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RELATION BETWEEN THE INITIAL KINETICS OF ATP SYNTHESIS AND OF CONFORMATIONAL CHANGES IN THE CHLOROPLAST ATPase STUDIED BY EXTERNAL FIELD PULSES

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Summary

ATP formation and the energy-dependent release of tightly bound [^{14}C]-adenine nucleotides from the chloroplast coupling factor CF_1 has been studied as a function of the time of energization of the membrane in the range of 500 μs up to 60 ms. The high time resolution was achieved because the energization was generated artificially by external electric field pulses. Applying external electric field pulses to a chloroplast suspension induces an electric potential difference across the thylakoid membrane. The following results were obtained:

(1) The amount of ATP generated increases linearly with the time of energization. The steady-state rate of ATP formation is reached in less than 500 μs.

(2) A fraction of the adenine nucleotides tightly bound to CF_1 is released on energization with a half-rise-time of about 2 ms. The size of the fraction, i.e., the amplitude of the fast phase of the release, increases with the magnitude of the induced transmembrane electric potential difference. A further slow release is superimposed.

(3) The initial rate of the release of adenine nucleotides is practically identical with the rate of ATP formation.

It is concluded that the release of tightly bound nucleotides monitors an initial conformational change by which the ATPase turns from an inactive into an activated state. For the explanation of the results a reaction scheme is proposed which takes into account a preceding activation of the ATPase.

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Abbreviations: AdN, adenine nucleotides; Chl, chlorophyll; CF_1, chloroplast coupling factor; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

In chloroplasts, phosphorylation can be induced by an artificially generated pH gradient across the thylakoid membrane, ΔpH [1] and/or by an artificially generated transmembrane electric potential difference, $\Delta\psi$ [2] without the need for a light-driven electron transport. According to the chemiosmotic theory [3] the flux of protons down their electrochemical potential gradient through the membrane-bound ATPase (often called ATP synthetase) provides the free energy necessary for ATP synthesis. The way in which the rate of ATP synthesis is controlled by the electric potential difference and the pH gradient has been the subject of several investigations [4—8]. Since: (1) no significant ATPase activity is observed in the dark on addition of ATP [9,10]; (2) ATP hydrolysis can be stimulated only by applying a transmembrane ΔpH or $\Delta\psi$ in the same direction as necessary for ATP syntheiss [11,12], and (3) a critical level of ΔpH or $\Delta\psi$ exists below which neither ATP synthesis nor ATP hydrolysis is observed [1,12,4,7] it must be concluded that an activation step, which depends on ΔpH and $\Delta\psi$, precedes the catalytic reaction (ATP synthesis or ATP hydrolysis). The activation, presumably the displacement of the ϵ inhibitor [13], may be coupled with a conformational change of the ATPase. Energy-dependent conformational changes in the ATPase have been demonstrated in a number of ways [14—16]. Among others, the energy-dependent release of nucleotides tightly bound to the coupling factor has been used to monitor conformational changes in the coupling factor, CF_1 [8,17—20]. As the light-induced nucleotide exchange and phosphorylation depend in the same way on different parameters, e.g. light intensity, uncouplers, etc. [21] it was concluded that both processes are correlated. Analyzing the dependence of AdN release and ATP synthesis on the transmembrane electric field strength, it was suggested that those coupling factors which exchange their tightly bound nucleotide turn into an active state and that the rate of ATP synthesis is controlled by the fraction of activated ATPases, i.e., the fraction of activated ATPases increases with increasing $\Delta\psi$ whereas the rate constant of ATP synthesis is practically independent of $\Delta\psi$ [8]. The purpose of the present work is to test by kinetic analysis whether the exchange of tightly bound nucleotides is sufficiently fast to be an initial step coupled with the preceding activation of the ATPase. Therefore, the exchange of tightly bound nucleotides and ATP formaton were measured as functions of the time of energization of the membrane in the range of $500\ \mu s \leqslant t \leqslant 60$ ms in order to analyze the initial kinetics of both processes.

The time resolution necessary for these investigations can be achieved by energization with an external electric field. The transmembrane potential difference induced by the external electric field has been measured directly by the electrochromic absorption changes [22]. Compared to conditions where the energization is induced by light, the major advantages of the external electric field method are:

(1) The transmembrane electric field induced by the external electric field can be switched on and off with a time constant of less than 10 μs independent of the magnitude of the induced $\Delta\psi$. Therefore, the time of energization is identical with the duration of the external electric field pulse and can be

adjusted in the range from 100 μs up to 60 ms [22].

(2) The induced membrane potential is proportional to the external electric field strength. The average value of $\Delta\psi$ has been estimated to be about 200 mV at an external field strength of 10^3 V/cm [22].

(3) The signal/noise ratio for the measurement of ATP synthesis can be increased by repetitive excitation so that even at extremely small energization times (e.g., 500 μs) a high sensitivity is achieved.

The light induced transmembrane potential difference can be generated in less than 1 ns [23,24] and only up to 50 mV (estimation of $\Delta\psi$ induced by a single turnover light flash). To increase $\Delta\psi$ above 50 mV illumination times are necessary long compared to the rate-limiting time of the turnover of the reaction centers. Therefore, it requires 4—8 ms to reach a potential difference of about 200 mV. The decay of $\Delta\psi$ varies between 10 ms and 200 ms, depending on the membrane conductivity, so that ATP synthesis can proceed after switching off the light (post-illumination ATP synthesis). Therefore, the time course of the energization induced by light is not determined by the duration of the illumination.

Materials and Methods

Broken chloroplasts were prepared as described elsewhere [25] from spinach either grown in a phytocell or obtained from the local market. Additionally, 10 mM ascorbate was present during grinding. The measurements of the exchange of tightly bound adenine nucleotides were carried out with chloroplasts which were prelabeled with [^{14}C]ADP in the light according to the procedure of Strotmann et al. [18]. Chloroplasts washed three times with medium I ($5 \cdot 10^{-2}$ M NaCl/10^{-3} M $MgCl_2$/$2 \cdot 10^{-2}$ M Tricine, pH 7.8) were illuminated and stirred for 1 min (white light, 10^6 erg/cm^2 per s in a medium containing: $5 \cdot 10^{-2}$ M NaCl, 10^{-3} M $MgCl_2$, $5 \cdot 10^{-4}$ M benzyl viologen, $2.5 \cdot 10^{-2}$ M Tricine, (pH 7.8), $3 \cdot 10^{-5}$ M [^{14}C]ADP (spec. act. $1.2 \cdot 10^5$ dpm/nmol) and chloroplasts giving a final chlorophyll concentration of $1.3 \cdot 10^{-3}$ M. After labeling the chloroplasts were washed in the dark four times with medium II ($5 \cdot 10^{-2}$ M NaCl/$2.5 \cdot 10^{-2}$ M Tricine, pH 7.8) to remove [^{14}C]ADP not tightly bound to coupling factor 1. These chloroplasts were resuspended in a medium containing 10^{-3} M NaCl/$2 \cdot 10^{-3}$ M $MgCl_2$/10^{-2} M Tricine, pH 7.8, and stored in an ice bath in the dark until use. The reaction medium comprised: $6 \cdot 10^{-4}$ M $MgCl_2$/10^{-3} M KH_2PO_4/$3 \cdot 10^{-3}$ M Tricine, (pH 7.8)/$3 \cdot 10^{-4}$ M ADP. After addition of the labeled chloroplasts giving a final chlorophyll concentration of $2.5 \cdot 10^{-4}$ M one aliquot was suspended in a cuvette between two flat platinum electrodes for application of the external electric field pulses. The other aliquot (corresponding control) was stored during this time.

The samples were then immediately centrifuged (5 min, 5000 $\times g$, 0°C). 500 μl of the supernatant was added to 10 ml Insta-Gel scintillator cocktail (Packard) and the radioactivity was counted in a scintillation counter (Tri-Carb, Packard). The release of ^{14}C-labeled adenine nucleotides in the control was about $1.0 \cdot 10^{-4}$ [^{14}C]AdN/Chl. This is corrected for in the data presented.

ATP formation was measured by the incorporation of ^{32}P. The reaction medium contained $5 \cdot 10^{-4}$ M $MgCl_2$, $5 \cdot 10^{-4}$ M KH_2PO_4, $3 \cdot 10^{-4}$ M ADP,

$5 \cdot 10^{-3}$ M Tricine, (pH 7.6), 5—10 μCi ^{32}P/ml and chloroplasts giving a chlorophyll concentration of $3 \cdot 10^{-4}$ M. After energization by external electric field pulses the chloroplasts (1 ml reaction volume) were denatured by addition of 0.2 ml 30% trichloroacetic acid and the samples were centrifuged at $5000 \times g$ for 10 min.

The assay for [^{32}P]ATP was carried out in a similar way to that described elsewhere [26]: to 0.5 ml of the supernatant of the denatured chloroplasts 0.5 ml of a freshly prepared ammonium molybdate solution was added (containing in 50 ml: 1.5 g $(NH_4)_6MO_7O_2 \cdot 4H_2O$/2.5 ml conc. HCl/0.5 ml triethylamine). The mixture was allowed to react for 15 min at room temperature and then centrifuged at $5000 \times g$ for 15 min. The radioactivity remaining in the supernatant (which corresponds to the amount of [^{32}P]ATP was counted in a scintillation counter using Cerenkov radiation (0.5 ml supernatant and 9.5 ml H_2O). The data presented are corrected for the corresponding control. Experiments in which the results have been analyzed additionally by the usual isobutanol-benzol extraction method [27], which we have used in former experiments [8], give essentially the same results. However, the precipitation method removes the phosphate more efficiently than the two-step extraction procedure and, furthermore, is experimentally easier and faster.

The principle of the external electric field method has been described elsewhere [2,22]. The chloroplast suspension is placed between two flat platinum electrodes (distance between the electrodes, 10 mm; area, 1 cm^2). The rectangular voltage pulses were generated by a high power pulse generator (Velonex V-2432) with the following specifications: (a) output voltage, 0—2000 V; (b) variable pulse polarity; (c) output current, $\leqslant$1.5 A; (d) rise and fall time, $\leqslant$10 μs; (e) pulse width, 300 μs—20 ms; (f) pulse frequency, $\leqslant$10 Hz and single pulse. Rectangular voltage pulses with a pulses width of 20 ms to 60 ms were provided by a different voltage pulse generator with the following specifications: (a) output voltage, 220 V; (b) variable pulse polarity; (c) output current, $\leqslant$12 A; (d) rise and fall time, $\leqslant$10 μs. For these experiments the chloroplasts were suspended in a cell between two flat platinum electrodes at a distance of 2 mm and an area of 5 cm^2.

The polarity of the voltage was changed after each pulse. The ion concentrations of the solution were limited to a level such that the heating per 30 ms pulse (external electric field strengths: 1000 V/cm) was less than 4°C. Furthermore, the cuvette was cooled and the time interval between the pulses was chosen to be long enough to allow reversal of the heating.

Results

Release of tightly bound nucleotides

The total amount of tightly bound ^{14}C-labeled nucleotides per chlorophyll, AdN_0/Chl, was determined after extraction of the nucleotides by heat denaturation or by denaturation with 40% trichloroacetic acid. Approx. $1.1 \cdot 10^{-3}$ AdN/Chl or 1.0 AdN/CF_1 (using a ratio of 860 Chl/CF_1 [28]) are bound. It has been shown elsewhere that this label is almost completely bound to CF_1 [18,29]. From these tightly bound nucleotides about $0.9 \cdot 10^{-3}$ AdN/Chl can be released upon energization with saturating continuous light under phos-

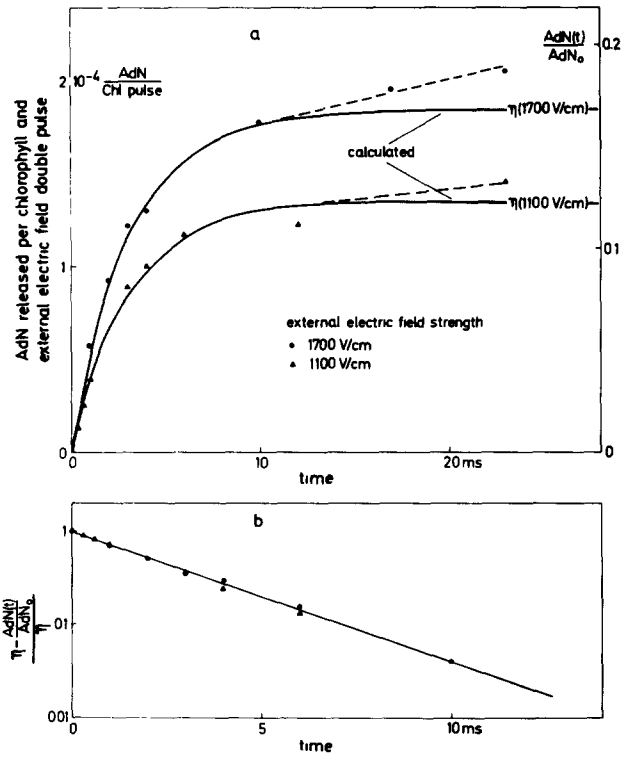

Fig. 1. (a). Amount of tightly bound adenine nucleotides per chlorophyll and external electric field double pulse released as a function of the duration of the external electric field pulse. At the right, the relative release of AdN, $AdN(t)/AdN_0$ is depicted (AdN_0/Chl = total amount of tightly bound nucleotides/chlorophyll = $1.1 \cdot 10^{-3}$ AdN/Chl). The curves have been calculated according to Eqn. 1. (b) Logarithmic plot of $[\eta - (AdN(t)/AdN_0]/\eta$ as a function of the duration of the electric field pulse with η = 0.17 at an external electric field strength of 1700 V/cm (•) and η = 0.12 at an external electric field strength of 1100 V/cm (▲).

phorylating conditions. As all experiments are carried out with a large excess of unlabeled ADP, the observed release reflects an exchange of labeled with unlabeled nucleotides at the coupling factor. The AdN release in continuous light show biphasic kinetics [8]. The kinetics of the fast phase can be measured time-resolved by energization with an external electric field. Fig. 1a shows the release of AdN per chlorophyll and external electric field double pulse as a function of the duration of the external electric field pulse at two different external electric field strengths.

Because in one external electric field pulse at only one half of the thylakoid the polarity of the transmembrane electric field corresponds to that induced by light (i.e., positive inside, negative outside [30]), the amount of AdN released in two external electric field pulses with opposite polarity (one external field double pulse) has been depicted.

The duration of the voltage pulse is practically identical with the time of energization of the membrane. This has been shown by measuring the transmembrane field indicating absorption changes induced by the external electric field [22]. At the right-hand side the relative AdN release, $AdN(t)/AdN_0$, is

depicted. Analyzing the results shown in Fig. 1a it follows:

(1) The amount of AdN released depends on the pulse duration.

(2) Only a fraction of the nucleotides tightly bound to CF_1 is released on energization within a few ms, i.e., only a fraction of the ATPases exchange their tightly bound nucleotides fast.

(3) The release of AdN as function of time can be described by the following equation

$$AdN(t)/AdN_0 = \eta(1 - e^{-kt}) \quad (1)$$

The amplitude of the fast release described by Eqn. 1 is called the fraction of ATPases, η. The curves in Fig. 1a have been calculated according to this equation. Fig. 1b shows a logarithmic plot of $[\eta - (AdN(t)/AdN_0)]/\eta$ as a function of the time of energization at two different field strengths. A rate constant, k, of about 320 s^{-1} corresponding to a half-rise-time, τ, of 2.1 ms can be calculated from the slope of the straight line. The rate constant is independent of the external electric field strength in this range, i.e., $k \neq f(\Delta\psi)$.

(4) The size of the fraction, η, increases with the magnitude of the external electric field strength and thereby depends on the magnitude of the induced transmembrane electric potential difference because both are proportional to each other, i.e., $\eta = f(\Delta\psi)$. The functional dependence of the size of the fraction on the transmembrane electric potential difference has been reported elsewhere [8].

(5) The observed AdN release showed deviations from the calculated curves when pulses of greater than 15 ms were used (see dashed lines). This indicates that a further slow AdN release is superimposed. Provided that the initial rate of this slow release is proportional to $(AdN_0 - \eta AdN_0)$, the rate constant of the slow release can be estimated very roughly to be about 1 s^{-1}.

Formation of ATP

Fig. 2 shows the amount of ATP per chlorophyll generated as a function of the number of the external electric field double pulses at the same field strength. At a pulse duration of 1 ms (Fig. 2a) or 20 ms (Fig. 2b) the amount of ATP generated increases linearly up to 100 or 25 external voltage pulses. From the slope of the straight lines the amount of ATP generated per chlorophyll as a function of the pulse duration, i.e., as function of the time of energization, has been determined. Because of the repetitive excitation, a high accuracy of the measurement of ATP formation as a function of time has bee achieved even at 1 ms pulse duration.

Fig. 3 shows the relative amount of ATP generated as a function of the duration of the external electric field pulse. The pulse duration was varied between 500 μs and 60 ms. In order to eliminate scattering due to different chloroplast preparations in each set of experiments, the values have been normalized to the ATP yield in a 30 ms pulse. The external electric field strength was 1100 V/cm. A linear dependence of ATP formation on time has been observed. Even at the shortest pulse duration the amount of ATP generated shows no deviation from this linear dependence; therefore, any initial lag phase larger than 500 μs can be excluded, i.e., ATP synthesis reaches the steady-state rate in less than 500 μs.

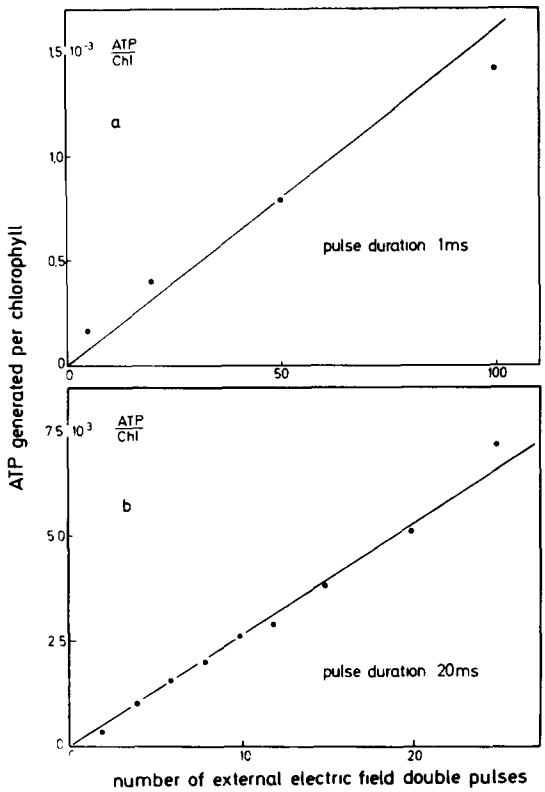

Fig. 2. Amount of ATP per chlorophyll generated as a function of the number of external electric field double pulses. Pulse duration: 1 ms (a) and 20 ms (b). Time between the pulses: ≈5 s (a) and ≈30 s (b).

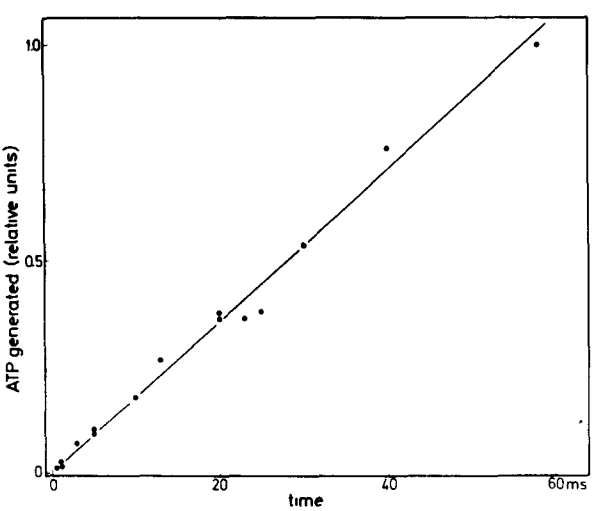

Fig. 3. Relative amount of ATP generated as a function of the duration of the external electric field pulse. The data are normalized to the ATP yield obtained in one external electric field double pulse of 30 ms duration (average value of different measurements: $5.5 \cdot 10^{-4}$ ATP/Chl pulse). External electric field strength, 1100 V/cm.

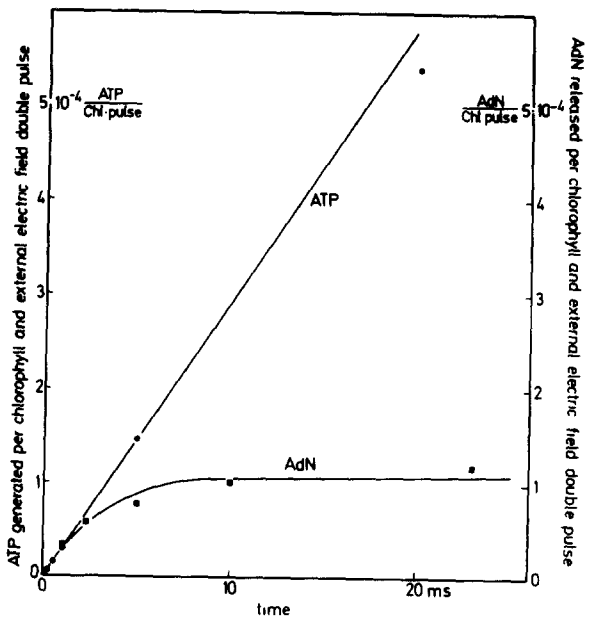

Fig. 4. Amount of ATP generated (•) and amount of AdN released (▪) per chlorophyll and external electric field double pulse as function of the duration of the external electric field pulse. External electric field strength, 1500 V/cm.

The result has been tested to be independent of the external electric field strength in the range between 1000 V/cm and 1700 V/cm. The dependence of the rate of ATP synthesis on the external field strength has been reported elsewhere [31,32]. To compare the initial kinetics directly, ATP formation and the release of tightly bound nucleotides have been measured under identical conditions at the same external electric field strength as a function of the time of energization. Fig. 4 shows that the initial rate of the exchange of AdN is within experimental error identical with the rate of ATP synthesis, i.e.,

$$\frac{\mathrm{ATP}(t)}{\mathrm{Chl}} \approx \left(\frac{\mathrm{AdN}(t)}{\mathrm{Chl}}\right)_{t\to 0} = \hat{k}t \tag{2}$$

Since for $t \to 0$ the expansion of the exponential function in Eqn. 1 reduces to $1-kt$, the initial exchange of AdN can be expressed as follows:

$$\left(\frac{\mathrm{AdN}(t)}{\mathrm{Chl}}\right)_{t\to 0} = k\eta \frac{\mathrm{AdN}_0}{\mathrm{Chl}}\, t \tag{3}$$

By comparison of Eqn. 2 and Eqn. 3 it results

$$\hat{k} = k \cdot \eta \cdot \frac{\mathrm{AdN}_0}{\mathrm{Chl}} \quad \text{with } \eta = f(\Delta\psi) \text{ and } k = 320\ \mathrm{s}^{-1} \tag{4}$$

Discussion

Evidence has been established elsewhere that the tight binding sites are different from the catalytic sites, because: (1) the K_M value for the tight binding

of ADP (2—6 μM [33—35]) is lower than the apparent K_M value for ADP in phosphorylation (25—80 μM [36—38]); (2) the substrate specificity for the exchange differs strongly from that of phosphorylation [34,39,40]; (3) tightly bound ADP is not directly converted into ATP but is first released into the medium upon energization and then probably phosphorylated at a different site [41,42]; (4) the exchange is uncoupler-sensitive as in phosphorylation but is not inhibited by inhibitors of phosphorylation (e.g., Dio-9, phlorizin) [8,21].

The function of the tightly bound nucleotides in the mechanism of phosphorylation is still a matter of discussion, but it is widely accepted that the exchange monitors a conformational change of the ATPase which seems to be not directly coupled with the catalytic reaction [33,43—45].

A correlation between the activation of the ATPase and the exchange of tightly bound nucleotides has been proposed on the basis of various pieces of evidence presented by several authors [8,43,44]. The results presented here here give evidence, by kinetic analysis, that the exchange of tightly bound nucleotides might reflect the activation of the ATPase. This can be concluded from the following reasons:

(1) The inital rate of the release of tightly bound nucleotides is practically identical with the rate of ATP formation (shown in Fig. 4).

(2) The rate constant for the AdN release was determined to be $k \approx 320\ s^{-1}$ (Fig. 1). Using this value, a rate of ATP synthesis, $\dot{ATP}$, is calculated to about 320 ATP/CF_1 · s = 370 · 10^{-3} ATP/Chl · s. Indeed, a higher rate of ATP synthesis has never been reported. The maximal rates which have been measured using conditions for a cyclic electron transport were, in fact, about 370 · 10^{-3} ATP/Chl · s [38,46]. These results clearly demonstrate that the exchange of AdN is sufficiently fast to monitor an initial, electric field controlled conformational change by which the ATPase turns from an inactive into an activated state. In this case η must be interpreted as the fraction of ATPases which have turned into the activated state. The size of this fraction strongly depends on the induced transmembrane electric potential difference (see Fig. 1 and Ref. 8).

Therefore, the maximal rate of ATP synthesis is expected if all ATPases are activated, i.e., $\eta = 1$ [47]. These results strongly support the model which has been developed on the basis of the measurement of ATP synthesis and AdN exchange as a function of the transmembrane electric potential difference induced by the external electric field [8]. This model proposes that the transmembrane electric potential difference controls the rate of ATP synthesis by a variable active fraction of ATPases, whereas the turnover number of ATP formation per active ATPase is approximately constant.

Recently, it was shown that the model is also valid for energization by artificially induced pH gradients performing acid-base jumps with the quenched flow method [48].

The results shown in Fig. 1 indicate that the fast phase of the exchange of tightly bound nucleotides is followed by a slow exchange of AdN with a half-rise time of approx. 1 s. On energization with light, a pronounced biphasic release of AdN has been observed ([8] and Gräber and Strotmann, personal communication), whereby the half-rise time of the slow phase has been determined to also be about 1 s. The kinetics of the fast phase reflects, in this case, mainly the time course of the energization with light. A biphasic release is con-

sistent with the proposed model assuming that the amplitude of the fast phase ($\tau \approx 2$ ms) indicates the size of the fraction, η, of active ATPases, whereas the slow phase ($\tau \approx 1$ s) indicates that this active fraction is not restricted to a special group of ATPases, but an activated ATPase can revert after several turnovers into the inhibited state whereas, correspondingly, an inhibited one becomes activated. It should be noted that a kinetic analysis of the AdN exchange under non-phosphorylating conditions [49] cannot be compared with results presented in this work, which have been obtained under phosphorylating conditions because, additionally, a metastable adenine nucleotide depleted CF_1 conformation is established at low ADP concentrations [33,49].

A detailed model concerning the kinetics of the exchange of AdN under phosphorylating conditions and of ATP synthesis must take into account the following observations: (a) an activation step precedes ATP synthesis; (b) an energy-dependent fraction of the tightly bound nucleotides is exchanged quickly i.e., within a few ms (Fig. 1); (c) further exchange occurs slowly, with a half-rise time of about 1 s (Fig. 1); (d) ATP synthesis reaches the steady-state rate in less than 500 μs (Fig. 3); (e) the initial rate of AdN exchange and the steady-state rate of ATP synthesis are practically identical (Fig. 4). A mechanism which meets these requirements is given by the following simplified scheme:

$$\begin{array}{ccccc} E_a + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E_aS & \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} & E_a + P \\ \Big\uparrow k_{-2} \quad \Big\downarrow k_2 & & \Big\uparrow k_{-3} \quad \Big\downarrow k_3 & & \\ E_i + S & \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} & E_iS & & \end{array} \tag{5}$$

The substrate, S, can bind with the activated enzyme, E_a, and with the inhibited enzyme, E_i. It is assumed that the substrate affinity is not altered due to bound inhibitor and vice-versa, i.e.,

$$\frac{k_{-1}}{k_1} = \frac{k_{-4}}{k_4} \text{ and } \frac{k_{-2}}{k_2} = \frac{k_{-3}}{k_3}.$$

The scheme is described by a set of coupled linear first-order differential equations:

$$\frac{d[E_a]}{dt} = k_{-2}[E_i] + (k_{-1} + k_5)[E_aS] - (k_1[S] + k_2)[E_a] \tag{6}$$

$$\frac{d[E_i]}{dt} = k_{-4}[E_iS] + k_2[E_a] - (k_{-2} + k_4[S])[E_i] \tag{7}$$

$$\frac{d[E_aS]}{dt} = k_{-3}[E_iS] + (k_1[S] + k_{-5}[P])[E_a] - (k_{-1} + k_3 + k_5)[E_aS] \tag{8}$$

$$\frac{d[E_iS]}{dt} = k_3[E_aS] + k_4[S][E_i] - (k_{-3} + k_{-4})[E_iS] \tag{9}$$

$$\frac{d[P]}{dt} = k_5[E_aS] - k_{-5}[P][E_a] \tag{10}$$

$$[E_t] = [E_a] + [E_i] + [E_aS] + [E_iS] \tag{11}$$

For the sake of simplicity the substrate concentration, [S], is regarded as a constant, and the concentration of the product, [P], is assumed to be so small that the reverse reaction or product inhibition can be disregarded ($k_{-5}[P][E_a] \approx 0$).

Under non-energized conditions all enzymes are assumed to be in the inhibited state, $E_t = E_i$. After energization the intermediates will reach their steady-state concentration determined by the extent of energization. It is assumed that the fraction of ATPases, η, i.e., the amplitude of the fast phase of the AdN release is practically identical with the steady-state concentration of E_aS, i.e., $E_aS >> E_a$. On the basis of this proposed scheme, the observed kinetics can be explained in two different ways, depending on which step the exchange of AdN is assumed to occur. (a) Since AdN release and ATP synthesis coincide kinetically, the AdN exchange can be assumed to occur simultaneously during the process $E_aS \overset{k_5}{\rightarrow} E_a + P$. For the calculation of AdN it was assumed that the exchange of labeled AdN occurs only once during the first turnover of each enzyme. Since ATP synthesis reaches the steady-state rate in less than 500 μs, it may be concluded that activation and formation of the E_aS complex are

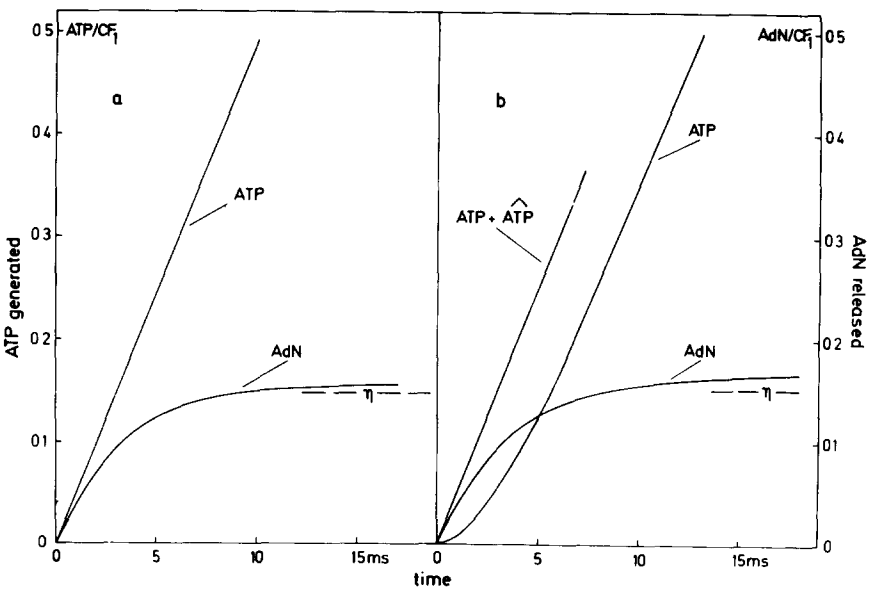

Fig. 5. Numerical solutions of the integrated rate equations (6—11) which have been obtained with the following rate constants: (a) $k_1[S] = 9.625\ 10^6\ s^{-1}$, $k_{-1} = 3500\ s^{-1}$, $k_2 = 5500\ s^{-1}$, $k_{-2} = 1000\ s^{-1}$, $k_3 = 0.55\ s^{-1}$, $k_{-3} = 0.1\ s^{-1}$, $k_4[S] = 5500\ s^{-1}$, $k_{-4} = 2\ s^{-1}$, $k_5 = 325\ s^{-1}$ (b) $k_1[S] = 9.625\ 10^5\ s^{-1}$, $k_{-1} = 3500\ s^{-1}$, $k_2 = 275\ s^{-1}$, $k_{-2} = 50\ s^{-1}$, $k_3 = 5.5\ s^{-1}$, $k_{-3} = 1\ s^{-1}$, $k_4[S] = 275\ s^{-1}$ $k_{-4} = 1\ s^{-1}$, $k_5 = 325\ s^{-1}$. Formation of ATP is identified with the calculated amount of product, P, as function of time. For the calculation of AdN it was assumed that the release of labeled AdN occurs only once during the first turnover of each enzyme either: (a) in the step $E_aS \overset{AdN}{\nearrow\!\!\rightarrow}_{k_5} E_a + P$, or (b) in the activation step $E_i \overset{AdN}{\nearrow\!\!\rightarrow} E_a$ and $E_iS \overset{AdN}{\nearrow\!\!\rightarrow} E_aS$. $\hat{ATP}$ is identical with the concentration of E_aS as function of time. For further details see text.

fast, preceding reactions. The calculated amount of product, P, as function of time is identified with the formation of ATP. A numerical solution of the rate equations, based on these assumptions, which account qualitatively for the observed results, is shown in Fig. 5a. The numerical integration has been performed on a digital computer using a variable-order Adams method. (b) The second approach is based on the assumption that the AdN release is directly coupled with the activation step, $E_i \rightarrow E_a$ or $E_iS \rightarrow E_aS$, respectively, and occurs only once during the first turnover of each enzyme. Under these conditions there exists an initial phase before the steady-state concentration of E_aS is reached. In this case, the time course for the formation of E_aS up to the steady-state concentration is practically identical with the time course of the initial release of AdN. Coincidentally, in time with the initial phase there will be a time lag of about 3 ms before the steady-state rate of product formation (corresponds to ATP formation) is established, because ATP formation is a consecutive reaction of the activation monitored directly by the exchange of AdN (Fig. 1). A numerical solution of the rate equations obtained under these assumptions is shown in Fig. 5b.

However, experimentally, a time lag of about 3 ms for ATP formation has not been observed. This discrepancy can be explained assuming that those coupling factors which have been activated and energized, i.e., enzymes which are still in the E_aS state after the external electric field is switched off, are able to complete their turnover. This might be the case because the duration of the external electric field pulse determines the time of energization and not the reaction time, which can be longer because of the dark periods between successive pulses. In this case the observed amount of product would be identical to $P + E_aS$ (corresponds to ATP + $\widehat{\text{ATP}}$). $\widehat{\text{ATP}}$ is identical with the concentration of E_aS as a function of time. The concentration of ATP + $\widehat{\text{ATP}}$, which is also depicted in Fig. 5b, would again account qualitatively for the measured amount of ATP generated as a function of time (Fig. 3). Therefore, a discrimination between case (a) and (b) is not possible on the basis of these experiments alone.

In acid-base jump experiments performed with the quenched-flow method, the time allowed for the reaction and the energization time are practically identical, provided that the reaction is stopped by denaturation as quickly as it is initiated. Under such conditions a time lag of about 3 ms up to 5 ms has been observed when studying the initial kinetics of ATP synthesis in acid-base jumps ($\Delta pH \approx 4$) with the quenched-flow method [50,51].

In light pulse experiments (saturating light intensity) a lag phase of up to 20 ms has been observed [52—54]. This large value reflects, at least to some extent, the time course of energization and de-energization of the membrane (see Introduction). Therefore, conclusions concerning the initial kinetics of ATP synthesis cannot be obtained from light pulse experiments.

To avoid these difficulties the transient phase of ATP formation in the light has been studied with the quenched-flow method using conditions of a cyclic electron transport. In such case energization occurs more quickly (compared to conditions of a linear electron transport), and the reaction can be stopped immediately if the quench is performed with perchloric acid, which denatures the chloroplasts. Under these conditions, a lag phase of 1—3 ms has been observed [55,56].

Using an EDTA or NH_4Cl quench which causes fast de-energization without denaturation, the experimental conditions are similar to those in the external electric field pulse experiments. In this case ATP formation is observed to start without any lag phase [56].

As discussed above, this might indicate that limited ATP formation is allowed to continue after de-energization and would support an interpretation of the results described in this work as illustrated in Fig. 5b.

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