flashes (reciprocal of flash frequency) (s) (see Fig. 5);
t	time elapsed since the first flash of the train (s) (see Fig. 5);
t_N	value of t at the Nth flash;
t^i_N	time elapsed since the Nth flash (s) (see Fig. 5);
t^e_N	value of t^i_N at which $\Delta\mu_{H^+} = T$ (for $T = -\Delta G_P/n$: see Fig. 5);
t^0_N	time elapsed between the first flash at which t^e_N would exceed i, and the Nth flash (see Fig. 5).

The following assumptions (partly made for the sake of simplicity) underly the model:

(1) Inward proton flux is assigned a positive value. ATP synthesis concurs with an outward proton flux through the ATPase. ATP hydrolysis with an inward proton flux.

(2) The increase of $\Delta\mu_{H^+}$ is linearly proportional to the inward proton flux.

(3) The flash-induced increase of $\Delta\mu_{H^+}$ is completed within a negligible time. (In practice, this time appeared to be about 50–200 ms (see Fig. 3A); the relevant values of $1/k_1$, $1/k_2$ an $1/k_3$ are in the range of seconds to tens of seconds.)

(4) Proton leakage is linearly proportional to $\Delta\mu_{H^+}$ (proportionality constant: k_1).

(5) Proton flux through the ATPase is linearly proportional to the driving force: $(\Delta\mu_{H^+} + \Delta G_P/n)$. Proportionality constants: k_2 and k_3.

(6) P, ΔG_P, n and the fraction of activated ATPases are constant throughout the flash train

(7) k_3 (at $\Delta\mu_{H^+} \geqslant T$) should be larger than k_2 (at $\Delta\mu_{H^+} \leqslant T$). Thus, ATP synthesis or hydrolysis proceeds intrinsically faster if $\Delta\mu_{H^+} \geqslant T$ than if $\Delta\mu_{H^+} \leqslant T$. This assumption is required in order to get an increase of Y at increased flash frequency (decreased i), as observed in practice. This stepwise transition of the rate constant which implies is discontinuity, can be seen as an approximation of a sharp, non-linear increase of the intrinsic rate which occurs around $\Delta\mu_{H^+} = T$ (c.f. Refs. 43–46). The critical transition level T can be chosen anywhere between the dark steady-state energization level ($\Delta\mu_{H^+} = -k_2\ \Delta G_P/c_2 n$, see below) and the flash-induced peak energization level, without essentially changing the mathematical model. However: (1) if $T \leqslant -k_2\ \Delta G_P/c_2 n + P$, $\Delta\mu_{H^+}$ will exceed T already after the first flash and 'situation I' (see below) does not exist; (2) if $T \leqslant -k_3\ \Delta G_P/c_3 n$, $\Delta\mu_{H^+}$

will not return spontaneously below T once it has exceeded this level and the system will thus be metastable. We will not consider this case below! If the critical transition level T is chosen to be $\Delta\mu_{H^+} = -\Delta G_P/n$ (c.f. Ref. 49), ATP synthesis, which occurs at $\Delta\mu_{H^+} \geqslant -\Delta G_P/n$, proceeds specifically faster than ATP hydrolysis, which occurs at $\Delta\mu_{H^+} \leqslant -\Delta G_P/n$. (B) In the dark steady state: $\Delta\mu_{H^+} \leqslant T$.

These assumptions yield the following set of equations:

$$\frac{d\Delta\mu_{H^+}}{dt} = -k_2\left(\frac{\Delta G_P}{n} + \Delta\mu_{H^+}\right) - k_1\Delta\mu_{H^+} \quad (\text{if } \Delta\mu_{H^+} \leqslant T) \tag{A-1a}$$

$$\frac{d\Delta\mu_{H^+}}{dt} = -k_3\left(\frac{\Delta G_P}{n} + \Delta\mu_{H^+}\right) - k_1\Delta\mu_{H^+} \quad (\text{if } \Delta\mu_{H^+} \geqslant T) \tag{A-1b}$$

For simplicity in the equations below, we define:

$$c_2 = k_1 + k_2,\ c_3 = k_1 + k_3,\ c_k = \frac{k_3}{c_3} - \frac{k_2}{c_2}$$

After a flash, the decay of $\Delta\mu_{H^+}$ can be described according to Eqn. A-1a, if $\Delta\mu_{H^+} \leqslant T$: $\Delta\mu_{H^+} = E_0 \cdot \exp(-c_2 t) - k_2\Delta G_P/nc_2$, or according to Eqn. A-1b, if $\Delta\mu_{H^+} \geqslant T$: $\Delta\mu_{H^+} = E_0 \cdot \exp(-c_3 t) - k_3\Delta G_P/nc_3$. Here, $-k_2\Delta G_P/nc_2$ is the steady state value of $\Delta\mu_{H^+}$ in the dark. This steady state is determined both by proton influx due to ATP hydrolysis and by proton leakage. E_0 is the flash-induced increase of $\Delta\mu_{H^+}$ above this steady state level. If another flash is fired at $t = i$, there is a sudden increase of $\Delta\mu_{H^+}$, up to $[\Delta\mu_{H^+(t=i)} + P]$. Thereafter, $\Delta\mu_{H^+}$ decays again according to the equations above, etc. The resulting ATP yield of the Nth flash can be calculated according to:

$$Y = \int_{t_N^i = 0}^{i} \frac{dATP}{dt} dt \tag{A-2}$$

Due to assumption 7, a discontinuity in the dissipation of $\Delta\mu_{H^+}$ (at $\Delta\mu_{H^+} = T$, i.e., at $t_N^i = t_N^c$) is introduced in the model. Therefore, Eqn. A-2 should be explicitly written as a combination (A-2 *) of three equations. Each equation corresponds to a different situation, determined by the level of $\Delta\mu_{H^+}$. This is indicated in parenthesis:

$$U\int_{t_N^i = 0}^{i} k_2\left(\frac{\Delta G_P}{n} + \Delta\mu_{H^+}\right)dt \quad (\text{if } \Delta\mu_{H^+} \leqslant T \text{ for } 0 \leqslant t_N^i \leqslant i) \tag{A-2 *a}$$

$$U\int_{t_N^i = 0}^{t_N^c} k_3\left(\frac{\Delta G_P}{n} + \Delta\mu_{H^+}\right)dt + U\int_{t_N^i = t_N^c}^{i} k_2\left(\frac{\Delta G_P}{n} + \Delta\mu_{H^+}\right)dt \quad (\text{if } \Delta\mu_{H^+} = T \text{ for any } 0 \leqslant t_N^i \leqslant i) \tag{A-2 *b}$$

$$U\int_{t_N^i = 0}^{i} k_3\left(\frac{\Delta G_P}{n} + \Delta\mu_{H^+}\right)dt \quad (\text{if } \Delta\mu_{H^+} \geqslant T \text{ for } 0 \leqslant t_N^i \leqslant i) \tag{A-2 *c}$$

Also as a consequence of the discontinuity, implied by assumption 7, three situations should be discerned (further disignated as I, II, III). Which of these situations applies depends on the flash frequency. In situation I, i.e., as long as inequality A-3 holds, the flash frequency is so low, that $\Delta\mu_{H^+}$ (see Eqn. A-1a) stays always below T during flashing and there is no transition of the rate constant k_2 of the ATPase-associated proton flux. Therefore, Y (see Eqn. A-2 *a) is independent of flash frequency in this situation. The curves in Fig. 2 are simulated according to the model described here: the small part of the solid curve in Fig. 2, below 0.05 Hz corresponds to this situation. In situation II, i.e., as long as inequality A-8 holds, $\Delta\mu_{H^+}$ will exceed T as soon as inequality A-9 holds. Thereafter, $\Delta\mu_{H^+}$ (see Eqn. A-1a,b) will oscillate around T until the end of the flash train. Each time $\Delta\mu_{H^+}$ crosses the level T, k_2 will change into

k_3 and vice versa. Therefore, Y (see Eqn. A-2 *b) increases sharply with flash frequency in situation II. The 'steepest' part of the simulated curve in Fig. 2, between 0.05 and 0.5 Hz, corresponds to this situation. Lastly, in situation III, i.e., as long as inequality A-3 holds, $\Delta\mu_{H^+}$ (see Eqn. A-1b) will stay above T as shown as $t_N^c \geqslant i$ (see Eqns. A-1 and A-2). This situation is illustrated in Fig. 5, for $T = -\Delta G_P/n$. In this situation, Y (see Eqn. A-2 *b) will become less dependent on the flash frequency as the flash frequency increases. The flat part of the simulated curve in Fig. 2, above 0.5 Hz, corresponds to this situation. In all these three situations, the ATP yield per flash becomes practically constant after a number of flashes. This number is smaller if the difference between T and the dark steady state level of $\Delta\mu_{H^+}$ ($-k_2\Delta G_P/nc_2$) is smaller. In case $T = -\Delta G_P/n$, the difference between T and the dark steady state level is equal to $k_1\Delta G n c_2$. Thus, a phosphate potential-dependent lag phase can occur in ATP synthesis [12]:

Situation I: If

$$i \geqslant -\frac{1}{c_2}\ln\left(1-\frac{P}{T+k_2\Delta G_P/c_2 n}\right) \tag{A-3}$$

or, in case $T = -\Delta G_P/n$, if

$$i \geqslant -\frac{1}{c_2}\ln\left(1+\frac{c_2 nP}{k_1\Delta G_P}\right) \tag{A-3*}$$

Then, $\Delta\mu_{H^+}$ never exceeds T and

$$Y_N = U\left[\frac{k_2 P}{c_2}\{1-e^{-c_2(t_N+i)}\}+\frac{k_1 k_2\Delta G_P i}{c_2 n}\right] \tag{A-4}$$

Here, $Uk_1k_2\Delta G_P i/c_2 n$ is the 'counteracting' effect of ΔG_P: in the dark, steady-state ATP hydrolysis equals

$$\frac{Uk_1k_2\Delta G_P}{c_2 n} \quad \text{pmol ATP per mg Chl per s.} \tag{A-5}$$

In practice, we corrected our measurements for dark ATP hydrolysis (analogously to [32]). Thus, the yield per flash should be

$$Y_N = \frac{UPk_2}{c_2}\{1-e^{-c_2(t_N+i)}\} \tag{A-6}$$

in order to bring it in line with our observations. After several flashes, Y_N becomes nearly independent of N ('pseudo steady-state'). Then,

$$Y_N \approx \frac{UPk_2}{c_2} \tag{A-7}$$

Situation II: If

$$-\frac{1}{c_2}\ln\left\{1-\frac{P}{T+\frac{k_2\Delta G_P}{c_2 n}}\right\} \geqslant i \geqslant -\frac{1}{c_3}\ln\left\{\left(1+\frac{P}{T+\frac{k_3\Delta G_P}{c_3 n}}\right)^{-1}\right\} \tag{A-8}$$

or, in case $T = -\Delta G_P/n$, if

$$-\frac{1}{c_2}\ln\left\{1+\frac{c_2nP}{k_1\Delta G_P}\right\} \geqslant i \geqslant -\frac{1}{c_3}\ln\left\{\left(1-\frac{c_3nP}{k_1\Delta G_P}\right)^{-1}\right\} \tag{A-8 *}$$

then $\Delta\mu_{H^+}$ will be alternatively smaller and larger than T, as soon as:

$$t \geqslant -i-\frac{1}{c_2}\ln\left\{1-\frac{(1-e^{-c_2 i})\left(T+\frac{k_2\Delta G_P}{c_2 n}\right)}{P}\right\} \tag{A-9}$$

or, in case $T=-\Delta G_P/n$, as soon as:

$$t \geqslant -i-\frac{1}{c_2}\ln\left\{1+\frac{(1-e^{-c_2 i})k_1\Delta G_P}{c_2 Pn}\right\} \tag{A-9 *}$$

in that case, and after correction for ATP hydrolysis as in Situation I (Eqns. A-4–A-6),

$$Y_N = U\left[\frac{k_3}{c_3}\left(P+\frac{c_k\Delta G_P}{n}\right)(1-e^{-c_3 t_N^e})+\frac{k_3}{c_3}\left(T+\frac{k_2 G_P}{c_2 n}\right)e^{-c_2(i-t_{N-1}^e)}\{1-e^{-c_3 t_N^e}\}\right.$$
$$\left.+\frac{k_2}{c_2}\left(T+\frac{k_2\Delta G_P}{c_2 n}\right)\{1-e^{-c_2(i-t_N^e)}\}+\frac{k_1 c_k \Delta G_P t_N^e}{n}\right] \tag{A-10}$$

or, in case $T=-\Delta G_P/n$:

$$Y_N = U\left[\frac{k_3}{c_3}\left(P+\frac{c_k\Delta G_P}{n}\right)(1-e^{-c_3 t_N^e})-\frac{k_1 k_3 \Delta G_P}{c_2 c_3 n}e^{-c_2(i-t_{N-1}^e)}\{1-e^{-c_3 t_N^e}\}-\frac{k_1 k_2 \Delta G_P}{c_2^2 n}\{1-e^{-c_2(i-t_N^e)}\}+\frac{k_1 c_k \Delta G_P t_N^e}{n}\right] \tag{A-10 *}$$

Here, t_N^e and t_{N-1}^e are the only time-dependent variables (at constant i) and can be found by iteration:

$$t_N^e = -\frac{1}{c_3}\ln\left(T+\frac{k_3\Delta G_P}{c_3 n X_N}\right) \tag{A-11}$$

where

$$X_N = P+\frac{c_k\Delta G_P}{n}+\left(T+\frac{k_2\Delta G_P}{c_2 n}\right)e^{-c_2(i-t_{N-T}^e)}$$

or, in case $T=-\Delta G_P/n$:

$$t_N^e = -\frac{1}{c_3}\ln\frac{1}{-\frac{c_3 nP}{k_1\Delta G_P}-\frac{c_3 c_k}{k_1}+\frac{c_3}{c_2}e^{-c_2(i-t_{N-1}^e)}} \tag{A-11 *}$$

If the inequality A-9 does not (yet) hold in situation II, Y_N is calculated according to Eqn. A-6. The first flash at which $\Delta\mu_{H^+}$ exceeds T, and at which Eqns. A-10 and A-11 should be used to calculate Y_N, occurs at the lowest value of t at which Eqn. A-9 holds and t/i is an integer. Let this be the Mth flash ($N=M$), then X_M in Eqn. A-11 corresponds to:

$$X_M = \frac{c_k\Delta G_P}{n}+P\frac{1-e^{-c_2(tM+i)}}{1-e^{-c_2 i}} \tag{A-12}$$

Starting from t^e_M, t^e_{M+1} (and any t^e_N) can be calculated according to Eqn. A-11. In the pseudo steady-state, $t^e_N \approx t^e_{N-1}$. (N.B.: In order to calculate t^e_N in the pseudo-steady-state, one can start at any value of t^e_N between o and i: by iteration of Eqn. A-11 t^e_N will always converge to its limiting value at $N \to \infty$). The simulated data shown in Fig. 2 have been calculated from the pseudo steady-state values of t^e_N, with $(t^e_N - t^e_{N-1}) < 10^{-11}$ s.

If $k_3 > k_1 > k_2$ (as in our simulations), most protons flow back via the ATPase as long as $\Delta\mu_{H^+} \geqslant -\Delta G_P/n$, and via leakage pathways as soon as $\Delta\mu_{H^+} \leqslant -\Delta G_P/n$. Consequently, the bulk of Y_N is generated between $t^i_N = 0$ and $t^i_N = t^e_N$. The net ATP synthesis occurs only between $t^i_N = 0$ and $t^i_N = t^e_N$.

Situation III: If

$$i \leqslant -\frac{1}{c_3} \ln\left(1 + \frac{P}{T + k_3 G_P/c_3 n}\right)^{-1} \tag{A-13}$$

or, in case $T = -\Delta G_P/n$, if

$$i \leqslant \frac{1}{c_3} \ln\left(1 - \frac{c_3 n P}{k_1 \Delta G_P}\right)^{-1} \tag{A-13*}$$

the value of t^e_N will exceed i (and $\Delta\mu_{H^+}$ will exceed T) after a few flashes. Thus, as soon as $t^e_N \geqslant i$ (see Eqns. A-11 and A-12), and after correction for ATP hydrolysis as in situation I (Eqns. A-4–A-6),

$$Y_N = U\left[\frac{k_3}{c_3}\left\{P + \frac{c_k \Delta G_P}{n} + \left(T + \frac{k_2 G_P}{n c_2}\right) e^{-c_2(i - t^e_L)}(1 - e)^{-c_3 i} e^{-c_3 t^0_N} + \frac{k_3}{c_3} P\left(1 - e^{-c_3(t^0_N + i)}\right)\right\} + \frac{k_1 c_k \Delta G_P}{n}\right] \tag{A-14}$$

or, in case $T = -\Delta G_P/n$:

$$Y_N = U\left[\frac{k_3}{c_3}\left\{P + \frac{\Delta G_P}{n}\left(c_k - \frac{k_1}{c_2} e^{-c_2(i - t^e_L)}\right)(1 - e^{-c_3 i}) e^{-c_3 t^0_N} + P\left(1 - e^{-c_3 t^0_N + i}\right)\right\} + \frac{k_1 c_k i \Delta G_P}{n}\right] \tag{A-14*}$$

Here, t^e_L is the largest real value of t^e_N which occurs in the train (i.e. the largest value of t^e_N calculated according to Eqns. A-11 and A-12 with $t^e_N \leqslant i$). In the pseudo steady-state (t^0_N large),

$$Y_N \approx U\left\{\frac{k_3 P}{c_3} + \frac{k_1 c_k i \Delta G_P}{n}\right\} \tag{A-15}$$

At very small i (very high flash frequency),

$$Y_N \approx \frac{U k_3 P}{c_3} \tag{A-16}$$

Comparing the pseudo steady-states of situation I, II, III, it appears that Y_N is independent of flash frequency both in situation I and in situation III at high flash frequency (see Eqns. A-4. A-6, A-7 and A-14–A-16 and the simulated data in Fig. 2). If $k_3 = k_2$ (no transition at $\Delta\mu_{H^+} = T$), Y_N is always equal to $Uk_2P/c_2 = Uk_3P/c_3$ at large values of t or t^0_N, irrespective of the flash frequency. Since this does not agree with our observations (Fig. 2, main figure), it follows that $k_3 \geqslant k_2$ in the above-described model, implying a transition of the intrinsic ATPase turn-over rate at $\Delta\mu_{H^+} = T$ (see assumption 7, above).

In principle, it is possible to obtain similar results as with the above-described model by assuming that a transition of the H^+/ATP ratio 'n' (instead of the rate constant 'k') occurs at T (c.f. Refs. 53 and 54). However, in order to fit the experimental data the value of n should then be changed by a factor 4–5,

which seems not realistic. Therefore, we will not enlarge upon this possibility.

Another quantity which could be variable is the flash-induced increase of $\Delta\mu_{H^+}$, denoted as 'P': a reduction of P could occur at increasing $\Delta\mu_{H^+}$ due to inhibition of electron transfer and proton translocation (feedback from $\Delta\mu_{H^+}$) or due to the occurrence of 'slip' in the electron transfer-driven pumping of protons [55]. However, this provides no explanation of our results: a decrease of P would tend to decrease the ATP yield Y at increasing flash frequency. If, in addition to such a decrease there would be no transition of the rate constant k (if $k_2 = k_3$), a net decrease of Y would occur at increasing flash frequency.

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