ATP synthesis with single turnover flashes in spinach chloroplasts

In situ monitoring with the firefly luciferase method

U. Schreiber and S. Del Valle-Tascon*

Lehrstuhl für Botanik I, Universität Würzburg, Mittlerer Dallenbergweg 64, 8700 Würzburg, FRG

Received 24 September 1982; revision received 20 October 1982

Single-turnover flash-induced ATP synthesis in chloroplasts was measured in situ with the luciferin luminescence method. In dark-adapted chloroplasts the first flashes only induce ATP hydrolysis. Once the reversible ATPase is fully activated, ATP hydrolysis persists for extended periods of darkness and flash-induced ATP-synthesis is optimal even at flash frequencies lower than 0.1 Hz. About one molecule of ATP is formed per 1000 chlorophyll and flash. In a low frequency flashing regime under steady state conditions, the newly formed ATP is stable. There is no threshold light intensity for flash-induced ATP synthesis. The data are in agreement with models involving short-range interaction between electron transport and the coupling factor.

ATP synthesis *ATP hydrolysis* *Single-turnover flash* *Thylakoidal ATPase* *Coupling mechanism*

1. INTRODUCTION

Under appropriate conditions the firefly luciferase method is suitable for in situ detection of ATP formation during photophosporylation [1]. This method has been successfully applied to measure the small amounts of ATP formed with single turnover flashes in bacterial chromatophores [2–5]. The results of these studies have led to considerable advancement in the understanding of the mechanisms governing the coupling of electron transport and ATP synthesis. Measurements in [3] suggested that in chromatophores from *Rhodospirillum rubrum* there is short-range interaction between electron-transport components and the coupling ATPase. So far, direct monitoring of single turnover flash-induced ATP synthesis in chloroplasts has not been reported. In chloroplasts the relative amount of reaction centers per chlorophyll is about one order of magnitude smaller than in chromatophores. Consequently, on a chlorophyll basis, in chloroplasts the expected ATP yield/flash is rather low. Furthermore, contrary to chromatophores, the thylakoidal ATPase appears to require a particular activated state and only a fraction of the total ATPase is supposed to be active at any instant of time [6–11].

Inoue et al. [12] measured flash-induced ATP formation with intact chloroplasts, applying repetitive flashing and determining the ATP accumulated over many flashes. These authors showed that flash illumination causes ATP hydrolysis as well as ATP synthesis, and only after several 'activating flashes' and with higher flash-frequen-

* Permanent address: Departamento de Botanica, Unversidad de Extremadura, Badajoz, Spain

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris-(hydroxymethyl)-aminomethane; DCCD, *N,N'*-dicyclohexylcarbodiimide; TNBT, tri-*n*-butyltin; CF_1, chloroplast coupling factor; chl, chlorophyll

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

cies there is net ATP synthesis. From these findings [12] it is clear that for any quantitative determination of light-induced ATP synthesis, the concomitant rate of ATP hydrolysis should be taken into account. While with repetitive methods accurate estimation of the rate of ATP hydrolysis is problematic, this is possible by the direct, single-flash detection method.

Here, we report on a measuring system which in spinach chloroplasts detects ATP formation upon single-turnover light flashes against a high background of endogenous ATP, the level of which is decreasing due to ATP hydrolysis. It is found that in freshly shocked intact chloroplasts the amount of ATP formed per flash is surprisingly high (~1 ATP/1000 chl molecules) even with extended dark times (>10 s) between the flashes. The flash-induced, increased level of ATP is stable, although the flash-induced increase of the membrane potential is only transient. Measurements at decreased flash intensities revealed that there is no threshold intensity for ATP synthesis. These findings raise the question of whether also in chloroplasts there may be short-range, direct interaction between electron transport and the coupling factors.

2. MATERIALS AND METHODS

Intact spinach chloroplasts capable of high CO_2-dependent O_2-evolution rates (~120 μmol $O_2 \cdot mg\ chl^{-1} \cdot h^{-1}$) were isolated according to standard methods [13,14]. Spinach leaves (*Spinacea oleracea* L.) were freshly harvested from the greenhouse before isolation. The degree of chloroplast intactness was 80–90%, as estimated by the ferricyanide method [15]. Following isolation, chloroplasts were stored in the dark at 0°C at ~2.3 mg chl/ml. Such chloroplasts retained full activity for at least 5 h. If not stated otherwise, shortly before each experiment 30 μl chloroplasts were osmotically ruptured by mixing with 5 ml shock medium consisting of 5 mM Hepes–Tris (pH 7.8) and 5 mM Mg-acetate. After 30 s hypotonic treatment, the suspension was made half-isotonic by mixing with 5 ml of the following buffer: 330 mM sorbitol, 2 mM KH_2PO_4, 10 mM Mg-acetate, 10 mM Hepes–Tris (pH 7.8), 2 mM dithiothreitol and 2 mg bovine serum albumin/ml. Final chlorophyll concentration was ~7 μM. The chloroplast suspension was continuously stirred in a 10 ml cuvette thermostatted at 10°C. For each experimental run 100 μl of a solution of 'ATP monitoring reagent' (LKB-Wallac; the content of one vial being dissolved in 1 ml double-distilled water) were added to the 10 ml cuvette. The reagent concentration was sufficiently low to avoid significant signal decay by product inhibition. ADP (Böhringer) was added to 2×10^{-5} M. Due to the presence of adenylate kinase in the shocked chloroplast preparation, part of this ADP was transformed into ATP and AMP. In a typical experiment ATP concentration was $\sim 5 \times 10^{-9}$ M before ADP-addition, was rapidly increased to 10^{-7} M upon ADP injection (presumably resulting from ATP present in ADP solution) and then increased slowly (within ~10 min) to $\sim 10^{-6}$ M in the dark (see also fig. 1). Following illumination part of this ATP is hydrolysed again (see below). ATP calibration was done with known amounts of a freshly prepared 10^{-5} M ATP solution (LKB-Wallac). There was strict linearity between the amount of ATP added and the increment of luminescence.

Luciferin luminescence was measured with a photomultiplier (Hamamatsu R 562) protected by the following filter set: 5 mm Schott BG 39 and SP 600 (Dichroic Optics). The anode current was pre-amplified within the photomultiplier housing and then further amplified, appropriately filtered and compensated. The system was sufficiently sensitive to detect changes in the order of 10^{-10} M ATP on a background of 10^{-6} M ATP. If necessary, signal drift was compensated with a laboratory-built variable voltage-ramp generator (opposite drift generator). Single-turnover light flashes of ~20 μs duration were applied with a General Electrics FT 230 xenon flash tube focused on a flexible light guide which was connected to the cuvette. Flash-light was filtered through 4 mm Schott RG 645. Flash-intensity was reduced by use of neutral density filters (Schott). The sample was contained in a 10 ml cuvette (cylinder-shaped, 50 mm wide and 5 mm high, covered with perspex windows) only separated by the blue filter set from the photomultiplier tube. The cuvette was continuously stirred with a small propeller, allowing rapid mixing (in $\leq$1 s) of added solutions. At 10°C, the overall response time of the measuring system was limited by the luciferin–luciferase reaction.

3. RESULTS AND DISCUSSION

Fig. 1 demonstrates the system performance for in situ monitoring of ATP concentration by the firefly luciferase method with spinach chloroplasts. Upon ADP addition to a dark-adapted sample there was a biphasic liminescence rise (see also section 2) due to contaminating ATP in the ADP solution (rapid phase) and to the action of adenylate kinase (slow phase) (fig. 1a). When the dark signal was stabilized, saturating flash illumination was given at 1 Hz repetition rate (fig. 1b) and the signal change was monitored on a 10-times more sensitive scale. It is apparent that the first flashes induced ATP uptake and only after ~6 flashes (not separately distinguishable in the presented graph) net ATP synthesis was observed, the rate of which increased with flashing time to a quasi-stationary value of ~50 nmol ATP.min^{-1} .mg chl^{-1}. When flashing light was interrupted there was ATP uptake with an initial high rate of ~40 nmol ATP.min^{-1}.mg chl^{-1}, slowing down to ~15 nmol ATP.min^{-1}.mg chl^{-1} after 3 min darkness and reaching within 20 min a quasi-stationary rate of ~2 nmol ATP.min^{-1}.mg chl^{-1} (not shown). When flashes were given at lower frequencies (e.g., 0.1 Hz in fig. 1c), the overall rate was governed by ATP uptake, although following each flash a rapid 'jump' in ATP concentration can be observed. By applying an 'opposite-drift generator' tuned to compensate the signal decay caused by ATP hydrolysis, flash-induced ATP synthesis can be accurately monitored (fig. 1d). This is depicted in more detail in fig. 2. It is apparent that the amount of ATP produced by a single flash is constant. This was true even after longer periods of darkness; the first flash in this series was, e.g., given after 30 s darkness. The newly formed ATP appears to be stable, i.e., following flash-induced ATP synthesis there is no significant stimulation of ATP hydrolysis. Increasing flashing frequency from 0.1–1 Hz did not change the total amount of ATP formed with 5 flashes. In this experiment the ATP yield/flash is 1.2 nmol ATP.mg chl^{-1}. This is equivalent to a yield of ~1 ATP molecule formed for every 1000 chl molecules, or ~0.5 ATP molecules electron-transport chain and flash, assuming a photosynthetic unit size of 500 chl molecules. Considering that there is ~1 CF_1 for every 1000 chl

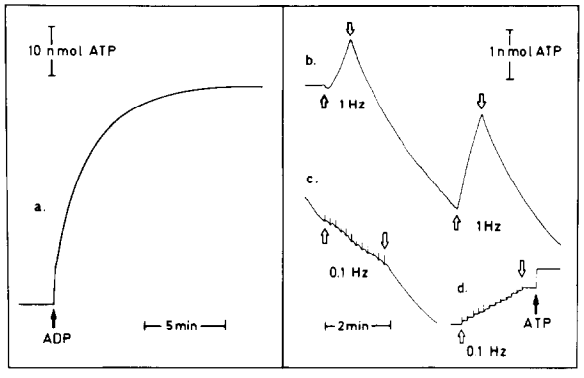

Fig. 1. In situ monitoring of ATP in osmotically shocked intact chloroplasts by the firefly luciferase method: (a) increase of ATP concentration induced injection of ADP (20 μl 10^{-2} M solution into the 10 ml cuvette). The rapid phase is due to contaminating ATP in the ADP solution, and the slow phase results from adenylate kinase activity. (b–d) Changes in ATP concentration induced by flash illumination. As compared to (a) sensitivity was increased by a factor of 10. Note also the changed time scale. In (d) and 'opposite-drift generator' was adjusted to compensate the drift caused by ATP uptake. The needle spikes are transient artifacts caused by the xenon flashes; at the given time scale they were not separated at 1 Hz flashing rate. Where indicated, 40 μl 10^{-5} M ATP were injected for calibration. The chloroplasts were dark adapted for 3 h following their isolation.

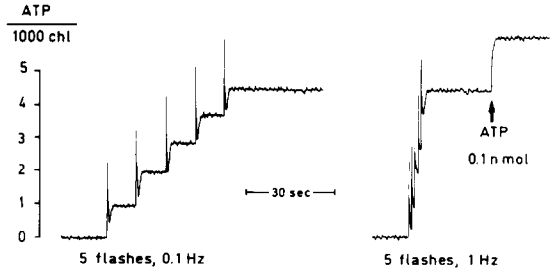

Fig. 2. ATP synthesis induced by single turnover light flashes. Five consecutive flashes were given at 0.1 and 1 Hz frequency. Conditions were as in fig. 1d, with sensitivity increased by a factor of 10. Where indicated, 10 μl 10^{-5} M ATP were injected for calibration. While the data for this figure were obtained with a sample hydrolysing ATP at a relatively high rate of 15 nmol ATP.min^{-1}.mg chl^{-1}, identical results were found when hydrolysis rate was slowed down to 2 nmol ATP.min^{-1}.mg chl^{-1}.

in spinach chloroplasts [16], this means that with each flash there is ~1 ATP formed/CF_1.

Single flash-induced ATP formation is affected in the expected way by uncouplers and ATPase inhibitors. Inhibition of 50% is observed following addition of 10^{-8} M gramicidin (uncoupler), 0.5 μg Dio-9/ml (ATPase inhibitor, affecting CF_1), 4×10^{-5} M DCCD (ATPase inhibitor, affecting CF_0) or 3×10^{-6} M TNBT (ATPase inhibitor, affecting CF_0). Hence, there can be no doubt that the flash-induced luminescence changes do reflect ATP formation.

These data demonstrate that with the firefly luciferase method the ATP produced by 1 single-turnover light flash can be reliably measured in chloroplasts. As already noticed in [12], with dark-adapted chloroplasts there is first ATP uptake before ATP synthesis occurs. This is presumably due to the action of the ATP hydrolase which becomes activated by the saturating light flashes. ATP hydrolase activity has been shown in intact and freshly shocked chloroplasts [17–20]. These data suggest that surprisingly few flashes are required to activate ATP hydrolysis and, consequently, ATP synthesis. Even following 8 h strict darkness 2 flashes given 1 s apart proved sufficient to induce considerable activation (not shown). In agreement with [17–20], once the ATP hydrolase is activated it remains active for extended periods of darkness. Obviously, ATP uptake occurs also in the dark times between flashes, a fact which may explain some of the flash frequency dependence on ATP yield in [7,12,21]. Under the conditions of fig. 1, ATP hydrolysis rate is ~15 nmol ATP. min^{-1}. mg chl^{-1}. Hence, with a yield of 1.2 nmol ATP. mg chl^{-1}, $flash^{-1}$, net ATP production will be only significantly (i.e., by >5%) affected by background ATP hydrolysis at flash frequencies below 10 Hz. At very low flash frequencies (e.g., 0.1 Hz in fig. 1) only net ATP uptake will be seen, although with appropriate kinetic resolution it is clear (fig. 2) that each flash produces the same 'burst' of ATP as at higher frequencies. Therefore, determining overall accumulated ATP with a repetitive flash technique, one may come to the erroneous conclusion that at low flash frequencies ATP synthesis is not possible.

Harris and Crofts [7] studies photophosphorylation in spinach chloroplasts, from the dark state, with saturating single turnover flashes applying a repetitive technique. These authors used broken chloroplasts of the class II type, which required rapid flashing rates for activating and maintaining ATP synthesis. Even at 100 Hz, only ~10% of the phosphorylation flash yield reported here with freshly shocked intact chloroplasts was observed. Chloroplast integrity plays an essential role in determining the properties of the coupling reactions and the yield of flash-induced ATP formation [12].

For bacterial chromatophores, ATP synthesis induced by single turnover flashes can be readily detected in situ with the firefly luciferase technique [3–5]. On the basis of such experiments it was first concluded [3] that direct interaction between electron transport and the coupling factor was likely. A decisive argument for this conclusion was that a plot of ATP formation *vs* flash intensity extrapolated through zero for decreasing light intensity. Fig. 3 shows the outcome of a similar experiment with spinach chloroplasts. Even at very low flash intensities, the ATP formed is proportional to flash energy. Apparently, also for flash-induced ATP synthesis in chloroplasts there is no threshold light energy below which the quantum yield will decline. For the evaluation of this finding it is important to take into account that under the given conditions there is high ATP hydrolase activity, which is known to establish considerable membrane gradients of ΔpH and $\Delta\psi$ [22–27]. Hence,

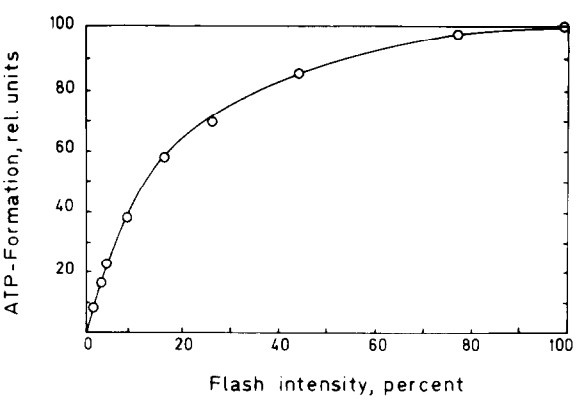

Fig. 3. Flash intensity dependence of single turnover flash-induced ATP formation. ATP formation was measured under the conditions of fig. 2. Flash intensity was reduced by use of neutral density filters. For each intensity 4 measurements were done and the mean value was calculated.

already in the dark the 'threshold energy' for ATP synthesis may be given, and even very small increments of the protonmotive force could result in formation of ATP, as predicted by the chemiosmotic theory [28]. However, such an interpretation would be based on the assumption that the coupling system is close to thermodynamic equilibrium. If this were the case, it is not clear why the increase in ATP level caused by a single flash is stable (fig. 2), although the flash-induced increase of the membrane potential is only transient. It was checked that under the given conditions, following appropriate activation by preillumination, ATP hydrolysis maintained a significant increased level of P515 absorbance, which was somewhat increased following a light flash and then decreased again with a half decay time of ~200 ms (see also [27]).

Despite the observed ratio of ~1 ATP formed/CF_1, this finding does not necessarily mean that all ATPase molecules are active under the given conditions and that there is 1 turnover/single CF_1 molecue. As shown in [9], only a fraction of the total GF_1 may be active at any instant of time, and this 'active fraction' may turn over several times within the lifetime of the flash-induced, stimulated membrane potential.

There is strict a complementarity between the light-induced and ATP-induced P515 absorbance increase [27], suggesting a localized membrane potential as a high-energy intermediate of the coupling reaction. 'Localized coupling', in [3,27,29–32], could also readily account for the findings of this study; i.e., the observed ATP flash-yield of 1 ATP/CF_1 and the observed flash-intensity dependence. Further studies with the direct ATP monitoring system and the application of single turnover flashes may help to clarify some of the controverial points concerning the mechanism of energy transduction in thylakoids.

ACKNOWLEDGEMENTS

We wish to thank the members of the electronic and mechanical workshops for skillful help with the building of equipment. Miss A. Weber is thanked for the preparation of excellent chloroplasts. Thanks are also due to Professor U. Heber for stimulating discussions. Financial support by EMBO for S.d.V.-T. is gratefully acknowledged. We also wish to acknowledge support by the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk.

NOTE ADDED

After submission of the above report we became aware of work by Graan, Th., Flores, S. and Ort, D.R. [in: Energy Coupling in Photosynthesis (1981) Selman, B.R. and Selman-Reimer, S. eds, pp. 25–84, Elsevier Biomedical, Amsterdam, New York] who determined single-turnover flash-induced ATP synthesis in chloroplasts with a sensitive ^{32}P-method. As in the present report these authors observed ATP yields close to 1 ATP/1000 Chl and flash, provided ~5 'activating flashes' were given. Contrary to the approach used here, their method does not allow one to monitor ATP hydrolysis parallel to ATP synthesis.

REFERENCES

[1] Lundin, A., Rickardsson, A. and Thore, A. (1976) Anal Biochem. 75, 611–620.
[2] Lundin, A., Thore, A. and Baltscheffsky, M. (1977) FEBS Lett. 79, 73–76.
[3] Del Valle-Tascon, S., Von Grondelle, R. and Duysens, L.N.M. (1978) Biochim. Biophys. Acta 504, 26–33.
[4] Petty, K.M. and Jackson, J.B. (1979) FEBS Lett. 97, 367–372.
[5] Melandri, B.A., Venturoli, G., De Santis, A. and Baccarini Melandri, A. (1980) Biochim. Biophys. Acta 592, 38–52.
[6] Bakker-Grunwald, and Van Dam, K. (1974) Biochim. Biophys. Acta 347, 290–298.
[7] Harris, D.A. and Crofts, A.R. (1978) Biochim. Biophys. Acta 502, 87–102.
[8] Strotmann, H. and Bickel-Sandkötter, S. (1977) Biochim. Biophys. Acta 460, 126–135.
[9] Gräber, P., Schlodder, E. and Witt, H.T. (1977) Biochim. Biophys. Acta 461, 426–440.
[10] Schlodder, E. and Witt, H.T. (1981) Biochim. Biophys. Acta 635, 571–584.
[11] Bickel-Sandkötter, S. and Strotmann, H. (1981) FEBS Lett. 125, 188–192.
[12] Inoue, Y., Kobayashi, Y., Shibata, K. and Heber, U. (1978) Biochim. Biophys. Acta 504, 142–152.
[13] Jensen, R.G. and Bassham, J.A. (1966) Proc. Natl. Acad. Sci. USA 56, 1095–1101.
[14] Heber, U. (1973) Biochim. Biophys. Acta 305, 140–152.

[15] Heber, U. and Santarius, K.A. (1970) Z. Naturforsch. 25 b, 718–728.
[16] Strotmann, H., Hesse, H. and Edelman, K. (1973) Biochim. Biophys. Acta 314, 202–210.
[17] Mills, J.D. and Hind, G. (1978) FEBS Lett. 87, 73–76.
[18] Mills, J.D., Mitchell, P. and Schürmann, P. (1980) FEBS Lett. 112, 173–177.
[19] Schreiber, U. (1980) FEBS Lett. 122, 121–124.
[20] Schreiber, U. and Rienits, K.G. (1982) FEBS Lett. 141, 287–291.
[21] Goldfeld, M.G., Dmitrovskii, L.G. and Blumenfeld, L.A. (1976) Biofizika 22, 357–358.
[22] Carmeli, C. (1970) FEBS Lett. 7, 297–300.
[23] Schreiber, U. and Avron, M. (1979) Biochim. Biophys. Acta 546, 436–447.
[24] Galmiche, J.M. and Girault, G. (1980) FEBS Lett. 118, 72–76.
[25] Schuurmans, J.J., Leuuwerik, F.L., Sin Oen, B. and Kraayenhof, R. (1981) in: Proc. 5th Int. Cong. Photosynthesis (Akoyunoglou, G. ed) vol. 1, pp. 543–552, Balaban Int. Sci. Serv. Philadelphia PA.
[26] Avron, M., Admon, A., Vinckler, C. and Korenstein, R (1982) in: Photosynthesis 1982 (Heber, U. et al. eds) pp. 93–106, Deutsche Forschungsgemeinschaft, Bad Godesberg.
[27] Schreiber, U and Rienits, K.G. (1982) Biochim. Biophys. Acta 682, 115–123.
[28] Mitchell, P. (1976) Biochem. Soc. Trans. 4, 399–430.
[29] Williams, R.J.P. (1978) Biochim. Biophys. Acta 505, 1–44.
[30] Ort, D.R., Dilley, R.A. and Good, N.E. (1978) in: Proc. 4th Inc. Cong. Photosynthesis (Hall, D.O. et al. eds) pp. 581–590, The Biochemical Society, London.
[31] Schuurmans, J.J., Peters, A.L.J., Leeuwerik, F.J. and Kraayenhof, R. (1981) in: Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F. et al. eds) Dev. Bioenerget. Biomembr. vol. 8, pp. 359–369, Elsevier Biomedical, Amsterdam, New York.
[32] Giersch, C., Heber, U., Kobayashi, Y., Inoue, Y., Shibata, K. and Heldt, H.W. (1980) Biochim. Biophys. Acta 590, 59–73.