Biochimica et Biophysica Acta, 292 (1973) 808–814

BBA 46519

THE ENERGY LEVEL ASSOCIATED WITH THE LIGHT-TRIGGERED Mg^{2+}-DEPENDENT ATPase IN SPINACH CHLOROPLASTS

TILLY BAKKER-GRUNWALD and KAREL VAN DAM

Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)*

(Received December 7th, 1972)

SUMMARY

Light-induced Mg^{2+}-ATPase activity of chloroplasts and the pH difference (ΔpH) across the thylakoid membrane maintained by this activity are measured simultaneously under varying conditions of preillumination time and dark decay time. It is shown that with increasing ATPase activity, ΔpH reaches a maximal level which is determined by the degree of uncoupling of the thylakoid membrane.

INTRODUCTION

As has been known for more than a decade[1], an ATPase reaction can be induced in chloroplasts by preillumination in the presence of a disulphydryl compound. This ATPase was shown, subsequently, to be able to drive processes like proton uptake[2], swelling in ammonium salts[3,4] and uptake of calcium salts[3]. Furthermore, several observations, notably the decay of ATPase activity in the dark in the absence of ATP and the effect of uncouplers, led to the suggestion that energy derived from ATP hydrolysis is needed for maintaining the light-triggered state, thus supporting continuous ATPase activity[5–8].

In order to describe the energetic aspects of the system in a more rigorous way, it can be considered to be composed of two capacities (Fig. 1): the energy-generative capacity of the ATPase enzyme, regulated by a positive feed-back of the energy level (I), and the dissipative capacity, determined by the degree of uncoupling of the thylakoid membrane (II).

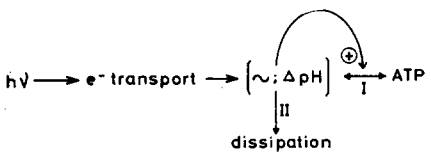

Fig. 1. Scheme for the light-triggered Mg^{2+}-ATPase.

Abbreviation: S_{13}, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide.

* Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

The object of the investigation described in this paper was to study the ATPase activity in relation to the energy level (as measured by the ΔpH) maintained during this activity under different conditions.

MATERIALS AND METHODS

'Type-B'[9] spinach chloroplasts were prepared by a rapid procedure: 15 g of leaves were suspended in 60 ml isolation medium containing 300 mM sucrose, 30 mM 2-(*N*-morpholino)propanesulphonic acid buffer (pH 7.4) and 3 mM $MgCl_2$, and homogenized in a small Waring blender for 5 s at top speed. The homogenate was filtered through 4 layers of Perlon net (mesh width, 56 μm), quickly accelerated in a MSE centrifuge up to $3000 \times g$ and decelerated within 1 min. 'Type-C'[9] spinach chloroplasts were prepared from Type-B chloroplasts by taking up the pellet described above in a medium containing 100 mM KCl, 5 mM $MgCl_2$ and 5 mM sodium tricine (pH 8.0) and homogenizing gently in a Potter–Elvehjem homogenizer. Total chlorophyll was determined according to the method of Whatley and Arnon[10]. The reaction vessel used was designed by Dr. J. W. T. Fiolet, and offered the possibility of simultaneously monitoring fluorescence (by an Eppendorf fluorimeter attachment) and ATPase activities (by the change in pH of the medium, measured with a sensitive pH-recording system). For illumination, light of saturating intensity was provided by a tungsten slide projector lamp through a side window in the reaction cuvette, covered by a red filter (630 nm).

ΔpH was calculated either from the quenching of 9-aminoacridine fluorescence[11] (filters: excitation 313+366 nm, emission 440–460 nm), or, in a parallel experiment, from the distribution of [^{14}C]methylamine[12] determined by centrifugation of the chloroplasts through silicone oil[13].

The reaction mixture contained, at 25 °C, in a final volume of 2.5 ml, 250 μmoles KCl, 12.5 μmoles $MgCl_2$, 12.5 μmoles sodium tricine (pH 8.0), 25 nmoles pyocyanin, 12.5 μmoles dithioerythritol, 12.5 μmoles Na–ATP and 125 μg chlorophyll, unless stated otherwise.

The electrode responses were calibrated with standard solutions of oxalic acid. The concentration of 9-aminoacridine was 3–6 μM. All chemicals were analytical grade.

[^{14}C]Methylamine was obtained from the Radiochemical Centre, Amersham; 9-aminoacridine from British Drug Houses. 5-Chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S_{13}) was kindly donated by Dr P. C. Hamm, Monsanto Comp., St. Louis, Mo. (U.S.A.).

RESULTS

Figs 2A, 2B and 2C show some typical pH traces: after a partial decay of the 'proton pump'[14], the proton production due to ATPase becomes manifest. Except for very short preillumination times (Fig. 2A) this acidification is linear in time within a few seconds. The velocity increases with illumination time[5,7]. Moreover, in the presence of ATP the effects of consecutive illumination periods on the rate of ATPase turn out to be additive, *e.g.* 2 min illumination followed by a few min of dark ATPase and another 2 min illumination gives the same final ATPase rate as 4 min illumination.

Figs 2D, 2E and 2F show traces of the 9-aminoacridine fluorescence quenching recorded simultaneously with Figs 2A, 2B and 2C, respectively. It can be seen that, with the exception of the experiment in Fig. 2D, after dark ATPase has started, the quenching adjusts to a new level within about 20 s.

In Fig. 3, ATPase velocities and ΔpH (calculated from the 9-aminoacridine

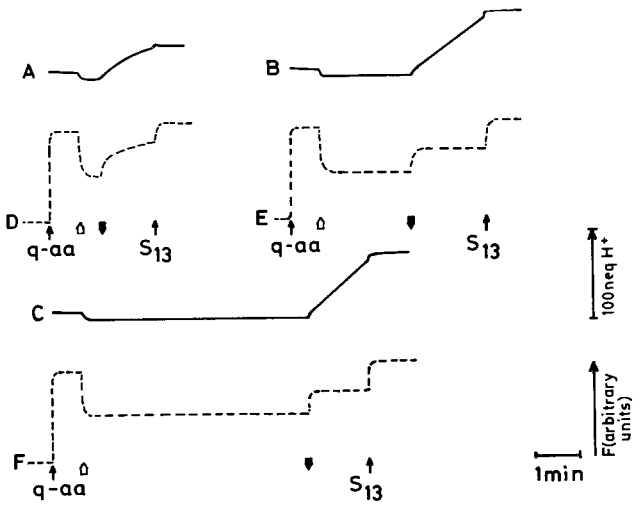

Fig. 2. Effect of preillumination time on ATPase and 9-aminoacridine fluorescence quenching. The reaction mixture was as described under Materials and Methods. The fluorescence after addition of a saturating amount of uncoupler (4 μM S_{13}) at the end of a dark ATPase period is taken as 100%. Preillumination times: A, 30 s; B, 2 min; C, 5 min. ———, pH; – – –, 9-aminoacridine fluorescence. 9-aa, 9-aminoacridine.

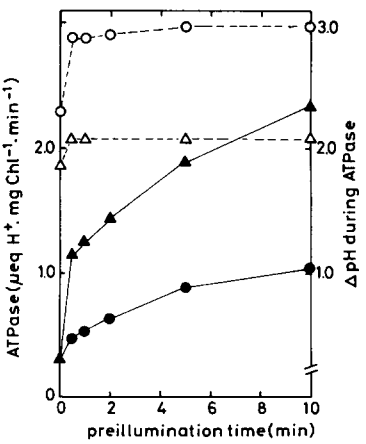

Fig. 3. ATPase and ΔpH maintained during ATPase as a function of the preillumination time. Values for ATPase and ΔpH were taken after 20 s ATPase in the dark. ΔpH was calculated from the 9-aminoacridine quenching as in ref. 11, assuming an osmotic space of 10 μl/mg chlorophyll, and obtaining a value for 100% fluorescence as in Fig. 2. In the light, ΔpH was 3.50 without, and 2.85 with 0.2 μM S_{13} added. Reaction mixture as described under Materials and Methods: ●—●, ATPase; ○---○, ΔpH during ATPase. Reaction mixture with 0.2 μM S_{13} added: ▲—▲, ATPase; △---△, ΔpH during ATPase.

quenching) are plotted as a function of the preillumination time (*cf.* refs 5, 7); because of the relaxation effects observed at short illumination times, the values after 20 s dark ATPase are arbitrarily taken. As can be seen, ΔpH reaches its saturation level much earlier than the ATPase rate. On the other hand, the dark decay of the ATPase is more rapid than that of ΔpH (Fig. 4; *cf.* refs 5–7, 15); this is still the case in the presence of P_i, where the decay of ATPase activity is slowed down[6,15].

A variation of preillumination time or dark decay time presumably does not influence the degree of coupling of the membrane; in terms of Fig. 1 this means that up to this point only the ATPase capacity has been influenced, the dissipative capacity remaining unchanged. In Fig. 3 the latter was increased by a limited amount of uncoupler (0.2 μM S_{13}). Relaxation effects at short illumination times were more pronounced here. Again ΔpH, although lower than in the absence of uncoupler, reaches its maximal level long before the (stimulated) ATPase activity associated with it.

From Figs 3 and 4 together, it seems that ΔpH maintained by ATPase is limited by a maximal level; judging from the decrease in this level caused by uncoupler (Fig. 3), it depends upon the dissipative capacity of the membrane. This becomes even

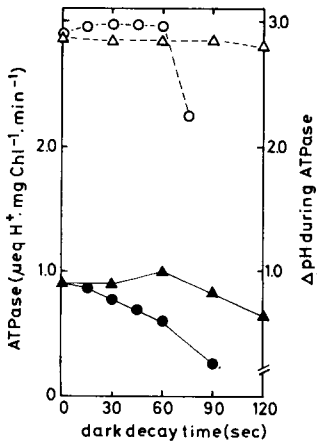

Fig. 4. ATPase and ΔpH during ATPase as a function of the dark decay time. After 5 min illumination, light was turned off. ATP (2.5 μmoles) was added after various time intervals as indicated. Values were calculated as described in the legend of Fig. 3. Reaction mixture as described under Materials and Methods (except for ATP, which was added after preillumination only): ●—●, ATPase; ○---○, ΔpH during ATPase. Reaction mixture with 0.4 mM P_i added: ▲—▲, ATPase; △---△, ΔpH during ATPase.

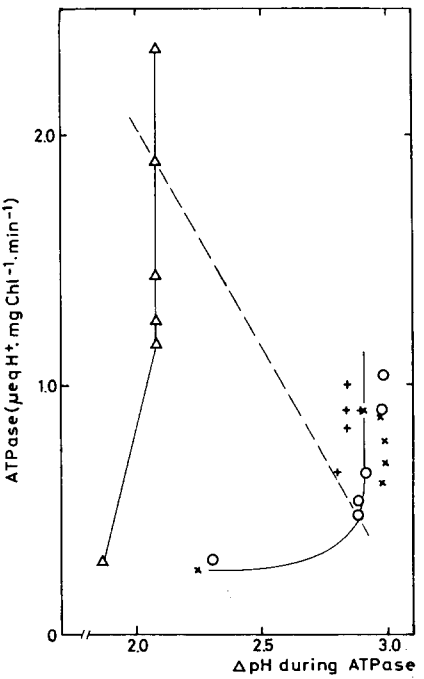

Fig. 5. ATPase rate as a function of ΔpH associated with it. The points were obtained by replotting the data of Figs 3 and 4. ○—○, preillumination time varied in reaction medium; ×—×, dark decay time varied in reaction medium; +—+, dark decay time varied in reaction medium with 0.4 mM P_i added; △—△, preillumination time varied in reaction medium with 0.2 μM S_{13} added. The two points, with and without uncoupler, exhibiting a decrease in ATPase rate of approx. 10% in the time interval between 20 s and 40 s dark ATPase have been connected by a dotted line. Points in the region above and to the right of it do not show significant relaxation; below and to the left of it relaxation is more pronounced.

more clear from Fig. 5, where all data from Figs 3 and 4 have been replotted so as to show ATPase rate as a function of ΔpH associated with it. The points in the upper right half of Fig. 5 correspond with linear ATPase rates; in contrast, the lower left half of Fig. 5 represents an 'unstable' ATPase region: starting from the dotted line, which connects the points showing a decrease in ATPase rate of about 10% during the interval between 20 s and 40 s dark ATPase, relaxation effects are increasingly strong towards the lower left.

DISCUSSION

Applicability of methods and calculations

In quantitizing the energy level maintained during ATPase two assumptions have been made: (1) the energy level is represented by ΔpH, (2) ΔpH can be measured by the distribution of ^{14}C-labelled or fluorescent amines across the thylakoid membrane. As to (1), in terms of the chemiosmotic theory[16], the energy level (protonmotive force) can be thought to be composed of two terms, an electrical potential and a ΔpH. It seems, however, that under steady-state conditions the electrical potential is low[17,12], so that it would be legitimate to take ΔpH as a measure for the energy level. In terms of the chemical theory[18], alternatively, ΔpH can be thought of as a pool in equilibrium with a high-energy intermediate. Indeed, for the interpretation of the experiments given here it is not relevant at all which hypothesis for photophosphorylation is adopted.

As to (2), upon comparison, ΔpH calculated from the 9-aminoacridine quenching[11] consistently gave higher values (up to 1 pH-unit) than that calculated from the [^{14}C]methylamine distribution[12] (not shown; *cf.* also ref. 4). Relatively, the results gave very similar pictures, however.

In all experiments mentioned, the influence of ADP formed during the reaction as a competitive inhibitor of ATPase[19] was neglected: this is justified by the results themselves, in the sense that in no case a linear ATPase rate would be expected in the presence of a considerable and increasing amount of a competitive inhibitor.

Feed-back between ΔpH and ATPase

In Fig. 5 we see that, above a certain ATPase level, ΔpH reaches a maximal value which is dependent only on the dissipative capacity. Furthermore, a region is indicated (the lower left half of Fig. 5) where ATPase rate declines considerably within the time course of the experiment.

Relaxation in ATPase rate caused by uncoupler is a well-established phenomenon[6,8]; in fact, as mentioned in the Introduction, it was one of the lines of evidence indicating that energy generated by ATPase activity is needed for maintaining that activity. However, in the absence of uncoupler, ATPase rates have been found to follow a linear time course (refs 5, 7; *cf.* Figs 2B, 2C). This seems natural enough but, in fact, for a complicated feed-back system like this (*cf.* Fig. 1) it is not obvious at all that even without uncoupler added the ATPase reaction should reach a steady-state rate within a short time (in the order of 30 s). The fact that this actually is the case for the points in the 'stable' upper right half of Fig. 5 means that every ATPase capacity in that region, at a given dissipative capacity, produces an energy level of the right magnitude for maintaining itself. Moreover, the fact that in this region ΔpH

is independent of the ATPase rate indicates that ΔpH needed for maintaining a certain ATPase capacity is independent of the magnitude of that capacity; presumably this would bear some relevance to any model to be proposed for ATPase activation. Also the additivity of preillumination times in the presence of ATP, mentioned above, can be explained in these terms.

In this respect it should be noted that, although ΔpH established by ATPase may be high enough for maintaining ATPase activity, it apparently never in itself is sufficient to activate ATPase molecules in an autocatalytic way: this would manifest itself in a relaxation towards a higher ATPase rate, a phenomenon that has never been observed. Possibly an energy barrier exists for ATPase activation, which is exceeded during illumination; indeed, ΔpH established in the light is higher than that maintained by ATPase activity (*cf.* Fig. 2), and so is the pH jump needed for acid–base ATPase triggering[20].

Alternatively, even in the absence of uncoupler, at very low ATPase levels ΔpH will not reach the value needed for maintaining ATPase activity, and ATPase rate will relax towards lower values. This is seen in Fig. 2A, and applies to the points on the lower left hand in Fig. 5. This also could explain the 'biphasic' ATPase behaviour observed by Kaplan *et al.*[20,21] after an acid–base jump instead of a light triggering: *e.g.* the curves in ref. 20, Fig. 3, just reflect the relaxation behaviour expected of a number of activated ATPase molecules that is too small to keep up the energy level needed for maintaining their activity.

The ΔpH maintained by a given ATPase activity is determined by the dissipative capacity; without uncoupler added, it reflects the 'natural' degree of uncoupling of the thylakoid membrane. The fact that the ΔpH associated with ATPase in the presence of P_i fits the same curve as in its absence (Fig. 5), indicates that in this case P_i only influences the decay of ATPase capacity, and not the dissipative capacity: it does not exert its action by improving the coupling of the membrane. This is in accordance with the explanations for the stabilization of the light-triggered state by P_i given in the literature[22,15].

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from The Netherlands Organization for the Advancement of Pure Research (Z.W.O.). We thank Drs P. W. Postma and J. M. Tager for critically reading the manuscript.

REFERENCES

1 Petrack, B. and Lipmann, F. (1961) *Symp. on Light and Life* (McElroy, W. D. and Glass, B., eds), pp. 621–630, The Johns Hopkins Press, Baltimore, Md.
2 Carmeli, C. (1970) *FEBS Lett.* 7, 297–300
3 Packer, L. and Crofts, A. R. (1967) in *Current Topics in Bioenergetics* (Sanadi, D. R., ed.), Vol. 2, 23–64
4 Gaensslen, R. E. and McCarty, R. E. (1971) *Arch. Biochem. Biophys.* 147, 55–65
5 Petrack, B., Craston, A., Sheppy, F. and Farron, F. (1965) *J. Biol. Chem.* 240, 906–914
6 Carmeli, C. (1969) *Biochim. Biophys. Acta* 189, 256–266
7 Packer, L. and Marchant, R. H. (1964) *J. Biol. Chem.* 239, 2061–2069
8 Rienits, K. G. (1967) *Biochim. Biophys. Acta* 143, 595–605

9 Hall, D. O. (1972) *Nature New Biol.* 235, 125–126
10 Whatley, F. R. and Arnon, D. I. (1963) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. VI, pp. 308–313, Academic Press, New York
11 Schuldiner, S., Rottenberg, H. and Avron, M. (1971) *Eur. J. Biochem.* 25, 64–70
12 Rottenberg, H., Grunwald, T. and Avron, M. (1971) *Eur. J. Biochem.* 25, 54–63
13 Harris, E. J. and Van Dam, K. (1968) *Biochem. J.* 106, 759–766
14 Neumann, J. and Jagendorf, A. T. (1964) *Arch. Biochem. Biophys.* 107, 109–119
15 Carmeli, C. and Lifshitz, Y. (1972) *Biochim. Biophys. Acta* 267, 86–95
16 Mitchell, P. (1968) in *Chemiosmotic Coupling and Energy Transduction*, Glynn Research, Bodmin, Cornwall
17 Schröder, H., Muhle, H. and Rumberg, B. (1971) in *Photosynthesis, Two Centuries after its Discovery by Joseph Priestley, Proc. 2nd Int. Congr. on Photosynthesis Research* (Forti, G., Avron, M. and Melandri, A., eds), Vol. 2, pp. 919–930, Junk, The Hague
18 Slater, E. C. (1967) *Eur. J. Biochem.* 1, 317–326
19 Bennun, A. and Avron, M. (1965) *Biochim. Biophys. Acta* 109, 117–127
20 Kaplan, J. H. and Jagendorf, A. T. (1968) *J. Biol. Chem.* 243, 972–979
21 Kaplan, J. H., Uribe, E. and Jagendorf, A. T. (1967) *Arch. Biochem. Biophys.* 120, 365–375
22 Carmeli, C. and Avron, M. (1967) *Eur. J. Biochem.* 2, 318–326