Mitochondrial ATP Contents During Phosphorylation

E. J. Harris

Department of Biophysics,
University College,
London, W.C.1

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Abstract

Using the method of quick separation by centrifugation through a layer of silicone the contents of ATP in mitochondria during active phosphorylation of external ADP have been determined. The rate of phosphorylation is linearly related to the ATP content (in state 3) and this relation is independent of the substrate. The rate of phosphorylation and the associated internal ATP content were both diminished as incubations were carried out using the mitochondrial protein at increasing concentrations.

Introduction

When ADP is added to a suspension of rat liver mitochondria it exchanges with the internal ADP and ATP.1 When phosphate and a substrate are present the internal ADP/ATP ratio is kept at a value depending on the phosphorylation process, so the exchange of the external ADP with the internal nucleotides ultimately leads to its replacement by a nearly stoichiometric amount of ATP. The aim of the present work was to establish the relation between the internal ATP content during phosphorylation and the rate of phosphorylation of the external ADP. According to the formulation of Heldt,2 the proportion of ATP, exported in the total nucleotides emerging in exchange for entering ADP, is strictly proportional to the internal ATP; hence the phosphorylation flux should be related to the internal ATP by a factor which has to be evaluated.

In making the series of experiments described below it was noticed that the rate of phosphorylation per unit of mitochondrial protein was inversely related to the concentration at which the suspension was being used. This suggests that some inhibitory factor is present in the mitochondrial preparation.

Methods

Rat liver mitochondria were prepared by a variant of Schneider’s3 method using 0.5 mM EGTA and 0.05% bovine serum albumin (defatted and dialysed) in the 250 mM sucrose solution for homogenization and two subsequent washes. The wash media also contained 5 mM KCl which improved the yield of mitochondria without causing loss of cytochrome or extensive co-sedimentation of reticular membranes. The final stock suspension was made at about 50 mg/ml in 300 mM sucrose. Protein was measured by a biuret method.4
Rates of phosphorylation and the associated internal ATP contents were found by taking samples from incubations in an open jacketed vessel exposed to a stream either of oxygen for the tris-buffered media or to 95% oxygen + 5% carbon dioxide for the bicarbonate buffered media. A glucose + hexokinase trapping system was used in the incubation so that the phosphorylation rate could be found from the amount of glucose-6-phosphate formed in a given time. This method ensured that the extramitochondrial ATP was kept at a low concentration. This would not have been the case if conventional oxygen electrode–ADP–cycles were used. The medium contained KCl, 150 mM, mannitol 60 mM, MgCl₂ 0.7 mM, tris-phosphate 5 mM and either tris-chloride pH 7-4 20 mM or NaHCO₃ 20 mM. Substrates were added as tris salts at 5 mM unless otherwise stated. Most experiments were made at 20° but the temperature of the vessel could be adjusted to 10° or 30°.

For the phosphorylation reaction the stock mitochondrial suspension was diluted with medium to protein concentrations between 0-7 and 6 mg/ml. After 3–4 min incubation a 0-5 ml sample was withdrawn. This was transferred to a polypropylene centrifuge tube (5 cm bore length, 0-4 cm diameter) which had previously been prepared by loading with 0-1 ml 1-5 M perchloric acid beneath about 3 mm thickness of silicone (G. E. Versilube F.50). As each sample was taken it was centrifuged for 45 sec in a Coleman Microfuge which sufficed to bring the mitochondria into the acid beneath the oil. A specially constructed miniature swing-out head was used.

The analysis of the acid extract from the first sample provided a value for the content of ATP before phosphorylation was initiated. After the sample had been taken, additions of hexokinase (5 units, Sigma type F.300 dissolved in 5% albumin) and ADP to 120 μM were made. Following this, 5 successive 0-5 ml samples were withdrawn at intervals of about 40 sec. Each was centrifuged as described above and the supernatants were acidified promptly with 0-1 ml 1-5 M perchloric acid. Later, the supernatants were transferred from above the silicone layers by a Pasteur pipette into tubes cooled in ice. The precipitated protein was removed by centrifugation and the fluids were brought to pH about 5 by addition of 5 M KOH + 1 M K acetate mixture. The mitochondrial acid extracts were recovered from beneath the silicone in each tube and transferred to cooled tubes and also brought to pH 5 as before. The supernatants were assayed enzymatically for glucose-6-phosphate in a spectrophotometer and the mitochondrial extracts were analysed fluorimetrically for ATP. Methods for both have been described by Williamson and Corkey.

**Results**

For measuring the rate of phosphorylation of glucose in presence of hexokinase and ADP tests were made to ensure that sufficient enzyme and ADP were present to stimulate the process maximally. At protein concentrations of about 1 mg/ml oxidative phosphorylation was half maximally stimulated with only 11 μM ADP (in presence of hexokinase and glucose) but 50–60 μM was required when the protein was at 4–6 mg/ml and rates of phosphorylation, rather than of respiration, were measured. Routinely 120 μM ADP was added. It was confirmed that with at least 1 unit hexokinase per 3 ml incubation medium the respiration was maximal but routinely 5 units were added.

When phosphorylation of the glucose was initiated by ADP + hexokinase the ATP