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CONFORMATIONAL CHANGE OF THE CHLOROPLAST ATPase INDUCED BY A TRANSMEMBRANE ELECTRIC FIELD AND ITS CORRELATION TO PHOSPHORYLATION

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SUMMARY

The energy-dependent release of bound [^{14}C]nucleotides trom the chloroplast coupling factor CF_1, has been used to monitor conformational changes in CF_1. The following results were obtained:

(1) Similar as in continuous light conformational changes of CF_1 are observed on energetization of the thylakoid membrane by short light pulses. Under these conditions the transmembrane electric potential difference induced is about 200 mV and the pH gradient set up across the membrane is about 1.0.

(2) Conformational changes are observed also in the dark when external voltage pulses are used for energization. Under these conditions the transmembrane electric potential difference induced is about 200 mV whereas the pH gradient between the inner and outer thylakoid space is zero.

(3) Only a fraction of the total number of coupling factors change their conformation. The size of this fraction depends non-linearly on the magnitude of the electric potential difference induced by light pulses or external voltage pulses.

(4) In a light or a voltage pulse of 30-ms duration, the amount of ATP generated is 5–8 times larger than the amount of CF_1 which have changed their conformation. This factor is independent of the magnitude of the electric potential difference.

If the observed conformational changes are coupled with phosphorylation these results may be explained tentatively by the following concept. The proton flux which is used for phosphorylation is focussed only to a fraction of the total number of ATPases. This fraction varies strongly with the electric potential difference (and probably also with the pH gradient). The variation occurs in such a way that the flux via these "active" ATPases and their turnover time is nearly constant (about 5 ms).

INTRODUCTION

In chloroplasts, the coupling factor CF_1 (a component of the ATPase) is

Abbreviations: AdN, [8-^{14}C]adenine nucleotides; CF_1, chloroplast coupling factor.

known to catalyse phosphorylation [1–3]. The free energy necessary for ATP synthesis is supplied by a transmembrane electric potential difference, $\Delta\Phi$, and a pH gradient, ΔpH, set up by the electron transport. The free energy stored in this electrochemical potential of H^+ is released through H^+ efflux via the ATPase pathway. This H^+ translocation is coupled with the ATP formation [4–6]. The mechanism of the coupling within the ATPase is subject to different hypotheses. A direct evolvement via protonations at the catalytic site of the ATPase has been proposed by Mitchell [7], whereas an indirect interaction of protons via conformationally induced changes of affinities at the catalytic site is assumed by Boyer et al. [8, 9] and Slater [10].

Conformational changes of CF_1 during illumination were detected by different methods, e.g. hydrogen exchange into protected sites [11, 12], inhibition of phosphorylation after preincubation with inhibitors [13], fluorescence changes of probes attached to CF_1 [14] and energy-dependent exchange of adenine nucleotides tightly bound to CF_1 [15–19, 34]. However, the correlation of these conformational changes with phosphorylation has not been demonstrated clearly.

All previous experiments have been carried out with continuous light or with acid-base transitions. Under these conditions the free energy for ATP synthesis is preferentially conserved in a pH gradient and a smaller part in $\Delta\Phi$. (Continuous light: $\Delta\text{pH} \cong 3.0$ [20], $\Delta\Phi \cong 100$ mV [21]). The high accumulation of protons inside the thylakoids, i.e. a low pH_{in} value, may influence the degree of protonation of CF_1, thereby inducing conformational changes of the enzyme which may be of no relevance for phosphorylation (side reaction).

The aim of this work is (1) to test if conformational changes occur also without a significant change of pH_{in}. This requires conditions where the energy is preferentially stored in $\Delta\Phi$, (2) to compare directly the number of coupling factors which change their conformation with the number of ATP molecules generated.

In order to realize these conditions we performed two types of experiments: (1) We used saturating light pulses of 30 ms duration. In this case the energetic contribution of $\Delta\Phi$ corresponds to about 200 mV, and that of ΔpH to about 60 mV. (2) We used external voltage pulses of 30 ms duration. In this case $\Delta\Phi$ is approx. 200 mV and $\Delta\text{ pH} = 0$. It was shown elsewhere that with such external voltage pulses the ATP yield is nearly the same as with saturating light pulses of the same duration [22].

For detection of the conformational change of CF_1 we used the release of tightly bound nucleotides which occur on energization of the membrane [15–19, 34]. This method has the advantage that no artificial indicators as fluorescamine [14] or *N*-ethylmaleimide [13] are necessary and it is experimentally easier than ^3H incorporation.

MATERIALS AND METHODS

Chloroplasts were prepared as described elsewhere [23] using spinach obtained from the local market. Additionally, 10 mM ascorbate was added during grinding. According to the procedure of Strotmann et al. [17] these chloroplasts were washed three times with the medium I ($5 \cdot 10^{-2}$ M NaCl, 10^{-3} M $MgCl_2$, $2 \cdot 10^{-3}$ M tricine, pH 7.8), incubated under continuous illumination for 60 s with [8-^{14}C]ADP (incubation medium $5 \cdot 10^{-2}$ M NaCl, 10^{-3} M $MgCl_2$, $2 \cdot 10^{-4}$ M benzyl viologen, $2.5 \cdot 10^{-3}$ M tricine, pH 7.8, $2 \cdot 10^{-5}$ M, [8-^{14}C]ADP (specific activity $1.2 \cdot 10^5$ dpm/

nmol), 10^{-3} M chlorophyll) for 1 min and then washed four times with medium II ($5 \cdot 10^{-2}$ M NaCl, $2.5 \cdot 10^{-2}$ M tricine, pH 7.8) to remove excess labelled ADP. These chloroplasts were stored in the dark in an ice bath until use. The thylakoid membranes were energized by (a) continuous light, (b) light pulses and (c) external voltage pulses.

Light pulses were realised by groups of single turnover flashes (duration 20 μs, wavelength 610–730 nm). Within the flash groups the dark time between the flashes was 2 ms. The darktime between these light pulses was 10–30 s.

The transmembrane electric potential difference induced by the pulses was measured by electrochromic absorption changes at 515 nm [24] with a repetitive flash photometer as described earlier [25]. Simultaneously, the release of the adenine nucleotides was measured. The electric potential difference has been calculated using the relation $\Delta\Phi = (\Delta A_{515\ nm} / {}^{1}\Delta A_{515\ nm}) \cdot 50$ mV* [26]. The saturating light pulses of 30 ms duration induce a transmembrane electric potential of $\Delta\Phi = 180$–220 mV and a ΔpH of 0.8–1.2. ΔpH has been estimated from the quenching of the aminoacridine fluorescence [27] regarding the restrictions outlined elsewhere [28]. In the following we use the average values $\Delta\Phi \cong 200$ mV and ΔpH $\cong$ 1.0, i.e. the electrochemical potential of $\Delta\Phi$ and ΔpH corresponds to about 260 mV. The reaction medium contained $2 \cdot 10^{-2}$ M tricine adjusted to pH 8 with NaOH, $5 \cdot 10^{-3}$ M $MgCl_2$, 10^{-2} M KCl, $5 \cdot 10^{-3}$ M K_2HPO_4, 10^{-2} M sucrose, 10^{-4} M benzyl viologen, chloroplasts giving a chlorophyll concentration of $2 \cdot 10^{-4}$ M, and when indicated $3 \cdot 10^{-4}$ M ADP. The reaction volume was 1 ml.

For application of the external voltage pulses, the chloroplasts were suspended in a cuvette between two platinum electrodes (distance 2 mm, area 5 cm^2) as described earlier [22]. The external electric field was $1.1 \cdot 10^3$ V/cm, i.e. a voltage pulse of 220 V was applied (duration of the pulse 30 ms, time between the pulses 15 s). At this field strength a transmembrane electric potential difference of about 200 mV is induced (see Results). For these experiments, the following reaction medium was used: $5 \cdot 10^{-3}$ M tricine adjusted to pH 8 with NaOH, $5 \cdot 10^{-4}$ M $MgCl_2$, $5 \cdot 10^{-4}$ M K_2HPO_4, 10^{-4} M KCl, $5 \cdot 10^{-2}$ M sucrose, chloroplasts giving a chlorophyll concentration of $4 \cdot 10^{-4}$ M and when indicated $3 \cdot 10^{-4}$ M ADP. The experiments were performed in the dark at about 5 °C. After energization the chloroplasts and the corresponding dark control were centrifuged immediately (5 min, $5000 \times g$, 0 °C). 100 μl of the supernatant was counted in a scintillation counter giving the total amount of adenine nucleotides (AdN) released. The nucleotide separation was performed by thin-layer chromatography on polyethyleneimine-coated cellulose plates (Polygram CEL 300 PEI/UV). 100 μl of the supernatant was directly applied to the plate and 1.2 M LiCl was used as eluant. The nucleotides were identified by co-migration of standard non-radioactive nucleotide markers.

Phosphorylation was measured by the ^{32}P method [29]. In these measurements an additional 1 μCi ^{32}P was present in the reaction medium.

RESULTS

Table I shows the amount of [^{14}C]AdN incorporated into the thylakoid

* ${}^{1}\Delta A_{515nm}$ absorption change at 515 nm in a single turnover flash.

membrane after incubation under continuous illumination for 60 s. It has been shown elsewhere that this label is almost completely bound to CF_1 [15, 17]. Approx. $10.5 \cdot 10^{-4}$ AdN are bound per chlorophyll. This corresponds to about 0.9 AdN per CF_1 using a value of 1 CF_1 per 860 chlorophyll [30]. Denaturation by boiling or by addition of trichloroacetic acid results in a release of the label to the medium. Separation of the released nucleotides into AMP, ADP and ATP shows that all three adenine nucleotides are present, the greater part being ADP.

Table II (top) shows that saturating continuous light (30 s) causes a release of $6 \cdot 10^{-4}$ mol [^{14}C]AdN/mol chlorophyll to the suspension medium in the presence of ADP and P_i. Most of the label in the dark control results from some smaller thylakoids remaining in the supernatant after centrifugation. Only about 60 % of the incorporated label is released (see Table I) as observed also by other authors [17]. It may be suggested that this non-releasable label is bound to ATPases of thylakoids which have been damaged during the washings. Omission of ADP results in a smaller release ($3.6 \cdot 10^{-4}$ mol [^{14}C]AdN/mol chlorophyll). Table II (center) shows the effect of 20 and 5 light pulses (30 ms each, $\Delta\Phi \cong 200$ mV, ΔpH $\cong$ 1.0, see Materials and Methods). 20 light pulses cause practically the same release of the incorporated label as in continuous light. Also the stimulation of the release by ADP is similar as in continuous light. Table II (bottom) shows that five external voltage double pulses (30 ms each, $\Delta\phi \cong$ 200 mV, ΔpH = 0, see Materials and Methods) cause a release of $3.5 \cdot 10^{-4}$ mol [^{14}C] AdN/mol chlorophyll. The yield is comparable with that obtained with five light pulses. Also the stimulation of the release by ADP is similar to that observed after energization with light. It should be pointed out that 10 voltage pulses, i.e. five voltage double pulses correspond to five light pulses (see below). Separation of the nucleotides shows that in all experiments, AMP, ADP and ATP are released. In continuous light the part of [^{14}C]ATP is increased in the presence of ADP in accordance with earlier observations [17]. It has been shown that this is caused by a subsequent phosphorylation of [^{14}C]ADP released before [31]. On the other hand it was demonstrated that a small part of the bound ADP (about 20 %) may be converted directly to ATP [38]. In the pulse experiments with addition of ADP the increase of the part of [^{14}C]ATP is small. This may reflect the rather low total amount of ATP synthesized compared to that in continuous light of 30 s.

Fig. 1 shows the amount of adenine nucleotides released per chlorophyll as a

TABLE I

AMOUNT OF ADENINE NUCLEOTIDES INCORPORATED INTO THE MEMBRANE AND RELEASED ON DENATURATION

For details, see text.

Conditions	$\frac{10^{-4} \text{ mol AdN}}{\text{mol chlorophyll}}$	$\frac{\text{mol AdN}}{\text{mol } CF_1}$	AMP (%)	ADP (%)	ATP (%)
Label incorporated into the thylakoid membrane	10.5	0.9	–	–	–
Label released by boiling	10.0	0.86	33	41	26
Label released by denaturation with 40 % trichloroacetic acid	9.0	0.78	–	–	–

TABLE II

AMOUNT OF ADENINE NUCLEOTIDES RELEASED BY EXCITATION WITH CONTINUOUS ILLUMINATION, LIGHT PULSES AND EXTERNAL VOLTAGE PULSES

The values are the difference between the amount released after excitation minus the corresponding dark controls (average $1.1 \cdot 10^{-4}$ mol AdN/mol chlorophyll). For details, see text.

Conditions	Adenine nucleotides released				
	$\frac{10^{-4} \text{ mol AdN}}{\text{mol chlorophyll}}$	$\frac{\text{mol AdN}}{\text{mol } CF_1}$	AMP (%)	ADP (%)	ATP (%)
30 s illumination + ADP + P_i	6.0	0.52	13	32	55
30 s illumination − ADP + P_i	3.6	0.31	34	56	10
20 light pulses (30 ms) + ADP + P_i	5.2	0.45	27	44	29
20 light pulses (30 ms) − ADP + P_i	2.8	0.24	25	50	25
5 light pulses (30 ms) + ADP + P_i	4.8	0.41			
5 light pulses (30 ms) − ADP + P_i	1.3	0.11			
5 external electric double pulses (30 ms) + ADP + P_i	3.5	0.3	50	32	18
5 external electric double pulses (30 ms) − ADP + P_i	2.0	0.17	46	40	14

function of the number of light pulses in the presence and absence of exogeneous ADP. In the first light pulse only about 25 % of the releasable label is lost. The curve in the presence of ADP is calculated (see Discussion).

In Fig. 2 the amount of adenine nucleotides released per chlorophyll is depicted as function of the number of external voltage pulses in the presence and absence of exogeneous ADP. The curve in the presence of ADP is calculated (see Discussion). In order to facilitate the comparison with Fig. 1, in Fig. 2 the number of external voltage double pulses is depicted. This has been done because in an external electric field only in one half of a thylakoid is the "correct" polarity of the transmembrane potential difference applied, i.e. positive inside, negative outside. Furthermore, in this half $\Delta\Phi$ induced is maximal only in the area of the poles because $\Delta\Phi$ declines to zero at the equator [22]. Therefore, the effect of at least two external voltage pulses corresponds to one light pulse. Taking this fact into account the amount of AdN released by light pulses corresponds to that released by external voltage pulses. A difference is the larger amount of AdN released in the first external voltage pulses in the absence of ADP. Comparing Fig. 1 and Fig. 2 it must be noted that the number of external voltage pulses was restricted to 10 because after 10 pulses, i.e. five double pulses, a partial deactivation of the enzyme was observed.

Fig. 3 shows the relative amount of AdN released as a function of the light-induced transmembrane electric potential difference. In a single turnover flash (50 mV) practically no AdN is released. A non-linear increase of AdN released can be seen up to 200 mV.

Fig. 4 shows the relative amount of AdN released as a function of the transmembrane electric potential difference induced by an external electric field. The external field strength was increased up to $1.1 \cdot 10^3$ V/cm. The transmembrane electric potential difference, $\Delta\Phi$, is proportional to the external electric field strength [22]. The absolute value of $\Delta\Phi$ induced by $1.1 \cdot 10^3$ V/cm was estimated as follows.

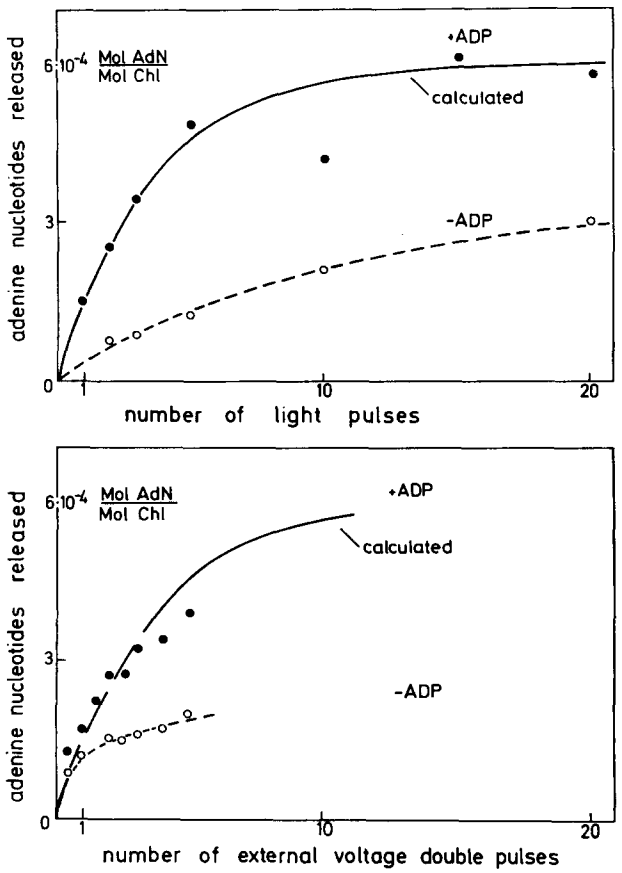

Fig. 1. Adenine nucleotides released per chlorophyll as a function of the number of saturating light pulses with and without exogeneous ADP. Pulse duration, 30 ms; time between the pulses, 10 s; induced transmembrane electric potential difference, $\Delta\Phi \cong 200$ mV; ΔpH $\cong 1.0$. For details, see text.

Fig. 2. Adenine nucleotides released per chlorophyll as function of the number of external voltage pulses with and without exogeneous ADP. Pulse duration, 30 ms; external field strength, 1100 V/cm; induced transmembrane electric potential difference, $\Delta\Phi \cong 200$ mV; time between the pulses 30 s. For details, see text.

The electrochemical potential induced by the light pulse corresponds to about 260 mV (see Materials and Methods). Because in one light pulse the amount of ATP generated as well as the amount of AdN released is comparable with the corresponding amounts generated by two external field pulses of $1.1 \cdot 10^3$ V/cm (see Table IV) we assume that this external field strength induces a $\Delta\Phi$ of the order of about 200 mV across the membrane (see scale on the top in Fig. 4). The dependence of the release of AdN on $\Delta\Phi$ induced by light (Fig. 3) is similar to that induced by the external voltage pulse (Fig. 4). It should be noted that the amount of ATP generated depends on $\Delta\Phi$ in a similar way (see below).

Table III shows the effect of uncouplers and ionophores on the relative release

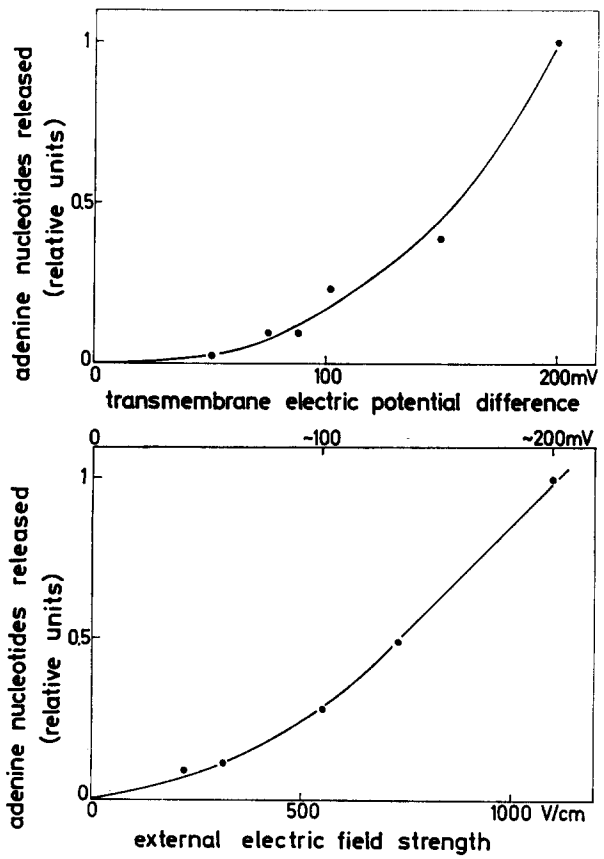

Fig. 3. Relative amount of adenine nucleotides released as function of the transmembrane electric potential difference induced by light pulses (three light pulses have been used). For details, see text.

Fig. 4. Relative amount of adenine nucleotides released as function of the transmembrane electric potential difference (scale on the top) induced by external voltage pulses (two external voltage double pulses have been used). The external field strength is indicated by the scale on the bottom. For details, see text.

TABLE III

INFLUENCE OF ADDED IONOPHORES (VALINOMYCIN (VMC) AND GRAMICIDIN D, (GmcD)) AND UNCOUPLERS (CARBONYLCYANIDE *p*-TRIFLUOROMETHOXYHYDRAZONE, FCCP) ON THE RELATIVE AMOUNT OF ADENINE NUCLEOTIDES RELEASED AND ON THE HALF LIFETIME OF THE ELECTRIC POTENTIAL DIFFERENCE

Light pulses of 10 ms duration have been used (dark time between pulses 30 s). Data for control, i.e. without additions. Adenine nucleotides released, $2.4 \cdot 10^{-4}$ mol AdN/mol chlorophyll; half lifetime of $\Delta\Phi$ of the control, 300 ms. For details, see text.

	Control	$5 \cdot 10^{-8}$ M VMC	10^{-10} M GmcD	10^{-6} M FCCP
Adenine nucleotides released	100 %	10 %	7 %	5 %
Half lifetime of electric potential decay	100 %	8 %	8 %	3 %

TABLE IV

AMOUNT OF ATP GENERATED AND AMOUNT OF AdN RELEASED IN LIGHT PULSES AND IN EXTERNAL VOLTAGE PULSES

For the measurement of the $\Delta\Phi$ dependence of ATP generated and AdN released in external voltage pulses (lower part) a different chloroplast preparation has been used. The accuracy of these data has been increased by measuring the dependence with one double pulse, two double pulses and five double pulses. For details, see text.

Conditions	ATP synthesized		AdN released	
	$\frac{10^{-4}\text{ mol ATP}}{\text{mol chlorophyll}}$	$\frac{\text{mol ATP}}{\text{mol } CF_1}$	$\frac{10^{-4}\text{ mol AdN}}{\text{mol chlorophyll}}$	$\frac{\text{mol AdN}}{\text{mol } CF_1}$
1 light pulse (30 ms, 200 mV)	12	1.0	1.5	0.13
5 light pulses (30 ms, 200 mV)	55	4.7	4.8	0.41
1 external voltage double pulse (30 ms, 1100 V/cm, $\cong$ 200 mV)	7.6	0.65	1.6	0.14
5 external voltage double pulses (30 ms, 1100 V/cm, $\cong$ 200 mV)	38	3.3	3.9	0.33
1 external voltage double pulse (30 ms, 1100 V/cm, $\cong$ 200 mV)	5.9	0.51	1.0	0.09
(30 ms, 730 V/cm, $\cong$ 130 mV)	3.7	0.32	0.52	0.05
(30 ms, 310 V/cm, $\cong$ 60 mV)	0.8	0.07	0.12	0.01

of AdN and on the half lifetime of the electric potential decay. The uncoupler, FCCP, increases the membrane permeability for protons, thereby quenching the pH gradient and accelerating the $\Delta\Phi$ decay from 300 to 10 ms. The ionophores, valinomycin and gramicidin, at the concentrations used increase the permeability for K^+, thereby accelerating only the $\Delta\Phi$ decay from 300 ms to about 25 ms. In parallel to this decrease also the amount of AdN released is decreased.

To compare pulse experiments with continuous light experiments, in Fig. 5 the relative amount of AdN released was measured as a function of illumination time. About 10–20 % of the total release occurs within 30 ms (time of the shortest light pulse used) and a slow release is observed which is half maximal at about 3 s. The slow phase of the release is in accordance with earlier observations [32] (see Discussion).

In Table IV the amount of ATP synthesized is compared with the amount of AdN released. In one light pulse of 30 ms duration ($\Delta\Phi \cong 200$ mV) about 1.0 ATP per CF_1 is generated but only 0.13 AdN per CF_1 is released. In one external voltage double pulse of 30 ms duration ($\Delta\Phi \cong 200$ mV) about 0.7 ATP per CF_1 is generated and only 0.14 AdN per CF_1 is released. Thus, there is a difference of a factor 8 or 5,

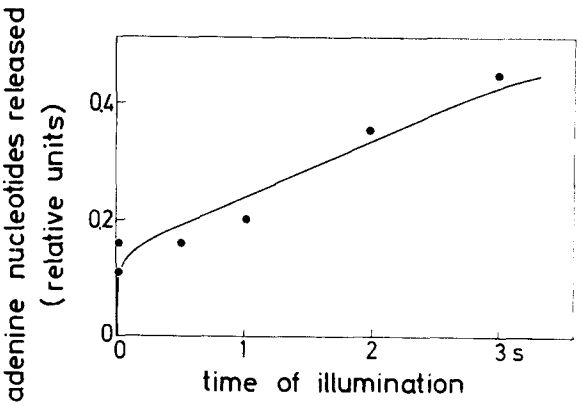

Fig. 5. Relative amount of AdN released in saturating continuous light as function of illumination time. Light duration was varied between 30 ms and 30 s with a mechanical shutter. Maximal release at saturation after 30 s: $4.4 \cdot 10^{-4}$ mol AdN/mol chlorophyll. For details, see text.

respectively. In five light pulses as well as in five external voltage double pulses about 3–5 ATP per CF_1 are generated whereas there occurs only about 70 % of the maximal release. This discrepancy is discussed below.

The amount of ATP generated and the amount of AdN released in one external voltage double pulse (30 ms) is shown also at different external field strength and at different $\Delta\Phi$ values, respectively (see Table IV, bottom). It can be seen that the amount of ATP generated is about a factor 6–7 higher than the amount of AdN released, i.e. the ratio ATP/AdN is approximately independent of $\Delta\Phi$.

It should be noted that the absolute values given for AdN release, ATP formation etc. can deviate in different preparations by about ± 30 %. The scattering of data within the same chloroplast preparation is about 15 %.

DISCUSSION

The existence of tightly bound nucleotides which can be released on energization of the membrane (by $\Delta pH + \Delta\Phi$ or ΔpH alone (acid-base transition)) has been demonstrated elsewhere [15–18]. In these experiments the amount of bound AdN ranges from about 0.4 to about 3 AdN/CF_1 [15–18, 34]. The distribution of the tightly bound nucleotides (AMP, ADP, ATP) shows also differences. Without discussing the differences, the correct value may be important for conclusions on the detailed mechanism of phosphorylation, we use the release of AdN as an indication that there are changes of the affinities of the nucleotides to CF_1. In accordance with other authors we assume that these changes of affinities reflect conformational changes in CF_1 [15–18, 34]. We assume furthermore that the number of AdN molecules released is proportional to the number of coupling factors which have changed their conformation. These are characterized in the following as "active" CF_1. Because in our experiments about 1 AdN is bound per CF_1 (see Table I) we can identify in our calculation the number of active CF_1 with the number of AdN released provided that the bound AdN molecules are distributed uniformly among all CF_1. We do not consider differences between the release of AMP, ADP or ATP.

Relation between the release of AdN and $\Delta\Phi$

In this work it is shown that the tightly bound nucleotides can be released from CF_1 by the action of an electric field across the thylakoid membrane. This is demonstrated by two types of experiments.

(1) A release of AdN is observed in light pulses under conditions where ΔpH is much smaller (ΔpH $\cong$ 1.0) than $\Delta\Phi$ ($\Delta\Phi \cong 200$ mV) compared to conditions of saturating continuous light (ΔpH $\cong$ 3.0, $\Delta\Phi = 100$ mV). Dissipation of the energy of $\Delta\Phi$ only through addition of valinomycin or gramicidin results in a strong inhibition of the release of AdN. Addition of FCCP which dissipates the energy of $\Delta\Phi$ and of ΔpH gives the same effect (Table III). These results show that the release of AdN under these conditions is preferentially caused by the electric potential difference.

(2) A release of AdN is observed after applying external voltage pulses to the chloroplast suspension in the dark. Under these conditions it is $\Delta\Phi \cong 200$ mV and ΔpH between the inner and outer thylakoid space is zero.

From these results it can be concluded: (a) The conformational change can be caused by a transmembrane $\Delta\Phi$ only. The mechanism of the interaction of the field with CF_1 is not known. One possibility may be an indirect action of the electric field by producing a local proton gradient across CF_1. However, also a direct interaction of the field with dipoles on the ATPase could be discussed. (b) The conformational change is not caused directly by the light-induced electron transport because a release of AdN is observed also in the dark in an external voltage pulse (i.e. without an electron transport from water to $NADP^+$) as may be concluded also from acid-base transition experiments [34]. (c) The conformational change is not caused only by an increase of H^+ concentration inside the thylakoids (see Introduction) because a release occurs also in an external voltage pulse, i.e. without an increase of the H^+ concentration in the inner thylakoid space.

Obviously, the conformational change can be caused by energy stored in $\Delta\Phi$ or ΔpH or $\Delta\Phi$ and ΔpH.

Fig. 3 and Fig. 4 show a non-linear dependence of the release of AdN on the magnitude of the transmembrane electric potential difference. Comparing the results of both experiments it must be mentioned that in the light pulse experiment the ΔpH changes by about 0.6 unit in the range from $\Delta\Phi = 50$ mV to 200 mV. This implies that the energetic contribution of ΔpH changes by 35 mV and that of $\Delta\Phi$ by 150 mV. Therefore, the curve in Fig. 3 reflects mainly the influence of $\Delta\Phi$ on the release of AdN. Fig. 4 shows the influence of $\Delta\Phi$ only because in this case ΔpH is zero. Because the number of [^{14}C]AdN molecules released is assumed to indicate the number of "active" ATPases (see above) it follows that the relative number, η, of ATPases which have carried out a conformational change is a function of the magnitude of $\Delta\Phi$: $\eta = N/N_{max} = f(\Delta\Phi)$. N = number of "active" ATPases, N_{max} = total number of ATPases. We assume that only these "active" ATPases are able to generate ATP. It should be noted that the ATP molecule formed may not be identical with the [^{14}C] ATP molecule released. The place for ATP synthesis may be different from the observed "tight binding site".

The effect of exogeneous ADP

Figs. 1 and 2 show a different release of tightly bound [^{14}C]nucleotides with and without exogeneous unlabelled ADP. Similar results have been reported in

continuous light [17]. It may be assumed that energetization of the membrane allows equilibration of the enzyme, AdN and the enzyme · AdN complex. This assumption which does not take into account the breakdown of the enzyme · AdN · P_i complex to enzyme and products seems to be justified because the greater part of the nucleotides released is ADP (see Tables I and II). (The increased part of [^{14}C]ATP in continuous illumination with added exogenous ADP is caused by phosphorylation of [^{14}C]ADP released before [31]). Under these assumptions the different release can be explained in the following simplified way:

(1) With exogenous ADP the release reflects an exchange of labelled with unlabelled ADP. The slope at the curve shows that the amount of AdN released per pulse decreases with increasing number of pulses. If we assume that in each pulse a constant fraction, η, of CF_1 is activated it follows that the amount of labelled AdN released in the nth pulse is $\eta \cdot N_{max} \cdot (1-\eta)^{n-1}$ if ADP $\gg$ [^{14}C]ADP. The curves in Fig. 1 and Fig. 2 are calculated using this equation with $\eta = 0.25$ and $N_{max} = 6 \cdot 10^{-4}$ mol AdN/mol chlorophyll. (The remaining label on the membrane at equilibrium is determined by the ratio of [^{14}C]ADP to unlabelled ADP, provided that all ATPases can be energized, i.e. no thylakoids are damaged during the extensive washings).

(2) In the absence of exogenous ADP the observed release reflects the dissociation of the enzyme · [^{14}C]AdN complex and the maximal release is determined by the equilibrium concentrations. The shape of the curve cannot be calculated as outlined before. In the absence of endogenous ADP there is practically no rebinding of ADP to CF_1 because of the low [^{14}C]ADP concentration [35]. Therefore, the time for equilibration is much longer than in presence of exogenous ADP. Equilibrium is obtained after illumination of about 5–10 min. From such an experiment the magnitude of the dissociation constant can be estimated and is in fair accordance with data published recently [35].

Relation between the release of tightly bound nucleotides and ATP synthesis

It is shown in Table IV that in one pulse of 30 ms duration about 5–10 times more ATP per CF_1 is generated than AdN per CF_1 is released. This result is practically independent of $\Delta\Phi$. Such a discrepancy between phosphorylation and AdN release can be read out also from experiments in continuous light but has not been discussed [32]: about 40 % (0.16 AdN/CF_1) of the maximal release occurs within about 2 s. The amount of ATP generated within this time is, however, about 25 ATP/CF_1. The greater difference observed in ref. 32 is not surprising: this reflects only the fact that the amount of labelled AdN runs into saturation whereas the amount of ATP increases, of course, linearly with time. It has been reported that the release of adenine nucleotides and phosphorylation depends in the same way on different parameters, i.e. on light intensity, on pH_{out}, on various inhibitors [32] and on the electric potential difference (this work). Therefore, it may be assumed that the conformational change observed here is involved in the mechanism of phosphorylation and is not a side reaction of energization. Under this assumption the outlined discrepancy can be explained by the following hypothesis.

A hypothetical model

If the release of one AdN indicates one "active" ATPase, we can conclude from our data that in each light or voltage pulse only a fraction of about 10–20 % of the

ATPase are "working". The 5–8 times higher amount of ATP synthesized can be explained if one assumes that each active ATPase carries out 5–8 turnovers in one pulse. The turnover time τ of the ATPase can be calculated from these data (Table IV) and the time of phosphorylation. In an external voltage pulse the time of energization and phosphorylation is identical with the pulse duration (30 ms) because practically no transmembrane gradients of ions are generated. In a light pulse the time of energization and phosphorylation can be estimated by considering the rise time of $\Delta\Phi$ and the half lifetime of the fast phase of $\Delta\Phi$ decay. (Only the fast phase of $\Delta\Phi$ decay is coupled to phosphorylation [36].) Under these conditions the time for phosphorylation is about 40 ms. It results in both cases a turnover time of about 5 ms. Because the ratio ATP/AdN is within the margin of error independent of $\Delta\Phi$ (see Table IV), it follows, furthermore, that τ is independent of $\Delta\Phi$.

In saturating continuous light (linear electron transport) the half maximal release occurs within about 1–3 s and a complete release is observed after about 10–30 s [32]. To compare pulse experiments with continuous light experiments the time course of the AdN release in the presence of ADP is shown in Fig. 5. A fast and a slow phase can be seen. The complete release after $\geqslant$ 10 s indicates that all CF_1 have been activated within this time. The shape of the curve can be explained if we assume that also in continuous light the complete work of ATP synthesis is carried out only by a fraction of CF_1 (10–20 %) working at their maximal rate but that this "active" fraction migrates statistically between all ATPases within about 1–3 s. With these assumptions the level of the break between the two phases in Fig. 5 indicates the "active" fraction, the slow phase indicates the migration of this state.

The consequences of this mechanism are in accordance with data obtained from independent experiments: (1) About the same turnover time has been measured directly (Schlodder, E. and Witt, H. T., unpublished results); (2) The rate of ATP synthesis in the pulse experiments is about $25 \cdot 10^{-3}$–$30 \cdot 10^{-3}$ mol ATP/mol chlorophyll per s, which is practically identical with the rate of phosphorylation in saturating continuous light and linear electron transport conditions at $pH_{out} = 8$ ($30 \cdot 10^{-3}$ mol ATP/mol chlorophyll per s). If this rate is obtained with only 10–20 % active ATPase, it must be concluded that under conditions where all ATPases are activated the rate of phosphorylation is expected to be about a factor 5–10 higher than our rate, i.e. about $150 \cdot 10^{-3}$–$300 \cdot 10^{-3}$ mol ATP/mol chlorophyll per s. Indeed, such rates of phosphorylation have been reported [29] if conditions for a cyclic electron transport are arranged ($375 \cdot 10^{-3}$ mol ATP/mol chlorophyll per s). (3) The fraction of active ATPases is a function of $\Delta\Phi$ (Fig. 3 and Fig. 4) and as it may be assumed also a function of ΔpH. Because of this dependency the higher rates of phosphorylation in a cyclic electron transport system results from a higher energization of the membrane. From the functional dependence of the rate of phosphorylation on $\Delta\Phi$ and ΔpH [28, 33] the energetization required for maximal rates can be estimated by extrapolation and should correspond to about 300 mV. This value represents the lower limit because this estimation does not regard that the curve must run into saturation, i.e. the turnover time becomes rate limiting. The fraction, η, of "active" ATPases as function of $\Delta\Phi$ is depicted schematically in Fig. 6. Under conditions of maximal phosphorylation, i.e. $\eta = 1$, it is expected that already in the first turnover all AdN is released and that this number is identical with the amount of ATP generated. Indeed, this has been demonstrated recently [34].

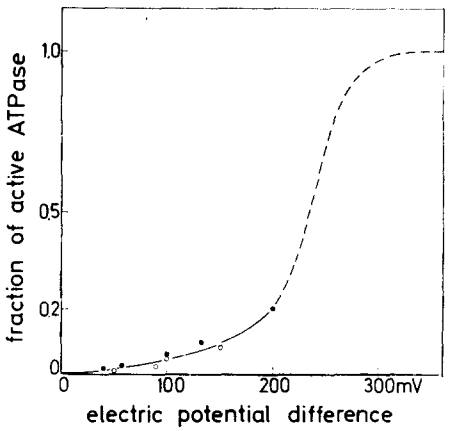

Fig. 6. Dependence of the fraction of active ATPases on $\Delta\Phi$. —, measurements according to Fig. 4 and Fig. 5; – – – –, assumption according to the hypothetical model (see Discussion).

The essential features of the outlined model can be summarized as follows: (1) The conformational changes of CF_1 observed is an event coupled with phosphorylation. Our results do not allow to distinguish between a conformational change as a catalytic step (i.e. without transmitting energy) or as energy transmitting step. (2) Under conditions of non-maximal energization only a fraction, $\eta = N/N_{max}$ of the total number, N_{max}, of ATPases is active (N = number of "active" ATPases). The fraction changes statistically between all ATPases within about 3 s. (3) The fraction, η, is a function of $\Delta\Phi$ (and ΔpH). For the activation of all ATPases about 300 mV are required. (4) The turnover time, τ, of the active ATPase is about 5 ms, and approximately independent of $\Delta\Phi$ (see Table IV) (and ΔpH).

A model of an "electrically gated ATPase" has been discussed by Junge [37] to account for threshold effects of ATP synthesis in flash light at low $\Delta\Phi$. The strong increase of phosphorylation with $\Delta\Phi$ above this range was explained by the decrease of the turnover time τ [37]. This is different from the results and conclusions presented in this work.

It was shown elsewhere that there exists a proton flux via a basal pathway, $\overline{\dot{H}_b{}^+}$, and one via the ATPase pathway, $\overline{\dot{H}_p{}^+}$. The overall rate of phosphorylation is $\overline{A\dot{T}P} = \overline{\dot{H}_p{}^+}/n$ (n = number of protons translocated via the ATPase pathway per ATP generated). Each flux, $\overline{\dot{H}_p{}^+}$ and $\overline{\dot{H}_b{}^+}$, depends in a different way on $\Delta\Phi$ and ΔpH [24]. The competition between both fluxes explains the observed variation in the H^+/ATP ratio as a function of $\Delta\Phi$ and ΔpH.

It may be asked in which way the $\overline{\dot{H}_p{}^+}$ flux is controlled as a function of $\Delta\Phi$ and ΔpH. $\overline{\dot{H}_p{}^+}$ and the rate of ATP synthesis can be written as follows:

$$\overline{\dot{H}_p^+}/n = \overline{A\dot{T}P} = N_{max} \cdot \frac{\eta}{\tau} = f(\Delta\Phi, \Delta pH)$$

n, number of protons translocated via the ATPase per ATP generated; N_{max}, total number of ATPases, η, "active" fraction of ATPases; τ, turnover time of the ATPase.

Two extreme cases can be discussed. (1) The proton flux, $\overline{\dot{H}_p{}^+}$, is distributed between all ATPases. One ATP is generated when n protons have passed the ATPase. Thus, the number of the "active" ATPases is constant and identical with the total number, N_{max} of ATPases, whereas the turnover time, τ, of each ATPase depends on $\Delta\Phi$ and ΔpH, i.e. $\tau = f(\Delta\Phi, \Delta pH)$. The results and the discrepancy between the number of ATP generated per pulse and the number of "active" ATPases observed in this work is, however, in contradiction to this mechanism and can only be explained by the outlined concept which is the other extreme. (2) The proton flux, $\overline{\dot{H}_p{}^+}$, is focussed only to the active fraction, η, of the ATPases. η is of such a size that each of these ATPases can work at the maximal rate. Thus, the turnover time, τ, of each ATPase is constant. The fraction, η, of "active" ATPases depends on $\Delta\Phi$ (and ΔpH), i.e. $\eta = f(\Delta\Phi, \Delta pH)$. The fraction changes statistically between all ATPases.(A slight dependence of τ on $\Delta\Phi$, about a factor 2 in the range of 60–200 mV, cannot be ruled out as yet).

In mechanism 1 the proton flux per ATPase changes with $\Delta\Phi$ and ΔpH whereas in mechanism 2 the proton flux per "active" ATPase is always constant and maximal. One advantage of mechanism 2 is the following. The time for gathering n protons in order to synthesize one ATP is minimal and constant and thereby independent on the light intensity. This is not the case in mechanism 1 and, therefore, gives rise to problems especially at low intensities. If the second concept is correct it indicates the possibility for the cooperation of all electron transfer chains with only a fraction of the ATPases. This is a further property which supports the chemiosmotic hypothesis.

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REFERENCES

1 Avron, M. (1963) Biochim. Biophys. Acta 77, 699–702
2 Vambutas, V. K. and Racker, E. (1965) J. Biol. Chem. 240, 2660–2667
3 McCarty, R. E. and Racker, E. (1966) Brookhaven Symp. Biol. 19, 202–214
4 Mitchell, P. (1966) Biol. Rev. 41, 445–502
5 Jagendorf, A. T. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 413–492, Academic Press, New York
6 Witt, H. T. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 493–554, Academic Press, New York
7 Mitchell, P. (1974) FEBS Lett. 43, 189–194
8 Boyer, P. D. (1975) FEBS Lett. 58, 1–6
9 Boyer, P. D., Cross, R. L. and Momsen, W. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2837–2839
10 Slater, E. C. (1974) in Dynamics of Energy Transducing Membranes (Ernster, L., et al., eds.), Vol. 13, pp. 1–20, Elsevier Press, Amsterdam
11 Ryrie, I. J. and Jagendorf, A. T. (1971) J. Biol. Chem. 246, 3771–3774
12 Ryrie, I. J. and Jagendorf, A. T. (1972) J. Biol. Chem. 247, 4453–4459
13 Magnusson, R. P. and McCarty, R. E. (1975) J. Biol. Chem. 250, 2593–2598

14 Kraayenhof, R. and Slater, E. C. (1975) in Proceedings of the 3rd International Congress on Photosynthesis, Rehovot/Israel 1974 (Avron, M., ed.), pp. 985–996, Elsevier, Amsterdam
15 Harris, D. A. and Slater, E. C. (1975) Biochim. Biophys. Acta 387, 335–348
16 Boyer, P. D., Smith, D. G., Rosing, J. and Kayalar, C. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E. et al., eds.), p. 361–372, North-Holland Publ. C., Amsterdam
17 Strotmann, H., Bickel, S. and Huchzermeyer, B. (1976) FEBS Lett. 61, 194–198
18 Magnusson, R. P. and McCarty, R. E. (1976) Biochem. Biophys. Res. Commun. 70, 1283–1289
19 Bachofen, R., Beyeler, W. and Pflugshaupt, C. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E. et al., eds.), pp. 167–172, North Holland Publ. Co., Amsterdam
20 Rumberg, B. and Siggel, U. (1969) Naturwissenschaften 56, 130–132
21 Gräber, P. and Witt, H. T. (1974) Biochim. Biophys. Acta 333, 383–392
22 Witt, H. T., Schlodder, E. and Gräber, P. (1976) FEBS Lett. 69, 272–276
23 Winget, G. D., Izawa, S. and Good, M. E. (1965) Biophys. Res. Commun. 21, 438–443
24 Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 244–254
25 Rüppel, H. and Witt, H. T. (1969) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.), Vol. 16, pp. 316–380, Academic Press, New York
26 Schliephake, W., Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 1561–1578
27 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 54–63
28 Gräber, P. and Witt, H. T. (1976) Biochim. Biophys. Acta 423, 141–163
29 Avron, M. (1960) Biochim. Biophys. Acta 40, 257–272
30 Strotmann, H., Hesse, H. and Edelmann, K. (1973) Biochim. Biophys. Acta 314, 202–210
31 Rosing, J., Kayalar, C. and Boyer, P. D. (1976) (Smith, D. J., Rosing, J. and Boyer, P. D., eds.), Xth International Congress of Biochemistry, Hamburg, 1976
32 Bickel-Sandkötter, S. and Strotmann, H. (1976) FEBS Lett. 65, 102–106
33 Portis, A. R. and McCarty, R. E. (1974) J. Biol. Chem. 249, 6250–6254
34 Smith, D. J. and Boyer, P. D. (1976) Proc. Natl. Acad. Sci. U.S. 73, 4314–4318
35 Strotmann, H. and Bickel-Sandkötter, S. (1977) Biochim. Biophys. Acta 460, 126–135
36 Junge, W., Rumberg, B. and Schröder, H. (1970) Eur. J. Biochem. 14, 575–581
37 Junge, W. (1972) Proceedings of the 2nd Internatioanl Congress on Photosynthesis Research, Stresa 1971 (Forti, G., Avron, M. and Melandri, A., eds.), pp. 1065–1074
38 Magnusson, R. P. and McCarty, R. E. (1976) J. Biol. Chem. 251, 7417–7422