A Continuous Luminometric Assay for the Determination of Bacterial Oxidative Phosphorylation

REIJO LAHTI and TUULA HÄMÄLÄINEN

Department of Biochemistry, University of Turku, SF-20500 Turku 50, Finland

Various radiochemical,[1,2] manometric and colorimetric[3] as well as potentiometric[4,5] methods have been used to measure oxygen and phosphate uptake coupled to oxidative phosphorylation. However, these methods are generally either inconvenient, indirect or insensitive to evaluate the rate of ATP synthesis in oxidative phosphorylation. By virtue of the great sensitivity of the photomultiplier tube to light, the firefly luciferase assay has been employed when extremely small quantities of ATP need to be measured.[6] The firefly luciferase method has been used to measure oxidative phosphorylation in mitochondria[7] and light induced photophosphorylation in the chromatophores of *Rhodospirillum rubrum*.[8] In addition, Tsuchiya[9] applied the luciferin–luciferase assay to investigate oxidative phosphorylation in the "right-side-out" (RSO-) vesicles of *Escherichia coli*. Because externally added ADP has no access to RSO-vesicles Tsuchiya[9] used RSO-vesicles artificially loaded with ADP. However, the ATP formed in the RSO-vesicles must be released by perchloric acid treatment prior to the ATP assay, and so ATP synthesis cannot be followed continuously in this system. Furthermore, perchloric acid treatment with the accompanying neutralization makes this assay rather complicated and results in low precision and sensitivity. These restrictions encouraged us to look for an alternative, and we found that application of the luciferin–luciferase assay to everted ("inside-out") vesicles of *E. coli* facilitates a direct, continuous and highly sensitive measurements of ATP synthesis in bacterial oxidative phosphorylation. The details of these studies are described in this paper.

Experimental. Bacterial strain, culture conditions and preparation of membrane vesicles. *Escherichia coli* K12 from the collection of our laboratory was used as a test organism. The cells were grown and the everted membrane vesicles were prepared as described by Hempfling and Hertzberg.[10] The vesicles were suspended in 250 mM sucrose, 10 mM morpholiopropane sulfonate (MOPS), 5 mM $MgCl_2$, 10 % methanol, 1 mM dithiothreitol, pH 7.5, to a concentration of about 5 mg protein per ml as determined by the method of Lowry *et al.*[11] with bovine serum albumine as the standard. The vesicles were stored in liquid nitrogen.

Assay of ATP synthesis. The formation of ATP in the oxidative phosphorylation was monitored as the light emitted in the luciferase reaction. Bioluminescence was measured with a Luminometer 1250 with a Potentiometric Recorder 2210 (LKB-Wallac, Finland). Purified firefly luciferase was donated as "ATP Monitoring Reagent" from LKB-Wallac, Finland. ADP was purified by ion-exchange chromatography as described by Lundin[12] to remove ATP appearing in most commercial preparations (1–2 %).

All the components of the reaction mixture were dissolved in 50 mM Tris/HCl (pH 7.75) with 250 mM sucrose, and the reaction mixture contained 10 mM $MgSO_4$, 2 mM potassium phosphate, and 0.04 mM ADP. For the routine assay of ATP formation, 3.5 μg of vesicles were generally used. ATP Monitoring Reagent was added 0.05 ml per reaction in a total volume of 0.25 ml. The reaction was performed at room temperature. It was initiated by the addition of potassium succinate (10 mM), and the light output from the reaction was measured continuously by a luminometer. ATP standard solution (10^{-9} M) was then added to the reaction tube for internal calibration, and the additional light output was measured. The rate of ATP synthesis in the reaction mixture was calculated by comparing the light output before and after addition of the ATP standard. The use of internal calibration

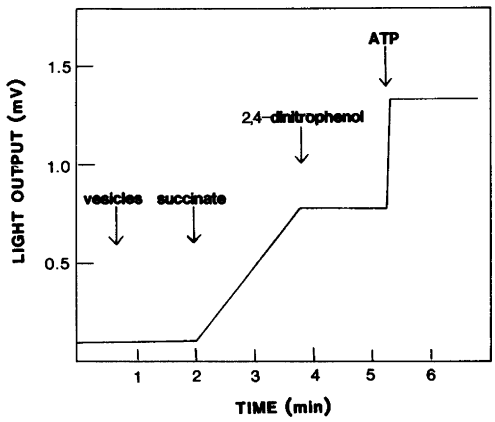

Fig. 1. Progress curve of succinate driven ATP synthesis and the effect of 2,4-dinitrophenol. Vesicles (3.5 μg), succinate (10 mM), 2,4-dinitrophenol (0.33 mM), and ATP-standard (10^{-9} M) were added at times indicated in the figure.

0302-4369/84 $2.50

Table 1. The rate of succinate driven ATP synthesis catalyzed by the everted membrane vesicles of *Escherichia coli* and the effect of some compounds known to affect respiration and/or oxidative phosphorylation.

Experimental conditions[a]	The rate of ATP synthesis nmol/min mg vesicles[b]
Control (=no extra additions)	85.8
No ADP	0.1
DCCD[c] (0.4 mM)	7.2
DCCD (0.8 mM)	0
Sodium azide (10 mM)	7.8
Potassium cyanide (1 mM)	14.3
Potassium cyanide (10 mM)	0.8
Malonic acid (5 mM)	5.1
Malonic acid (10 mM)	0
2,4-dinitrophenol (0.33 mM)	0.2
2,4-dinitrophenol (0.66 mM)	0

[a] Details of the experimental conditions are described in Experimental. [b] The rate of ATP synthesis in each case was calculated from the light output observed with the internal ATP standard, and so the minor effects that some compounds exerted on the ATP Monitoring Reagent were taken into account. [c] DCCD=*N,N'*-dicyclohexylcarbodiimide.

is recommended to correct the effect of inhibitors and other disturbing agents on light output.

Results. The progress curve of ATP formation catalyzed by *E. coli* membrane vesicles is presented in Fig. 1. Practically no activity is observed in the absence of succinate, which is the substrate of electron transport chain (Fig. 1). The reaction proceeds linearly up to several millivolts unless the reaction is artificially disturbed; *e.g.* by the addition of 2,4-dinitrophenol, the classical uncoupler of oxidative phosphorylation, the reaction stops immediately (Fig. 1). Table 1 summarizes the effects of various well-known inhibitors of oxidative phosphorylation and/or respiration on ATP formation. It is clearly seen that each inhibitor severely decreased the rate of ATP synthesis. In the absence of ADP, the acceptor of orthophosphate, little activity could be demonstrated (Table 1).

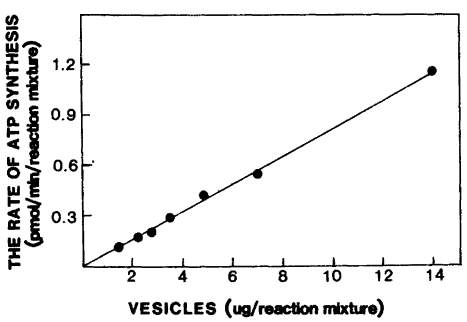

Fig. 2. Dependence of ATP synthesis on the concentration of membrane vesicles in the reaction mixture.

The rate of ATP synthesis increased linearly with the increase in the concentration of vesicles in the reaction mixture (Fig. 2). 3.5 μg of membrane vesicles were generally used for routine assays.

The precision of this kinetic assay was determined by using aliquots of the same preparation of vesicles in ten different reaction mixtures. The arithmetic mean of the measured activities was 83.9 nmol/min mg vesicles, the range was 9.1 % of the mean value, and the standard deviation was 2.3.

Discussion. In this paper we describe a convenient, precise, and highly sensitive luminometric method for the determination of ATP synthesis catalyzed by the "inside-out" membrane vesicles of *E. coli.* That the measured ATP synthesis is due to oxidative phosphorylation coupled to the electron transport chain is supported by the following observations. First, succinate, the substrate of the electron transport chain, was necessary for the reaction. Furthermore, in the presence of the well-known inhibitors of oxidative phosphorylation and respiration, ATP synthesis was completely inhibited.

Tsuchiya[9] has also described a method based on the luciferin–luciferase reaction for the determination of ATP synthesis catalyzed by *E. coli* membrane vesicles. However, our new method has several advantages over that presented by Tsuchiya.[9] First of all, owing to the everted membrane vesicles our assay is continuous, and so accurate kinetic studies are possible. ADP concentration of the reaction mixture cannot be accurately controlled by the Tsuchiya's[9] assay. Furthermore, in that method it is difficult to change the concentration of ADP in the reaction mixture; for each ADP concentration different membrane vesicles have to be made, and this naturally decreases the precision. By the "right-side-out" vesicles used by Tsuchiya[9] the ATP synthesized remains inside the vesicles, inaccessible to the luciferin–luciferase system, until it is extracted by perchloric acid. The extraction procedure with the accompanying centrifugation and neutralization steps complicate the assay and decrease the precision. The perchloric treatment also decreases the sensitivity of the ATP assay considerably.

Owing to the right-sidedness of the vesicles the method presented by Tsuchiya[9] should be used whenever the experiments have to be carried out at strictly physiological conditions. On the other hand, when reliable and accurate kinetic information are required our method should be preferred. Hence these two methods for the assay of ATP synthesis in oxidative phosphorylation are complementary and not exclusive ones.

Acknowledgement. The authors gratefully acknowledge Dr. Timo Lövgren (LKB Wallac, Finland) for donating the ATP Monitoring Reagent.

1. Boyer, P. D. and Bryan, D. M. *Methods Enzymol. 10* (1967) 60.
2. McCarthy, J. E. and Ferguson, S. J. *Eur. J. Biochem. 132* (1983) 425.
3. Slater, E. C. *Methods Enzymol. 10* (1967) 19.
4. Nishimura, M., Ito, T. and Chance, B. *Biochim. Biophys. Acta 59* (1962) 177.
5. Cox, G. B., Newton, N. A., Gibson, F., Snoswell, A. M. and Hamilton, J. A. *Biochem. J. 117* (1970) 551.
6. Strehler, B. L. *Methods Biochem. Anal. 16* (1968) 99.
7. Lemaster, J. J. and Hackenbrock, C. R. *Eur. J. Biochem. 67* (1976) 1.
8. Lundin, A., Thore, A. and Baltscheffsky, M. *FEBS Lett. 79* (1977) 73.
9. Tsuchiya, T. *J. Biol. Chem. 251* (1976) 5315.
10. Hempfling, W. P. and Hertzberg, E. L. *Methods Enzymol. 55* (1979) 164.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. K. *J. Biol. Chem. 193* (1951) 265.
12. Lundin, A. *Methods Enzymol. 57* (1978) 55.

Received September 23, 1983.