SENSITIVE MEASUREMENT OF FLASH INDUCED PHOTOPHOSPHORYLATION IN BACTERIAL CHROMATOPHORES BY FIREFLY LUCIFERASE

A. LUNDIN and A. THORE

National Defence Research Institute, Department 4, S-172 04, Sundbyberg 4

and

M. BALTSCHEFFSKY

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-104 05 Stockholm, Sweden

Received 13 April 1977

1. Introduction

The use of firefly luciferase for measurement of electron transport-linked ATP formation in chloroplasts and mitochondria has been previously described [1–6]. Most of the observations were of a qualitative rather than quantitative nature. However, Lemasters and Hackenbrock [5,6] have described the use of firefly luciferase for quantitative, continuous measurement of oxidative phosphorylation in mitochondria and submitochondrial particles. Under the conditions employed in their experiments the bioluminescence intensity gradually decreased by end product inhibition of luciferase. This effect could be corrected for by the use of internal standards, i.e., the addition of known concentrations of ATP.

In contrast to the brief flash usually obtained when mixing ATP and luciferase reagent, it was recently demonstrated that under appropriate conditions the intensity of the bioluminescence may be maintained essentially constant for several minutes using a purified luciferase reagent [7]. With this reagent any given ATP concentration up to 10^{-6} M results in a constant light emission directly proportional to the ATP concentration. The luciferase reagent consumes only negligable amounts of ATP and may be added to ATP converting systems using the bioluminescence intensity for continuous measurement of the ATP concentration in the system. The kinetic properties of the reagent eliminates elaborate calibrations and the reagent has been proven useful for continuous monitoring of several ATP converting reactions [7].

In the present paper we show that the purified luciferase reagent may be used for sensitive measurement of photophosphorylation in *Rhodospirillum rubrum* chromatophores following single flashes or continuous illumination. In contrast to the 'pH-method' [8] and the '^{32}P-method' [9] the luciferase technique allows accurate determination of the amount of ATP formed after a single 1 ms flash.

2. Materials and methods

The equipment used was a cuvette chamber from a Johnson Foundation Dual beam spectrophotometer with a photomultiplier tube (EMI 9492B) operated at 1200–1300 V. The signal was amplified, balanced with a bucking voltage and read out on a storage oscilloscope.

The photomultiplier was provided with a guard filter (Corning 9782) blocking red light. The cross illumination consisted of a 1 ms Xe-flash triggered with a delay circuit. The light was filtered through triple layers of Wratten 88 A gelatin filter and entering at 90° angle from the photomultiplier. Continuous illumination for 10–30 s was obtained from a 20 W halogen lamp, with the light passing

through a 875 nm interference filter. Light intensity was adjusted with a rheostat to saturation for the conditions employed.

Chromatophores were prepared and stored as described previously [10,11]. Luciferase was purified from FLE-50 (Sigma) as described previously [7]. For measuring the ATP formation the reaction mixture contained in a final volume of 1.5 ml: 0.1M glycyl–glycin buffer, pH 7.75, 10 mM $Mg(Ac)_2$, 0.1 mM succinate, 2 mM inorganic phosphate, 0.02 mM ADP, 0.14 mM luciferin, 0.1% bovine serum albumin, luciferase preparation corresponding to a protein concentration of 1–5 μg/ml and chromatophores corresponding to 0.1–40 μM BChl. Each experiment was calibrated by the addition of a known concentration of ATP.

Acid extractable ATP in the reaction mixture was determined as follows: an equal volume of ice-cold 10% trichloroacetic acid was rapidly injected into the mixture, while the bioluminescence was measured. Thus ATP concentrations were estimated by both methods in the same sample.

After addition of 6.25 mM EDTA and extraction with diethyl ether to remove trichloroacetic acid [12] the extracts were diluted 100-times and assayed for ATP by the firefly method [13].

3. Results

Figure 1 shows the time course of the bioluminescence in a mixture containing chromatophores, ADP, P_i and luciferase reagent after illuminating with a single flash and after the addition of ATP. In both cases the half-time of the rise in bioluminescence is approximately 200 ms, similar to the half-time of the luciferase reaction reported by DeLuca and McElroy [14]. Thus the luciferase reaction appears to be rate limiting. In agreement with previous work light emission was directly proportional to ATP concentrations up to 10^{-6} M [7]. In the experiment depicted in the figure the yield of ATP corresponds to one ATP/208 BChl. In other experiments the yield was one ATP/189–330 BChl.

Figure 2 demonstrates that the luciferase technique may also be used for monitoring of steady state photophosphorylation. The rate of ATP formation was 3.5 ATP min^{-1} $BChl^{-1}$. In this and several other

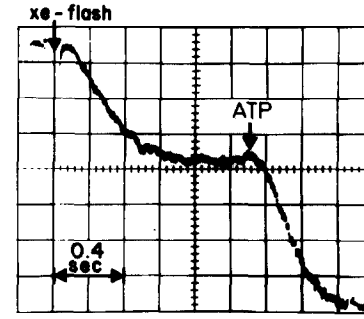

Fig.1. Measurement of photophosphorylation after a single flash. The first increase (downwards) of the bioluminescence represents the ATP formed after a single flash and the second increase the addition of 20 μl ATP to a final concentration of 2.4×10^{-7} M. Chromatophore concentration corresponded to 39 μM BChl, in a complete reaction mixture as described in Materials and methods.

experiments the rate obtained by the luciferase technique agreed within 5% with the values obtained with the pH-method performed as described by Nishimura et al. [8].

The next series of experiments were performed to confirm that the luciferase technique actually measures photophosphorylation.

In the absence of luciferase reagent or chromatophores illumination did not result in any increase of emitted light. Table 1 shows that in the absence of ADP and P_i the amount of ATP formed by single flashes was strongly reduced. Half maximum yield

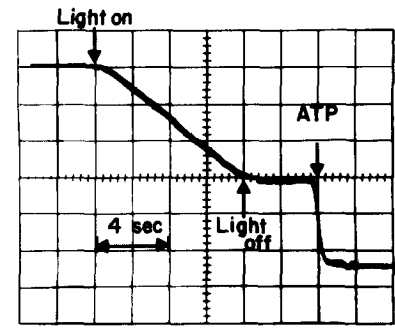

Fig.2. Measurement of photophosphorylation during continuous illumination. After the illumination 10 μl ATP to a final concentration of 1.1×10^{-6} M was added for calibration of the experiment. Chromatophore concentration corresponded to 0.39 μM BChl. Other conditions as in fig.1.

Table 1
Amount of ATP formed by single light flashes in the absence or presence of some components known to affect photophosphorylation in *Rhodospirillum rubrum* chromatophores

Experimental conditions[a]	ATP formed/1.000 BChl
Control	5.3 (100%)
No ADP	0.09 (2%)
No ADP and no P_i	0.03 (0.6%)
FCCP (0.3 μM)	3.1 (58%)
FCCP (4 μM)	0.2 (4%)
Antimycin A (1.2 μM)	1.7 (32%)
Antimycin A (6 μM)	1.3 (25%)
Valinomycin (0.33 μM) + KCl (4 mM)	4.0 (75%)
Valinomycin (10 μM) + KCl (4 mM)	0.6 (11%)

[a]Chromatophore concentration corresponded to 35 μM BChl

was obtained with approximately 1 μM ADP confirming the value obtained for flash phosphorylation as indirectly indicated by Saphon et al. [9]. Table 1 also shows that flash induced phosphorylation was sensitive to uncouplers, ionophores and electron transport inhibitors in low concentrations. These compounds had no effect on the luciferase reaction itself.

In order to further ascertain the reliability of the continuous bioluminescence method ATP determinations were made by addition of trichloroacetic acid and assay of ATP in the extracts. Table 2 shows that under conditions used for single flash experiments the higher ATP value obtained by the extraction method was similar irrespective of whether the chromatophores had been subjected to phosphorylating conditions (ADP + light) or not. The most likely explanation is that chromatophores contain a small amount of ATP not accessible to luciferase. This ATP corresponds to one ATP/233 BChl and was too little

Table 2
Comparison between ATP measured directly in the chromatophore suspension and ATP measured after extraction of the suspension with trichloroacetic acid

Experimental conditions		BChl (μM)	ATP concentration (μM)[a]		
			Measurement done in		
Light	ADP (20 μM)		Suspension	Extract	Difference between extract and suspension
–	–	35	0.00	0.15	0.15 ± 0.02
Flashes	+	35	0.56	0.76	0.20 ± 0.06
–	–	0.12	0.00	0.00	0.00 ± 0.00
Continuous	+	0.12	0.60	0.60	0.00 ± 0.01

[a]Average for 2–5 experiments. Maximal limits of variation are given for the difference between extract and suspension

to be measured under the conditions used for steady state photophosphorylation experiments.

4. Discussion

The method described in the present paper was found to be sensitive and convenient for measuring photophosphorylation in chromatophores. Firefly luciferase has high specificity for ATP [15]. However, nucleotide converting enzymes in the chromatophore suspension such as adenylate kinase may cause analytical problems in some types of experiments. Since concentrations of ATP down to 10^{-11} M may easily be measured with simple equipment [13] these problems may be overcome by using low concentrations of chromatophores.

Though to some extent limited by the half time of the luciferase reaction (200 ms) [14] a main application of the luciferase method as described in the present paper will undoubtedly be measurement of photophosphorylation following single flashes causing only one turnover of the cyclic electron flow. This type of measurement is an important approach to obtain a detailed picture of the events involved in photophosphorylation.

Acknowledgements

This work was supported by the Swedish Board for Technical Development and by a grant from the Swedish Natural Science Research Council (to M.B.).

References

[1] Strehler, B. L. and Totter, J. R. (1952) Arch. Biochem. Biophys. 40, 28–41.
[2] Strehler, B. L. and Hendley, D. D. (1961) in: Light and Life (McElroy, W. D. and Glass, B. eds) pp. 601–608, Johns Hopkins Press, Baltimore.
[3] Strehler, B. L. (1968) in: Methods of Biochemical Analysis (Glick, D. ed) Vol. 16, pp. 99–181, Wiley, New York.
[4] Kukarskikh, G. P., Krendeleva, T. Ye. and Rubin, A. B. (1972) Biophysics 17, 88–95.
[5] Lemasters, J. J. and Hackenbrock, C. R. (1973) Biochem. Biophys. Res. Commun. 55, 1262–1270.
[6] Lemasters, J. J. and Hackenbrock, C. R. (1976) Eur. J. Biochem. 67, 1–10.
[7] Lundin, A., Rickardsson, A. and Thore, A. (1976) Anal. Biochem. 75, 611–620.
[8] Nishimura, M, Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177–182.
[9] Saphon, S., Jackson, J. B. and Witt, H. T. (1975) Biochim. Biophys. Acta 408, 67–82.
[10] Baltscheffsky, H. (1960) Biochim. Biophys. Acta 40, 1–8.
[11] Baltscheffsky, M. (1967) Nature 216, 241–243.
[12] Lundin, A. and Thore, A. (1975) Appl. Microbiol. 30, 713–721.
[13] Lundin A. and Thore, A. (1975) Anal. Biochem. 66, 47–63.
[14] DeLuca, M., and McElroy, W. D. (1974) Biochemistry 13, 921–925.
[15] McElroy, W. D. and Green, A. (1956) Arch. Biochem. Biophys. 64, 257–271.