FORMATION OF CREATINE PHOSPHATE FROM CREATINE AND ^{32}P-LABELLED ATP BY ISOLATED RABBIT HEART MITOCHONDRIA

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Summary

With ATP [γ-^{32}P] we have demonstrated directly that mitochondrial creatine phosphokinase catalyzes the formation of large amounts of creatine phosphate with mitochondria generated ATP as substrate rather than added extramitochondrial ATP.

Introduction

The mitochondrial bound creatine phosphokinase (ATP-creatine phosphotransferase EC2.7.3.2.) has been demonstrated electrophoretically to be a separate isoenzyme (1,2,3). The control of creatine in mitochondria respiration was suggested by Bessman in 1960 (4) and verification of the respiratory control of muscle mitochondria was published by Bessman and Fonyo (5) in 1966. The functional significance of this bound creatine phosphokinase has also been emphasized in relation to the effect of creatine on muscle cell growth (6). Saks et al (7,8) on theoretical and kinetic grounds suggested that mitochondrial creatine phosphokinase has a preference for mitochondria generated ATP as compared to extramitochondrial ATP and furthermore, that the mitochondrial creatine phosphokinase may be coupled to the nucleotide translocase. With ^{32}P-labelled ATP, and using our rapid automated method of analysis of phosphorylated compounds (9,10), we have demonstrated directly that the mitochondrial creatine phosphokinase catalyzes the formation of larger amounts of creatine phosphate with the mitochondria generated ATP as substrate rather than added ATP as substrate.

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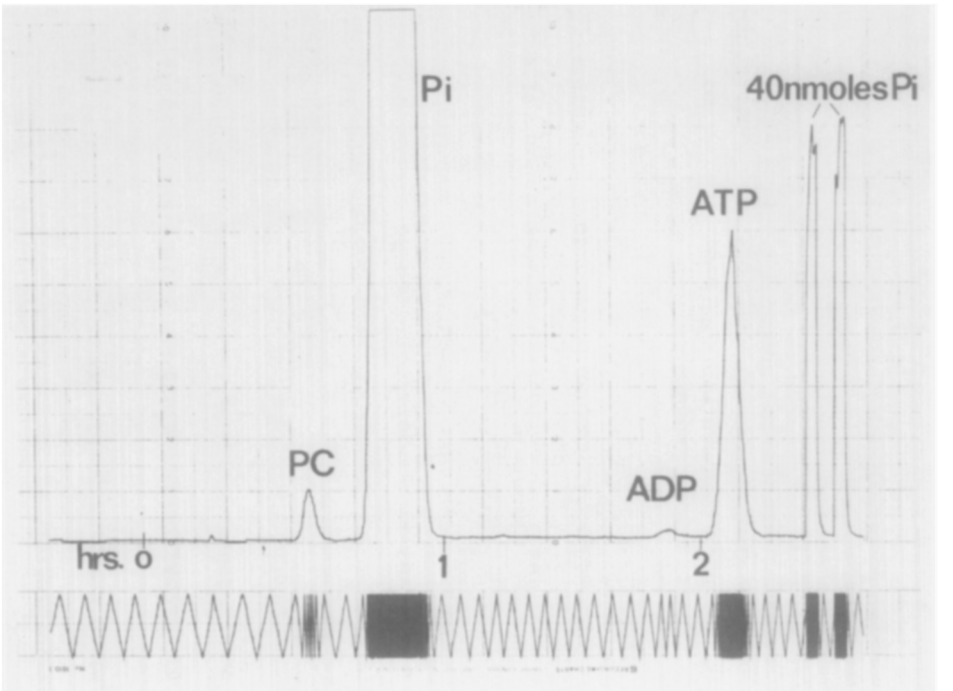

Figure 1. Typical chromatogram illustrating separation of the compounds of interest: PC, P_i, ADP, ATP. The 40-nmol P_i standards used for quantitation of the individual peaks were measured in duplicate in order to improve precision. The integrator tracing is also shown at the bottom of the figure.

Methods

Preparation of rabbit heart mitochondria: Mitochondria were isolated from the rapidly excised ventricles of New Zealand albino rabbits according to the method of Von Korff (11). The homogenization medium was of the following composition: mannitol, 0.35 M; EDTA, 0.1 mM; Tris phosphate, 10 mM at pH 7.2. The mitochondria suspending medium contained, in addition to the above, 0.5% dialyzed bovine serum albumin. A section of the prepared mitochondrial pellet observed with the electron microscope contained mostly intact mitochondria. Respiratory control ratios were consistently 9 or higher in the absence of Mg^{2+} or 3 and higher with Mg^{2+}. The mitochondrial protein content was determined by the method of Lowry et al (12).

Experiments were carried out as follows. The incubation mixture contained mannitol, 0.25 M; KCl, 10 mM; α-ketoglutarate, 5.0 mM; creatine, 10 mM; EDTA, .01 mM; dithiothreitol, 0.1 mM; P_i, 5.0 mM; mitochondria, 0.1 ml containing 0.8 to 1.5 mg of protein; ATP, 0.8 to 1.0 mM. The vessels containing the mixture, except ATP, were incubated in a Dubnoff shaker for 5 minutes to allow temperature equilibration. Then, 0.1 ml of ATP (Sigma) containing up to 20 μCi of ^{32}P-labelled ATP (New England Nuclear) was added to start the reaction. The temperature was 30°C and the total volume of incubation mixture in each vessel was 1.0 ml. At the designated times, (5, 10, 15 sec.), 0.3 ml of 3 M perchloric acid ($HClO_4$) was added to stop the reaction. Subsequent manipulations were then carried out at 0 to 4° C.

Table I

Effect of Atractyloside and CCCH on mitochondrial CP formation*

	Incubation Time (sec.)	CP nmol	CP cpm/nmol	CP Total Counts	ATP nmol	ATP cpm/nmol	ATP Total Counts	ADP nmol	Total Nucleotides nmol
Control	5	8.58	8644	74167	29.4	24039	706728	2.40	31.8
	10	12.20	10133	123625	28.5	19870	566294	2.86	31.4
CCCH 4×10^{-6} M	5	2.26	26475	59833	26.0	30673	797494	4.98	31.0
	10	3.49	30424	106181	24.7	29187	702705	6.43	31.1
Atractyloside 5×10^{-6} M	5	2.81	24953	70119	25.9	29866	773521	4.38	30.2
	10	3.31	30890	102274	24.8	29977	743432	6.65	31.5

*One ml of incubation mixture contained α-KG, 5mM; ATP [γ-^{32}P], 0.8 mM; Mg^{++}, 1.5 mM; P_i, 5 mM; Cr, 10 mM; DTT, 0.1 mM. Incubation temperature, 30°C.
CCCH is Carbonylcyanide m-chlorophenyl hydrazone, DTT is Dithiothreitol.
Values shown were contained in 200 µl of neutralized acid extract.

The acidified mitochondrial mixture was kept for a few minutes on ice and was then transferred to a centrifuge tube and the protein removed by centrifugation for 15 min. at about 2000 x g. The resulting supernatant solution was removed, the precipitate was washed once with ice water and the washing was added to the supernatant solution. The whole was then neutralized to a bromothymol blue end-point with K_2CO_3; the $KClO_4$ precipitate formed was removed by centrifugation and washed twice with ice water, the washings being pooled with the supernatant and the whole brought to a final volume of 4 ml.

A portion of this neutralized extract, usually about 200 µl, was placed on a 150 mm x 3 mm anion exchange column connected to the automatic phosphate analyzer (9, 10) and eluted according to methods described in detail elsewhere (13). The chromatogram in Figure 1 illustrates the kind of routine separation obtained in many runs that were made. The degree of separation shown was required in order to provide adequate quantitation of the phosphate compounds by integration of the chromatographic peaks and also to permit precise quantitation of the radioactivity in each of the peaks (13). The 40-nanomole standard used for calculating the amount of phosphate in each peak is also shown in the figure.

The radioactivity in each peak was quantified by collecting the effluent from the colorimeter in plastic minivials (0.5 ml per vial) with the aid of a fraction collector set for one-minute fractions. About 5.5 ml of ACS scintillation fluid (Amersham-Searle) was added to each vial with vigorous mixing and all vials were capped tightly and counted in a Delta-300 scintillation counter (Searle Analytic). Specific activities (cpm/nanomole) were calculated after suitable corrections had been applied to allow for the decay of ^{32}P.

Results

The results of one experiment are summarized in Table I. This is representative of 5 similar experiments, and the consistency of the results of these experiments is illustrated in Fig. 2.

As is shown in Table I, the formation of creatine phosphate was rapid in the control experiments. In 5 seconds about 8.6 nmoles of creatine phosphate were formed in a 200 µl aliquot of the diluted extract of reaction mixture. A "zero time" experiment produced no observable creatine phosphate. After 5 sec. in the control experiment the specific activity of the creatine phosphate was about 36 percent of the specific activity of the medium ATP. Therefore, the mitochondrially generated ATP which is not labelled (since P_i is not labelled) must have been used to form the other 64 percent of the creatine phosphate measured. It is clear that the amount of mitochondrially generated ATP per ml of reaction mixture must be relatively small in comparison with the medium ATP concentration as it is limited by the amount of ADP formed by the forward reaction: $ATP + C \rightleftarrows ADP + CP$, with the possible addition of some ADP from the

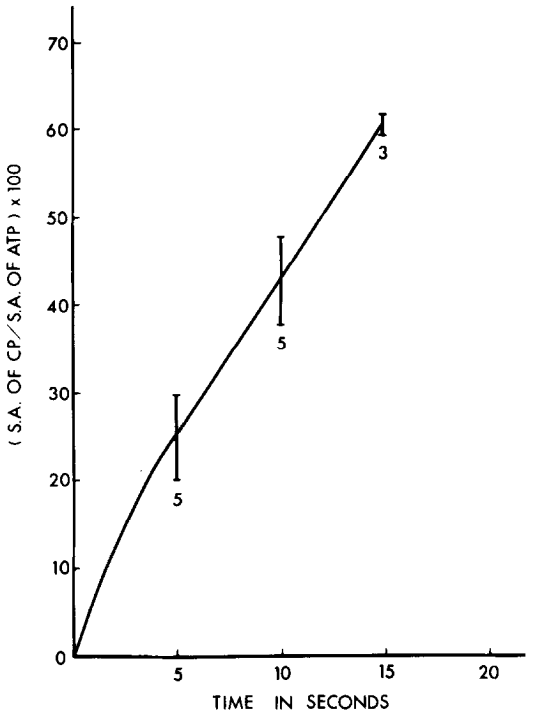

Figure 2. Increase of the relative specific activity of creatine phosphate with time. Numbers under each point indicate the number of experiments performed.

adenylate kinase reaction. We therefore conclude that the large percentage of unlabelled creatine phosphate formed was derived from the reaction of mitochondrial creatine phosphokinase with ATP newly generated by oxidative phosphorylation.

When the mitochondrial phosphorylation was uncoupled by a low concentration of carbonyl cyanide chlorophenylhydrazone (CCCH), or inhibited by the translocase inhibitor, atractyloside, the amount of creatine phosphate formed was decreased to about one-third or less (5 sec. interval, Table I) than that without such inhibitors. This provides further evidence for the relatively high efficiency of mitochondrial creatine phosphate formation using mitochondria generated ATP as substrate. As expected, the specific activity of creatine phosphate formed in the presence of CCCH or atractyloside was close to the specific activity of

the added ATP. When the total counts of the creatine phosphate formed in the control experiment and in the presence of CCCH or atractyloside are compared, they are observed not to be significantly different. The contribution of added extramitochondrial ATP in the formation of creatine phosphate thus remained constant in the presence or absence of the inhibitors.

The contribution of externally added ATP to the formation of creatine phosphate did increase with time as shown in Fig. 2 indicating most probably that the external ATP has access to at least a high proportion of the active sites of mitochondrial creatine phosphokinase.

Discussion

The present communication directly confirms the conclusions of Saks et al (7,8) that mitochondrial creatine phosphokinase can utilize ATP newly generated by the mitochondria more effectively than ATP added externally, and that a coupling of mitochondrial creatine phosphokinase with the nucleotide translocase is a distinct possibility. The preference of mitochondrial creatine phosphokinase for mitochondria generated ATP also strengthens our contention (4,5) that the creatine-creatine phosphate system has a shuttle function in high energy phosphate bond transport in addition to its energy storage and buffering role.

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