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A Spectrophotometric Assay of ATP Synthesized by Sarcoplasmic Reticulum

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Abstract

The problems encountered with a coupled enzyme assay for ATP using glucose, hexokinase and glucose-6-phosphate dehydrogenase are discussed and a modification where fructose and glucose-phosphate isomerase were substituted for glucose is described. This modified assay was used successfully to measure the ATP synthesized by reversal of the sarcoplasmic reticulum ATPase. ATP synthesized by adenylate kinase contaminating the sarcoplasmic reticulum was easily corrected for by a subtraction procedure.

Introduction

It is now well established that the calcium pump of the sarcoplasmic reticulum (SR) can work in the reverse direction under certain conditions. Under these conditions calcium is pumped out of the SR and ATP is synthesized at the rate of 1 mol per 2 mol of Ca^{2+} transported through the membrane (Makinose and Hasselbach 1971; Panet and Selinger 1972).

The synthesis of ATP is normally assayed by measuring the amount of ^{32}P-labelled inorganic phosphate that is incorporated into ATP. The labelled phosphate group of the ATP is transferred to glucose by the use of hexokinase (EC 2.7.1.1). The labelled glucose-6-phosphate is isolated by paper chromatography and its ^{32}P content measured with a scintillation counter.

This procedure is laborious and time consuming especially when the rate of ATP synthesis is studied. A continuous spectrophotometric assay would have many advantages. The ATP content of many tissues and fluids has been assayed spectrophotometrically with the use of glucose, hexokinase, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and $NADP^+$ according to the following coupled reactions:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP},$$

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphogluconate} + \text{NADPH}.$$

The amount of $NADP^+$ converted to NADPH is measured at 340 nm in a spectrophotometer (Lamprecht and Trautschold).

In this paper the modifications of this system needed to allow a continuous assay of ATP production by SR in the presence of ADP are described.

Methods

SR was prepared from the psoas muscles of laboratory rabbits essentially by the method of Martonosi *et al.* (1968). In some cases, the SR was further purified by centrifugation for 14 h at 25 000 rev/min on a linear sucrose gradient (0·74–1·4 M) in a Beckman Spinco SW 25·1 rotor.

Calcium uptake by the SR was carried out using 2 mM acetyl phosphate as substrate essentially as described by Makinose and Hasselbach (1971). Efflux of calcium was started by the addition of EGTA and ADP.

Protein concentrations were estimated by the method of Lowry *et al.* (1951).

Hexokinase, glucose-6-phosphate dehydrogenase and glucosephosphate isomerase (EC 5.3.1.9) were purchased from Boehringer, Mannheim. Acetyl phosphate was obtained from Calbiochem. B.D.H. Aristar grade glucose and B.D.H. glucose-free D-fructose were used.

Results

Preliminary experiments showed that even in the absence of SR and ATP, or ADP, significant reaction rates were obtained with the glucose, hexokinase, glucose-6-phosphate dehydrogenase, $NADP^+$ assay system. These reaction rates were independent of calcium and hexokinase concentrations but dependent on glucose and glucose-6-phosphate dehydrogenase concentrations. Although not investigated in depth these reactions appeared not to be due to contamination of either of the coupling enzymes or the glucose but to the glucose-6-phosphate dehydrogenase acting on the glucose.

To avoid this problem, fructose was used instead of glucose and glucosephosphate isomerase was used to convert the fructose-6-phosphate to glucose-6-phosphate. The assay system for ATP was now as follows:

$$\text{ATP} + \text{fructose} \xrightarrow{\text{hexokinase}} \text{fructose-6-phosphate} + \text{ADP},$$

$$\text{fructose-6-phosphate} \xrightarrow{\text{glucosephosphate isomerase}} \text{glucose-6-phosphate},$$

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphogluconate} + \text{NADPH}.$$

This system proved to be most satisfactory for estimating small quantities of ATP as there was no detectable reaction rate in the absence of SR.

When this assay system was used to measure the ATP synthesis associated with the efflux of calcium from loaded SR vesicles, a biphasic reaction rate was recorded (see Fig. 1). The first part of the reaction was dependent on the amount of calcium initially present in the vesicles, whilst the second part was not. This latter portion is due to a second reaction catalysed by the SR preparation. As this reaction was inhibited by AMP it is almost certainly due to adenylate kinase (EC 2.7.4.3), a known contaminant of SR preparations (Weber *et al.* 1966). When the SR was purified by sucrose density gradient centrifugation the rate of this reaction was substantially reduced.

By extrapolating the second linear portion of the reaction rate (the adenylate kinase reaction) back to zero time (when EGTA was added) it is possible to calculate the amount of ATP synthesized during the efflux of calcium. The initial rate minus the rate of the second part measures the rate of formation of ATP by calcium efflux.

Fig. 2 shows the results of an experiment where SR was loaded with varying amounts of calcium, and ATP synthesis was measured as above after the addition of

EGTA and ADP. The slope of the line is very close to the theoretical value of 1 mol of ATP synthesized per 2 mol of calcium transported across the membrane (Makinose and Hasselbach 1971; Panet and Selinger 1972). The rate of formation of ATP varied from 112 nmol min^{-1} (mg protein)$^{-1}$ at the highest calcium content to 45 nmol min^{-1} (mg protein)$^{-1}$ at the lowest calcium content tested.

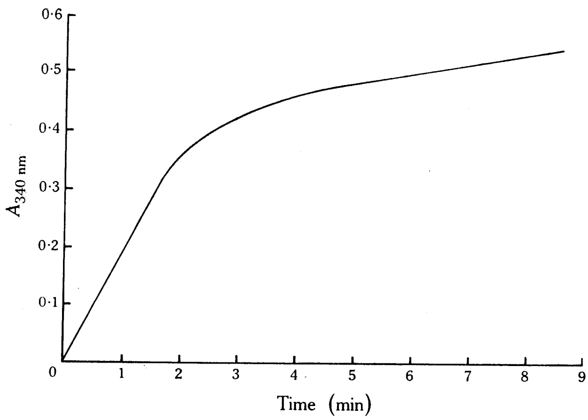

Fig. 1. Spectrophotometric recording of ATP synthesis after addition of EGTA to SR vesicles (0·32 mg protein/ml) previously loaded with 250 nmol calcium using 2 mM acetyl phosphate as substrate (pH 7·0, 25°C). Efflux of calcium was started by adding 2 mM ADP plus 2 mM EGTA to the SR vesicles in the initial reaction medium after allowing 15 min for uptake. The concentrations of other reactants in the 2 ml reaction were: fructose, 10 mg/ml; $NADP^+$, 0·5 mg/ml; hexokinase, 1·4 units/ml; glucose-6-phosphate dehydrogenase, 1·7 units/ml; glucosephosphate isomerase, 2·1 units/ml; P_i, 20 mM; KCl, 40 mM and Mg^{2+}, 7 mM.

Discussion

A slow reaction rate in the absence of hexokinase has been reported for the ATP assay involving glucose, coupling enzymes and $NADP^+$. This slow rate was ascribed to hexokinase contamination of the glucose-6-phosphate dehydrogenase and occurred in the presence of ATP (Lamprecht and Trautschold 1974). The slow reaction rate measured in the present study was of a similar magnitude to that described above but occurred in the absence of either ATP or ADP. The rates were dependent on the glucose concentration and were increased when the glucose-6-phosphate dehydro-

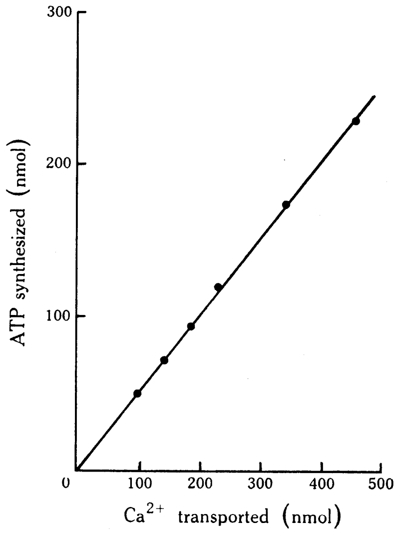

Fig. 2. Relationship between amount of ATP synthesized by calcium efflux from SR and the amount of calcium previously loaded into the SR (0·64 mg protein) using 2 mM acetyl phosphate substrate. The amount of calcium loaded into the SR was varied by altering the total amount of Ca^{2+} in the initial uptake medium. Efflux and other assay conditions were as in the legend to Fig. 1.

genase concentration was increased but not when hexokinase was added. The magnitude of the reaction measured over a 24-h period was far too large for the reaction to be due to a contaminant of the glucose and therefore was probably due to the action of glucose-6-phosphate dehydrogenase on glucose itself. Anderson and Nordlie (1968) have shown that under certain conditions the glucose dehydrogenase activity of glucose-6-phosphate dehydrogenase can be up to 20% of the glucose-6-phosphate dehydrogenase activity. When fructose and glucosephosphate isomerase were substituted for the glucose the slow reaction was eliminated. This was important as a method capable of measuring slow rates of synthesis of ATP was required.

The presence of adenylate kinase in SR preparations (Weber *et al.* 1966) is a potential source of error in this coupled enzyme type of assay for ATP. Fortunately the contamination can be reduced by further purification of the SR by sucrose density gradient centrifugation. The amount of ATP synthesized by the remaining adenylate kinase can be easily determined as described in the Results section and separated from the ATP synthesized by calcium ion movement.

Fig. 2 shows that when allowance is made for the adenylate kinase component the ATP synthesis measured by this technique for a range of calcium ion movements is very close to the previously reported value of 1 mol of ATP for every 2 mol of Ca^{2+} transported (Makinose and Hasselbach 1971; Panet and Selinger 1972). The method therefore offers a quick and accurate means for the estimation of small quantities of ATP synthesized over a period of time by membrane systems such as SR. The substitution of fructose and glucosephosphate isomerase for glucose should also improve the accuracy of ATP determinations in non-membrane systems.

Acknowledgments

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