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PHOSPHORYLATION OF 1,N6-ETHENO-ADP IN FLASH GROUPS AND THE CONCOMITANT DECAY KINETICS OF THE ABSORPTION CHANGE AT 515 nm

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SUMMARY: The accelerated decay of the transmembrane electric field under phosphorylating conditions as well as the concomitant generation of [^{32}P]-ATP after excitation with flash groups has been measured. An acceleration of the decay and a corresponding synthesis of ATP is found on addition of ADP and 1,N6-etheno-ADP (ε-ADP). Both effects are smaller in the presence of optimal concentrations of ε-ADP than in the presence of an optimal ADP concentration. A quantitative evaluation of H^+/ATP ratio under comparable conditions gives values of 3.3 for ADP and 3.5 for ε-ADP. The results are consistent with the interpretation that the accelerated decay of the transmembrane field is caused directly by a proton flux via the ATPase.

INTRODUCTION: The decay of the electrochromic absorption changes (measured e.g. at 515 nm) is caused by the flux of ions across the thylakoid membrane (1,2). After excitation with ms-flashes or flash groups of single turnover flashes, the decay of the absorption change at 515 nm is accelerated under phosphorylating conditions (3,4). This has been interpreted in the following way: under phosphorylating conditions there is, parallel to the basal flux of ions (e.g. K^+ or Cl^-) a flux of protons via the ATPase which is linked to ATP synthesis (3,4,5).

However, two results have been reported which are not consistent with this interpretation (6):

1. the acceleration of the decay of the 515 nm absorption change is observed also on addition of ATP;

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2. on addition of ε-ADP no acceleration is observed but, on the other hand, ε-ADP can be phosphorylated.

It has been suggested, therefore, that the accelerated decay of the 515 nm absorption change was not directly linked to the phosphorylating reaction but reflects a modification of the membrane permeability by the newly synthesized ATP (6). However, the effect of ATP may be explained by a binding of ATP to the enzymatic centrum with a corresponding equilibrium between bound ATP, ADP and P_i. Energization may then lead to resynthesis of ATP. Therefore, the observed effect of ATP on the decay of the 515 nm absorption change may be explained by a proton flux directly linked with the resynthesis of ATP as proposed earlier (3,5,7).

On the other hand, synthesis of ε-ATP from ε-ADP without the occurrence of an accelerated 515 nm decay is in direct contradiction with the interpretation of this effect as a phosphorylating proton flux. The experimental basis of this conclusion, however, was a comparison of the rate of phosphorylation of ε-ADP in continuous light with the decay kinetics of the 515 nm absorption change after a flash group (6). In view of the mechanistic importance of this conclusion, in this work the synthesis of ε-ATP and the kinetics of the electrochromic absorption change after excitation with a flash group have been both measured in the same experiment.

MATERIALS AND METHODS: Spinach chloroplasts were prepared as described elsewhere (8) and used within two hours after preparation. The reaction medium contained 20 mM tricine adjusted to pH 8 with NaOH, 10 mM KCl, 5 mM $MgCl_2$, 1 mM P_i, 10 μCi/ml ^{32}P, 20 mM sucrose, 0.1 mM benzylviologen, chloroplasts giving a chlorophyll concentration of 0.4 mM and ADP or ε-ADP as indicated in the figures.

Illumination of 1 ml of this suspension was carried out in an optical cell (optical pathlength 1.4 mm) either by continuous light (wavelength>610 nm, intensity 0.1 W/cm^2) for 30 s or by flash groups (wavelength>610 nm). Each group consists of 5 saturating single turnover flashes (darktime between the flashes 2 ms, between the flash groups 10 s). Between 10 and 30 flash groups were fired in order to get an accumulation of sufficient ATP^{32}.

Simultaneously, the absorption change at 515 nm was measured in a flash spectrophotometer (9) and the signals from each flash group were added in an averager in order to increase the signal to noise ratio. After illumination the chloroplasts were denatured by addition of 0.3 ml 30% TCA.

The assay for ATP^{32} was carried out in the following way, similar to that described elsewhere (10): to 0.5 ml of the supernatant of the denatured chloroplasts, 0.5 ml of a freshly prepared ammoniummolybdate solution was added (50 ml ammoniummolybdate solution contained: 1.5 g $(NH_4)_6Mo_7O_2 \cdot 4\ H_2O$, 2.5 ml conc. HCl and 0.5 ml triethylamine). The mixture was allowed to react for 15 min. at room temperature and then centrifuged at 5000 g for 15 min. The radioactivity remaining in the supernatant (which corresponds to the amount of ATP^{32}) was determined in a scintillation counter, Packard Tri-carb) using the Cerenkov-radiation (0.5 ml supernatant and 9.5 ml H_2O). The corresponding dark control was handled in exactly the same way except that it was not illuminated. The total amount of radioactivity was determined in the same way except that instead of ammoniummolybdate solution, 0.5 ml of H_2O was added and that from the resulting solution only 20 µl were used for counting.

Experiments in which the results were also determined by the usual isobutanol-benzol-extraction method (11) and which we have used in former experiments (12), 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 number of chlorophyll molecules per electron transport chain was measured by the oxygen yield per flash as described elsewhere (13). A value of 700 Chl per electron transport chain was obtained for the preparations used.

RESULTS: First, the rate of phosphorylation in saturating continuous light was measured as a function of the concentration of ADP and ε-ADP. In accordance with earlier results (14), the half maximal rate was obtained at $5 \cdot 10^{-5}$ M for ADP and $1.1 \cdot 10^{-4}$ M for ε-ADP. The maximal rates were $40 \cdot 10^{-3}$ ATP/Chl · s and $15 \cdot 10^{-3}$ ε-ATP/Chl · s under our conditions (see Materials and Methods). The following flash experiments were carried out where the optimal rates had been determined in continuous light.

Fig. 1 shows the flash-induced absorption changes at 515 nm under different conditions. An accelerated decay was observed on addition of ADP and ATP. A smaller effect was also obtained on addition of ε-ADP. In order to obtain the maximal effect, a saturating concentration for the acceleration effect was used in each case. The amount of ATP generated in the same experiment

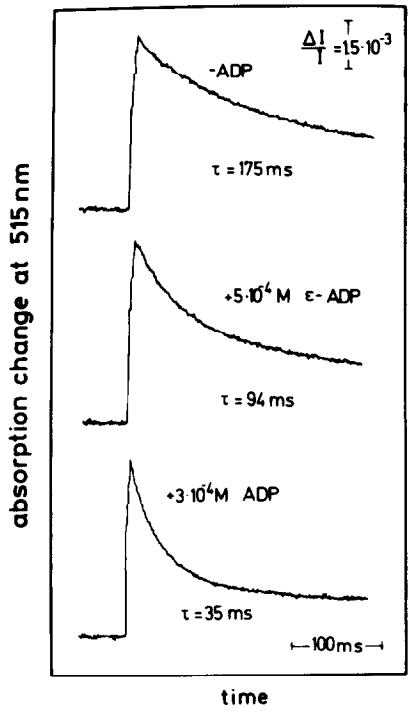

Fig. 1. Kinetics of the transmembrane electric potential difference as measured by the absorption change at 515 nm after excitation with a flash group. Reaction medium as described in Materials and Methods. Top: without adenine nucleotides. Center: $+5 \cdot 10^{-4}$M ε-ADP. Bottom: $+3 \cdot 10^{-4}$ ADP.

was shown in Table I. Without addition of any nucleotide, a small amount of ATP was found which results from endogeneous nucleotides. The effect observed with ε-ADP was about half of that seen in the presence of ADP. This may be already expected from the data obtained in continuous light (see above).

Table I - ATP yield per flash group as measured by the incorporation of ^{32}P. Reaction medium as described in Materials and Methods with addition of different adenine nucleotides.

Addition	-	$3 \cdot 10^{-4}$M ADP	$5 \cdot 10^{-4}$M ε-ADP
$\frac{\text{ATP}}{\text{Chl} \cdot \text{flash}} \cdot 10^4$	1.7	14.0	7.5

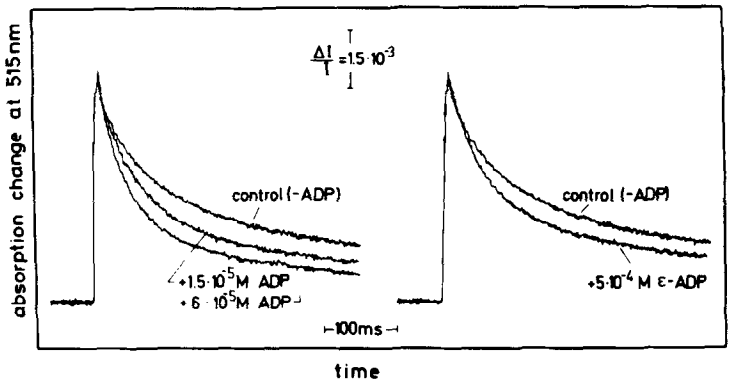

Fig. 2. Kinetics of the transmembrane electric potential difference as measured by the absorption change at 515 nm after excitation with a flash group. Reaction medium as described in Materials and Methods. Right: effect of addition of $5 \cdot 10^{-4}$M ε-ADP. Left: effect of addition of different concentrations of ADP.

To compare the decay kinetics directly with the addition of either ADP or ε-ADP, one must use conditions in which comparable amounts of ATP or ε-ATP are generated. In Fig. 2a, an experiment with increasing concentration of ADP is depicted, and it can be seen that the amplitude of the fast phase of the decay kinetics and its rate constant increase with increasing amounts of ADP. Fig. 2b shows a similar experiment with ε-ADP. Comparison of the kinetics in the presence of ε-ADP ($5 \cdot 10^{-4}$M) with that in the presence of a series of different ADP concentrations show that practically identical kinetics are observed in the presence of $1.5 \cdot 10^{-5}$ M ADP.

Table II shows the amount of ATP generated under the different conditions. Practically the same amount of ATP was found in the presence of $1.5 \cdot 10^{-5}$ M ADP as ε-ATP in the presence of $5 \cdot 10^{-4}$ M ε-ADP. For a quantitative comparison between the accelerated decay of the 515 nm absorption change and the amount of ATP generated, the amount of protons translocated via the phosphorylating pathway,

Table II - ATP yield per flash group and electron transport chain as measured by the incorporation of ^{32}P, amount of protons via the phosphorylating pathway calculated as described in the text, and ΔH^+_p/ATP at different ADP concentrations and optimal ε-ADP concentration.

Addition	-	$3 \cdot 10^{-4}$M ADP	$6 \cdot 10^{-5}$M ADP	$1.5 \cdot 10^{-5}$M ADP	$5 \cdot 10^{-4}$ ε-ADP
$\frac{\text{ATP}}{\text{flash e-chain}}$	0.05	0.6	0.47	0.24	0.23
$\frac{\Delta H^+_p}{\text{flash e-chain}}$	-	1.38	1.30	0.63	0.63
ΔH^+_p/ATP	-	2.5	3.1	3.3	3.5

ΔH^+_p, was calculated similarly as described elsewhere (4). The decay kinetics is due to a basal flux of ions $(\frac{d \Delta A}{dt})_{basal}$ (which is observed under non-phosphorylating conditions) and a parallel flux of phosphorylating protons $(\frac{d \Delta A}{dt})_{phosph}$

$$(\frac{d \Delta A}{dt})_{tot} = (\frac{d \Delta A}{dt})_{phosph} + (\frac{d \Delta A}{dt})_{basal}$$

The total amplitude due to the phosphorylating proton flux is

$$\Delta A_{H^+_p} = \int_0^\infty \left\{ (\frac{d \Delta A}{dt})_{total} - (\frac{d \Delta A}{dt})_{basal} \right\} dt$$

Using the calibration that the amplitude observed in a single turnover flash, $^1\Delta A$, corresponds to two charges translocated via the membrane per electron transport chain (15,16), ΔH^+_p is calculated:

$$\Delta H^+_p = 2 \frac{\Delta A_{H^+_p}}{^1\Delta A}$$

The slopes $(\frac{d \Delta A}{dt})_{total}$ and $(\frac{d \Delta A}{dt})_{basal}$ are obtained by graphical differentiation of the curves in Figs. 2a and 2b, plotting the difference as a function of time and by integration of the resulting curve graphically. ΔH^+_p is given in Table II. Before calculating the ΔH^+_p/ATP ratio, the amount of ATP^{32} generated in the absence of added nucleotides was subtracted. The error in the determination of H^+_p/ATP is estimated to be about 0.7 so that within the error limits the same H^+_p/ATP ratio is found for ADP and ε-ADP.

DISCUSSION: The results show that an acceleration of the decay of the absorption change at 515 nm after excitation with flash groups can be observed on addition of ε-ADP, under phosphorylating conditions (Fig. 1). The observed acceleration is small compared to that which occurs on addition of ADP (Fig. 1). However, the rate of phosphorylation in continuous light was also found to be about a factor of 2.5 smaller. Similarly, the ATP yield obtained from flash groups was a factor of about 2-3 smaller (Table I and II). After adjusting the ADP concentration in such a way that the same amount of ATP or ε-ATP was generated, the same acceleration was observed (Fig. 2).

A quantitative evaluation of the additional amount of charges (which are assumed to be protons) translocated during the accelerated decay shows that ΔH^+_p/ATP is practically identical for ADP and ε-ADP. It can be concluded that the results obtained with ε-ADP are consistent with the interpretation that the accelerated decay of the transmembrane field under phosphorylating conditions reflects directly a proton flux via the ATPase.

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1. Junge, W. and Witt, H.T. (1968) Z. Naturforsch., 23b, 244-254.
2. Witt, H.T. (1979) Biochim. Biophys. Acta, 505, 355-427.
3. Rumberg, B. and Siggel, U. (1968) Z. Naturforsch., 23b, 239-254.
4. Junge, W., Rumberg, B. and Schröder, H. (1970) Eur. J. Biochem., 14, 575-581.
5. Junge, W. (1970) Eur. J. Biochem., 14, 582-592.
6. Girault, G. and Galmiche, J.M. (1976) Biochem. Biophys. Res. Comm., 68, 724-729.
7. Saphon, S., Jackson, J.B. and Witt, H.T. (1975) Biochim. Biophys. Acta, 408, 67-82.
8. Winget, G.D., Izawa, S. and Good, N.E. (1965) Biochem. Biophys. Res. Comm., 21, 438-443.
9. Rüppel, H. and Witt, H.T. (1970) Methods Enzymol., 16, 316-380.
10. Sugino, Y. and Nigoshi, Y. (1974) J. Biol. Chem., 239, 2360-2364.
11. Avron, M. (1960) Biochim. Biophys. Acta, 40, 257-272.

12. Gräber, P. and Witt, H.T. (1976) Biochim. Biophys. Acta, 423, 141-163.
13. Renger, G. (1972) Biochim. Biophys. Acta, 256, 428-439.
14. Shahak, Y., Chipman, D.M. and Shavit, N. (1974), Proc. 2nd Int. Congr. Photosynth., Rehovot, (Avron, M., ed.) 859-866, Elsevier, Amsterdam, the Netherlands.
15. Schliephake, W., Junge, W. and Witt, H.T. (1968) Z. Naturforsch., 23b, 1571-1578.
16. Tiemann, R., Renger, G., Gräber, P. and Witt, H.T. (1979) Biochim. Biophys. Acta, 546, 498-519.