Compartmentation of Mitochondrial Creatine Phosphokinase

I. DIRECT DEMONSTRATION OF COMPARTMENTATION WITH THE USE OF LABELED PRECURSORS*

Susan Erickson-Viitanen§, Paul Viitanen§, Paul J. Geiger, William C. T. Yang, and Samuel P. Bessman

From the Department of Pharmacology and Nutrition, University of Southern California School of Medicine, Los Angeles, California 90033

Mitochondrial creatine kinase was first proposed to act as a functional component in respiratory control in 1966 (Bessman, S. P., and Fonyo, A. (1966) Biochim. Biophys. Res. Commun. 22, 597-602). Since that time, evidence has accumulated to support the theory of a creatine-phosphorylcreatine shuttle mechanism involved in supplying energy for aerobic muscle contraction (Bessman, S. P., and Geiger, P. J. (1981) Science 211, 448-452). To demonstrate directly the interaction between mitochondrial oxidative phosphorylation and that of creatine phosphate synthesis, we have studied the labeling of adenine nucleotides and creatine phosphate with $^{32}$P$_2$P0$_4$ or [$\gamma$-32P]ATP over a range of adenine nucleotide concentrations incubated with rabbit cardiac and rat skeletal muscle mitochondria. An apparent direct mitochondrial ATP contribution to creatine phosphate synthesis was observed that varied inversely with the total adenine nucleotide present in the reaction system. This reaction of de novo synthesized ATP with creatine phosphokinase prior to equilibration with the total ATP pool was observed regardless of the entry point of electrons from oxidizable substrate into the electron transport chain. This special relation was not observed for added yeast hexokinase in forming glucose 6-phosphate. Mitochondria could not synthesize creatine phosphate in the presence of atractylloside, thus underscoring the requirement for adenine nucleotide translocase-linked transport of ATP prior to reaction with the bound creatine phosphokinase. These studies show that there is coupling or compartmentation of ATP synthesis and transport with creatine phosphate formation in heart and skeletal muscle mitochondria.

The energy demands of cardiac and skeletal muscle are substantial. Estimates suggest, for instance, that the contraction-relaxation cycle in heart muscle may utilize up to 80% of the total ATP turnover (1). At the same time, there is need for regulation and coordination of energy production and utilization—reviewed in Refs. 2 and 3). The localization of distinct isoenzymes of creatine phosphokinase at sites of energy production (mitochondria) (4) and energy utilization (myofibrils (5), sarcoplasmic reticulum (6), and plasma membrane (7), apparent compartmentation of adenine nucleotides (8, 9), and the strict correlation of muscle function with content of creatine phosphate (2, 10) are among the factors that have led to the proposal of a central role for creatine phosphokinase in the transport of energy in muscle (2, 10-13).

That the localization of creatine phosphokinase within myofibrils bears functional importance was suggested on kinetic grounds (7, 14) and has recently been directly demonstrated in our laboratory (15).

Bessman and Fonyo's polarographic studies of the creatine acceptor effect observed with muscle mitochondrial (16) established that efficient creatine phosphate synthesis occurred under conditions of oxidative phosphorylation. Other workers have corroborated and extended these findings (17, 18). On the basis of a kinetic analysis of creatine phosphokinase under conditions where the rate of oxidative phosphorylation was varied, a functional or physical coupling between mitochondrially bound creatine phosphokinase and the adenine nucleotide translocase was proposed (19, 20). There is also a recent report of a decreased apparent $K_m$ for ATP for bound creatine phosphokinase during respiration compared to inhibited mitochondria with ATP supplied through an external regenerating system (13).

In our laboratory, apparent creatine phosphokinase compartmentation has been suggested from the use of labeled precursors (21). Comparison of mass action ratios and published $K_m$ values have also been used by others to infer compartmentation (22). However, both the kinetic and isotopic approaches have been criticized (22-24) and the range of apparent $K_m$ values obtained for ATP with the enzyme studied under different conditions and stages of purification (13, 17, 19, 25) has led to some confusion as to the significance of this parameter for compartmentation (see, for example, discussions in Ref. 23).

We report here studies on the interaction of mitochondrially bound creatine phosphokinase with oxidative phosphorylation as measured by isotope incorporation into newly synthesized ATP and creatine phosphate. Our results provide strong evidence for a compartmentation of creatine phosphate synthesis which was found to be dependent on the total concentration of adenine nucleotides and involves competition between de novo synthesized ATP and external ATP pools for interaction at the active site of creatine phosphokinase.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of Grant HL 07042 from the Department of Health, Education and Welfare, United States Public Health Service. The work described here is from a dissertation submitted to the Graduate School of the University of Southern California in partial fulfillment of requirements for the degree of Doctor of Philosophy, 1981. Present address, Department of Physiological Chemistry and Pharmacology, Roche Institute of Molecular Biology, Nutley, NJ 07110.

$ Recipient of Grant HL 07042 from the Department of Health, Education and Welfare, United States Public Health Service. Present address, Department of Biochemistry, Roche Institute of Molecular Biology, Nutley, NJ 07110.
EXPERIMENTAL PROCEDURES

Materials—Adenine and pyridine nucleotides, creatine, yeast hexokinase type P-300, pyruvate kinase type III, P-enolpyruvate, atracylsamide, Hepes, TMPD, 2-amino-2-hydroxymethyl-1,3-propanediol base, Ap,A, and p-chloromercuribenzoate were purchased from Sigma. Alamine 336 was obtained from McKersor Corp., Minneapolis, MN, and Freon-TF from Miller-Stephenson Co., Los Angeles. The [3P]P, [3P]PO, and [3P]ATP were purchased from New England Nuclear.

Isolation of Mitochondria—Mitochondria from the ventricles of New Zealand white rabbits (4-6 lbs) were prepared as described previously (21) with substitution of Tris-Cl for Tris/PO as buffer. Mitochondria were suspended to a concentration of between 8 and 12 mg/ml in suspending medium containing 0.35 m mannitol, 10 mM Tris-Cl (pH 7.2), 0.1 mM EDTA, and 0.5% bovine serum albumin. Mitochondria from the hind leg muscles of male Sprague-Dawley rats (200-300 g) were isolated after homogenization of tissue with a Polytron homogenizer (Brinkmann Instruments, Inc.). Animals were killed and exsanguinated and the hind leg muscles were immediately removed and cooled in chilled homogenization medium containing 160 mM KCl, 10 mM Hepes (pH 7.4), and 0.25 mM EDTA. The tissue was minced with scissors, passed through a Harvard tissue press and weighed. 4-4.5 g of muscle were again minced with scissors in 10 volumes of homogenization medium and the tissue was homogenized with a Polytron for 15 s at a setting of 4.0 followed by 15 s at a setting of 4.7. The homogenate was centrifuged at 500 x g for 15 min and the supernatant was passed through a layer of fine nylon mesh. Mitochondria were isolated after centrifugation at 10,000 x g for 10 min. Mitochondria were washed once by resuspension in 20 ml of homogenization medium and centrifuged with a few drops of homogenization medium to remove the thin white layer covering the pellet. Mitochondria were washed once more by suspension in 20 ml of homogenization medium and centrifuged again at 10,000 x g. Washed mitochondria were suspended to a concentration of between 6 and 10 mg/ml in suspending medium containing 0.35 m mannitol, 10 mM Tris-Cl (pH 7.2), 0.1 mM EDTA, and 0.5% bovine serum albumin. Protein was determined by standard methods (26, 27).

Polarographic Determinations—The rates of oxygen consumption, ADP/O ratio, and respiratory control ratio were determined with the use of a standard Clark electrode and a GME Model KM Oxygen. The reaction medium contained 0.25 m mannitol, 10 mM KCl, 10 mM Tris-Cl, 0.1 mM EDTA, 3.5 mM substrate (ketoglutarate, succinate, or ascorbate plus 0.75 mM TMPD), and 5.0 mM potassium phosphate buffer (pH 7.2). Small volumes of nucleotide and MgCl2 were added to achieve final concentrations of 0.054-0.298 mM ADP and 1.4 mM MgCl2. All reactions were carried out at 28 °C.

Incubation Conditions—All reactions were carried out individually in polystyrene test tubes (16 x 100 mm). The 1 ml-reaction mixture was that of Yang et al. (21) except for 10 mM Hepes, 2.5-5 mM pyruvate, and 0.035-0.7 mM TMPD, final pH 7.2. The MgCl2 concentration was 1.0 mM in the presence of 0.2 mM or less nucleotide and 2.0 mM for nucleotide above 0.2 mM. Atracylsamide was prepared fresh in water the day of the experiment and added to a final concentration of 10 μM.

The external ATP-regenerating system consisted of 30 units of pyruvate kinase and 2.4 mg P-enolpyruvate. This level of pyruvate kinase was 3- to 4-fold excess over that necessary to maintain levels of ATP and ADP for mitochondrial respiration in the presence of creatine and 80 μM ATP.

Yeast hexokinase, when added as an external kinase, was added to tubes just before the addition of mitochondria.

Reactions were all initiated with a mixture containing 12-15 μCi of [3P]P, experiments being started with isotope plus creatine, labeled ATP experiments with nucleotide. Individual experiments are further described in the legends to figures and tables (see "Results"). For all experiments, aliquots of mitochondria equivalent to 0.5-1.2 mg of protein were temperature-equilibrated with the reaction mixture minus isotope and creatine phosphokinase substrate for 3 min at 0°C. Starting mixture was added and the tube was shaken by hand in the water bath for 5-s reactions were terminated with 1.0 ml of 1.4 M HClO4. The 5-s reactions were reproducible to within ±5% in terms of net creatine phosphate formed. Quantities and specific activities of creatine phosphate and nucleotide present at zero time were determined on samples to which acid had been added prior to addition of the starting mixture.

Jacobus (28) reported that in contrast to rat heart mitochondria, solubilization of rabbit heart mitochondrial creatine phosphokinase by phosphatase was significant in 5-10 mM phosphate in as short an incubation as 5 min. A spectrophotometric assay was used to estimate the amount of creatine phosphate released after 5 min in a reaction medium containing 5 mM potassium phosphate. Only 9.0 ± 0.9% of the total enzyme was solubilized under these conditions. The discrepancy between our finding and that of Jacobus (28) is perhaps due to the different incubation medium used (0.25 m mannitol, etc., versus 125 mM KCl (28)). Vidal et al. (29) found that phosphate solubilization of pig heart mitochondrial creatine phosphokinase was enhanced in 100 mM KCl compared to 250 mM sucrose. The effect of the incubation conditions used in the compartmentation studies described below could, therefore, amount to only an insignificant loss of bound enzyme.

Separation of Mitochondrial from Extramitochondrial Compounds—Measurements of the incorporation of [3P]P into mito- and extramitochondrial ATP and inorganic phosphate were carried out on reactions terminated by the inhibitor stop technique (30). Incubations were carried out as described above except that the stop solution was 0.40 m of ice-cold homogenization medium containing 5.5 μM atracylsamide to inhibit adenine nucleotide translocase (21), 14 μg/ml of oligomycin to inhibit ATPase (32), 0.7 mM p-chloromercuribenzoate to inhibit phosphate transport (33), and 70 μM ApA to inhibit mitochondrial adenylate kinase (34). The final pH was 7.4.

We utilized the inhibitor stop method to verify the use of total ATP specific activity as a measure of ATP synthesis activity and to attempt to show that the specific activity of inorganic phosphate equaled the specific activity of newly synthesized mitochondrial ATP. This assumption as to the equality of inorganic phosphate and newly synthesized ATP specific activities is supported by experiments utilizing labeled ATP and unlabeled phosphate. The specific activity of P, and newly synthesized ATP was zero over the entire time course of the experiment. The apparent mitochondrial contribution to creatine phosphate synthesis obtained in the "reciprocal" experiment was identical with that observed with labeled inorganic phosphate as substrate. Thus, it seems that mixing of phosphate between mitochondrial and extramitochondrial spaces must be extremely rapid.

In regard to the stop solution per se—the chosen concentrations of atracylsamide and ApA were studied separately in the oxygraph where they yielded essentially 100% inhibition of ADP- and AMP-induced oxygen consumption, respectively. The stop solution containing all components caused respiration to cease as rapidly as detection with the Clark electrode permits. ApA had no significant effect on creatine phosphate synthesis. When the ApA was omitted from the stop solution, ATP, ADP, and AMP were found in approximately equal concentrations whereas with ApA in the medium, AMP was nearly undetectable. The same preparation of mitochondria was used for measurement of all nucleotide pool sizes.

After addition of stop solution, test tubes were vortex-mixed and the contents were rapidly transferred to microfuge cups and immediately added to 2 ml of neutralizing reagent (see "Materials"). After addition of the starting mixture, samples were handled in the same way except that the stop solution was extracted with Freon-Alamine. Since this procedure could, therefore, result in an insignificant loss of bound enzyme. The release of creatine phosphokinase after 5 min in our incubation medium containing 5 mM phosphate was significant in 5-10 mM phosphate in as short an incubation as 5 min.
ATP was recovered as glucose 6-phosphate.

Content and specific activities of creatine phosphate, P, glucose 6-phosphate, ADP, and ATP were determined using the phosphate analyzer (Alab Scientific Products, Inc., Los Angeles, CA) described previously (21, 37).

RESULTS

Analysis of Specific Activity Data—The specific activities of ATP and creatine phosphate were observed to approach that of inorganic phosphate asymptotically after addition of labeled P, to respiring mitochondria. Conversely, in the presence of added [γ-32P]ATP and unlabeled phosphate, specific activities of ATP and creatine phosphate decreased with time. With sufficiently short periods of assay, the specific activity increases in ATP and creatine phosphate were nearly linear and formation of ATP from creatine phosphate (CP) via the reverse reaction of creatine phosphokinase (CPK) was minimal. Furthermore, the total concentration of ATP was maintained at a constant level through oxidative phosphorylation acting on ADP produced by the action of Mg2+-ATPases.

Diagrammatically this situation can be described by the following reaction sequence:

\[
\begin{align*}
\text{OX PHOS} & \xrightarrow{k_1} \text{CPK} \\
P + ADP & \xrightarrow{k_2} \text{ATP} + Cr. \\
\text{Mg}^{2+} \cdot \text{ATPase} & \xrightarrow{h} \text{CP} + ADP
\end{align*}
\]

where the back reaction of creatine phosphokinase governed by \(k_3\) is negligible at small creatine phosphate levels formed in short incubation periods. The increase in ATP specific activity is thus a function of both the pool size into which counts from P, are incorporated and the concentration of mitochondria which determines the rates of oxidative phosphorylation, Mg2+-ATPase, and creatine phosphokinase activities present.

In a typical experiment, the rate of increase in ATP activity can be written

\[
\frac{\text{cpm ATP}}{\text{dt}} = k
\]

where \(k\) is an observed pseudo-first order rate constant describing the flux of counts into ATP at a constant pool size. As the specific activity of P, is also virtually constant over the time course of the experiment, integration yields simply:

\[
\text{cpm ATP} = kt
\]

Unlike the constant pool size of ATP, creatine phosphate changes both in the counts/min incorporation and nanomoles formed, i.e. counts originating from P, are trapped in creatine phosphate (CP).

\[
\frac{\text{dnmol CP}}{\text{dt}} = k' \text{ (nmol ATP)}
\]

Integration is likewise simple because (nmol ATP) is virtually constant and therefore:

\[
\text{nmol CP} = k'^{1/2} \text{ (nmol ATP)t}
\]

Where \(k'\) is a pseudo-zero order rate constant dependent upon the concentration of ATP and creatine present in a given experiment.

The change in counts/min incorporated into creatine phosphate (CP) with time is a function of both the changing ATP specific activity and the rate of creatine phosphate synthesis. Considering a single pool of ATP, i.e. complete and instantaneous mixing of mitochondrially synthesized and pre-existing ATP pools, the rate of change of creatine phosphate counts/min can be written as:

\[
\frac{d(\text{cpm CP})}{dt} = k' \text{ (cpm ATP)}
\]

which is integrated to give

\[
\text{cpm CP} = 1/2k'it^2
\]

Therefore, the specific activity (SA) (counts/min/nmol) of creatine phosphate (CP) at time \(t\) is equal to Equation 6 divided by 4.

\[
\text{SA}_{CP} = \frac{\text{cpm CP}}{\text{nmol CP}} = \frac{1}{1/2k'it^2} = \frac{1}{k'it^2}
\]

and with substitution of Equation 2 into this result:

\[
\text{SA}_{CP} = 1/2 \text{SA}_{ATP}
\]

This analysis shows that if there were no compartmentation of ATP pools during the initial phase of the reaction, the specific activity of creatine phosphate would be equal to one-half of that of the specific activity of the precursor, ATP. Furthermore, the specific activity of creatine phosphate formed by the end of a given interval of time \(t\) is simply a function of the average ATP specific activity (SA) present during that same time interval \(\Delta t\), i.e. (\(\text{SA}_{CP}\) = \(1/2 \text{SA}_{ATP}\)\(\text{\Delta t}\)).

The location of creatine phosphokinase within the intermembrane space, bound to the outer side of the inner mitochondrial membrane (38) perhaps juxtaposed to the adenine nucleotide translocase (31), suggests that two pools of ATP must be considered: that supplied by the incubation medium and that synthesized \textit{de novo} by respiring mitochondria and exported to the intermembrane space via the adenine nucleotide translocase. Significant deviation from the predicted relationship (Equation 7) would suggest incomplete equilibration, i.e. compartmentation of the two nucleotide pools.

A quantitative estimate of the extent of compartmentation was calculated as follows. Equation 8 describes the specific activity of nascent creatine phosphate formed in time interval \(t\) as the sum of fractional contributions from two ATP pools having specific activities equal to that of precursor P, and total ATP:

\[
\text{SA}_{CP} = x(\text{SA}_{P}) + y(\text{SA}_{ATP})
\]

where \(\text{SA}_{P}\) and \(\text{SA}_{ATP}\) are the average specific activities of P, and ATP over time interval \(t\), and where \(x\) and \(y\) are fractional contributions (\(x + y = 1\)). This equation can be solved for \(x\) to give

\[
x = \text{fractional contribution} = \frac{(\text{SA}_{P}) - (\text{SA}_{ATP})}{(\text{SA}_{P}) - (\text{SA}_{ATP})}
\]

The assumptions implicit in this representation include the following.

1. Only these two pools of ATP are able to give rise to creatine phosphate.
2. \textit{De novo} synthesized and exported ATP has a specific activity equal to its precursor P,.
3. External ATP specific activity is adequately represented by total ATP specific activity.
4. Synthesis of creatine phosphate is linear and hence the back reaction of creatine phosphokinase (creatine phosphate + ADP \(\rightarrow\) ATP + creatine) is negligible.

The small amount of nucleotide contained within the mitochondria (39), relative to added nucleotide, and the rapid rates of P, transport (40) and ATP synthesis characteristic of mitochondria support assumptions 2 and 3 above.

Equation 9 thus gives an indication of the extent of compartmentation of adenine nucleotide interaction with creatine phosphokinase. If the specific activity of creatine phosphate were equal to the average specific activity of ATP present for
that reaction interval, \( x = 0 \) and compartmentation would be absent. If, however, nascent creatine phosphate specific activity was found to be equal to that of P, compartmentation would become complete, i.e. creatine phosphate synthesis would occur entirely from a (small) pool of de novo synthesized and exported ATP prior to equilibration of total ATP.

**Effect of Exogenous ATP Concentration on Apparent Compartmentation of Creative Phosphokinase**—Experiments with rabbit heart mitochondria and exogenous ATP concentrations of 0.7 mM and higher were reported to yield creatine phosphate specific activities that were consistently but only slightly greater than one-half the specific activity of ATP (23). In terms of two ATP pools, this finding could represent an overwhelming number of direct mitochondrial contributions by the exogenous ATP pool, i.e. exogenous concentration of ATP is so great that creatine phosphokinase sites are virtually all occupied by it. Decreasing the competition by the exogenous pool would therefore increase the possibility of direct interaction by a mitochondrially derived pool.

Table I shows the effect of total adenine nucleotide pool size on the quantities and specific activities of creatine phosphate and ATP formed with respiring heart mitochondria in the presence of labeled inorganic phosphate. As mentioned above, short time intervals were used to avoid “inhibition” by creatine phosphate (13) and to ensure that ATP specific activity changes were linear. Levels of creatine phosphate are routinely 5–10% lower at 10 s than would be expected from 5–s rates, independent of the concentration of creatine phosphate or ADP, both of which increase the rate of the reverse creatine phosphokinase reaction, hence decreasing net creatine phosphate synthesis (19). This difference reflects the actual time required to terminate reactions (0.2–0.5 s) which has a greater effect on 5-s than on 10-s reactions. With reaction periods of 5–60 s and ATP concentrations up to 0.65 mM, creatine phosphate synthesis is linear for up to 30 s, or until a concentration of about 0.26 mM is reached. At the largest ATP concentration, 2.0 mM, the ratio of creatine phosphate specific activity to that of ATP is close to 0.5 as expected. Smaller ATP concentrations yield increasingly larger specific activity ratios. When the creatine phosphate formed between 0 and 5 or 5 and 10 s is compared to the average ATP specific activity measured during these intervals, ratios of creatine phosphate specific activity to average ATP specific activity increase as the total ATP concentration is decreased. However, this simple analysis does not take into account the specific activity of the second ATP pool, that of de novo synthesized ATP, which defines the upper limit of the specific activity ratio for a given total nucleotide level.

Solution of the contribution equation (Equation 9) for this and other experiments over the 5–10-s interval is summarized in Fig. 1 for ATP concentrations ranging from 0.04–0.75 mM ATP. The 5–10-s interval was chosen to allow for equilibration of added \(^{32}P\), within mitochondria and initial export of pre-existing unlabeled mitochondrial ATP as discussed by Vignais (41). Average ATP specific activities were determined from the specific activity versus time plots for each experiment. Selected samples were treated with an excess of yeast hexokinase and glucose to distinguish \( \alpha \)-from \( \beta \)-phosphate of ATP. The \( \beta \)-phosphate contributed 5% or less of the total radioactivity of the ATP.

The concentration dependence of mitochondrial contribution (Fig. 1) reflects the competition between ATP in the medium and newly exported mitochondrial ATP for the ATP binding site on creatine phosphokinase. Apparent contributions increase sharply at ATP concentrations of 0.2 mM and below. Contribution of 100% would theoretically occur at a level of ATP equal to that contained in isolated mitochondria. For rabbit heart mitochondria, this corresponds to a value of 7.50 \pm 0.53 nmol/mg (mean \pm S.E.; \( n = 6 \)) measured in the absence of added nucleotide. Qualitatively, the concentration-effect curve constructed from isotope incorporation data is similar to that derived by kinetic analysis of rat heart mitochondrial creatine phosphokinase based on a functional coupling of enzyme to the adenine nucleotide translocase (20). The concentration-effect curve for rat skeletal muscle mitochondria was found to be essentially identical, with apparent mitochondrial contributions increasing sharply at ATP concentrations below 0.2 mM (data not shown).

**Effect of ATP concentration on the apparent mitochondrial contribution to nascent creatine phosphate production by rabbit heart mitochondria.** Values represent solutions to Equation 9 (see “Experimental Procedures”) for 3–7 preparations of mitochondria at each ATP concentration (mean \pm S.E.). The starred values were obtained with the use of \(^{32}P\)ATP as the source of label. Values with this reverse method have been published (Ref. 21).

---

**Table I**

**Effect of nucleotide concentration on specific activities and levels of phosphorylated intermediates in rabbit heart mitochondria**

Mitochondrial incubations and measurements of nanomoles and specific activities of phosphorylated intermediates were carried out as described under “Experimental Procedures.” A mixture of \(^32\)P plus creatine (20 mM final concentration) was added to initiate the incubations. Values represent 75 \( \mu \)l of the original 1-ml reaction volume. Nanomoles of creatine phosphate (CP) present before the addition of P, and 0.078 mM CP were subtracted from 5- and 10-s values. Values in the mitochondrial pellet (after precipitation) were about 20% of the value for the pool.

<table>
<thead>
<tr>
<th>Total nucleotide pool size</th>
<th>CP</th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00 mM</td>
<td>7.38</td>
<td>13.86</td>
<td>5.08</td>
<td>9.48</td>
<td>3.92</td>
</tr>
<tr>
<td>0.224 mM</td>
<td>7.54</td>
<td>2.80</td>
<td>5.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.128 mM</td>
<td>7.54</td>
<td>2.80</td>
<td>5.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.078 mM</td>
<td>7.54</td>
<td>2.80</td>
<td>5.38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total pool size</th>
<th>5 s</th>
<th>10 s</th>
<th>10 s</th>
<th>10 s</th>
<th>10 s</th>
<th>10 s</th>
<th>10 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>580</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
</tr>
<tr>
<td>AMP</td>
<td>580</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
</tr>
<tr>
<td>ADP</td>
<td>580</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
</tr>
<tr>
<td>ATP</td>
<td>580</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>392</td>
</tr>
<tr>
<td>Specific activity</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** Effect of ATP concentration on the apparent mitochondrial contribution to nascent creatine phosphate production by rabbit heart mitochondria. Values represent solutions to Equation 9 (see “Experimental Procedures”) for 3–7 preparations of mitochondria at each ATP concentration (mean \pm S.E.). The starred values were obtained with the use of \(^{32}P\)ATP as the source of label. Values with this reverse method have been published (Ref. 2).
activity as measured by the difference in initial rates of ATP cleavage and creatine phosphate synthesis in actrylcoside-inhibited mitochondria (see for example Fig. 4B). The above estimated rate of P$_i$ turnover is sufficient to account for ATP synthesis at 87% of maximal, assuming an oxygen consumption rate of 350 nmol at 0/min/mg and an ADP/O ratio of 3 (see accompanying paper (57)). Although rates of nucleotide transport via the translocase are much lower than rates of P$_i$ transport at 0 °C (3-6 nmol/min/mg for rat liver, 41), the large temperature coefficient of ATP-ADP exchange (43) predicts values in excess of 100 nmol/min/mg for liver at 37 °C (44). Rates for heart mitochondria would be even higher as the number of adenosylate transporters per mg of mitochondrial protein is about 4 times greater than in liver (45).

The content of P$_i$ in pellets derived from the experiments shown in Fig. 2 was 14.1 ± 1.8 nmol/mg of protein (mean ± S.E., n = 6), yet the apparent 10-s specific activity was only 20% of that of the external phosphate and hence too low to account for the total ATP specific activity even at 5 s. Internal compartmentation of P$_i$ into exchangeable and nonexchangeable pools (40) and compartmentation of mitochondrial de-novo nucleotides such as synthesis and export of new ATP are coupled (41) could account for the low value observed for P$_i$ specific activity. Clearly, however, the specific activity of newly exported ATP cannot exceed that of the total P$_i$, and thus our method of analysis sets a lower limit on possible fractional contributions obtainable. The nature of the experiment, in which total nucleotide level is varied, imposes a maximum on the respiration rate possible. As total nucleotide is decreased, the concentration of ADP decreases as well. As stated in the text, the creatine-stimulated rate of respiration in the presence of 80 μM ATP is 80-85% of the state 3 rate. With less nucleotide, for example, 50 μM, creatine-stimulated respiration is about 70% state 3. This decrease in the rate of respiration naturally offsets the magnitude of the observed contribution.

An alternative experimental design (21) in which ATP is already labeled and uptake and incorporation of isotope by oxidative phosphorylation are not required should yield identical results in terms of mitochondrial contributions if the supposition of two compartments is valid. When [γ-32P]ATP was used as the isotope source, the specific activity of P$_i$, and hence of newly synthesized mitochondrial ATP was virtually constant and zero over the time course of the experiment. This result reflects the relatively large pool size of P$_i$ (2.5-5.0 mM) compared to that of the labeled ATP. In this case, a deficit of radioactive counts in creatine phosphate relative to the average (interval) ATP is indicative of direct mitochondrial ATP contributions. In the case of rabbit heart mitochondria respiring with 70 μM ATP, an apparent mitochondrial contribution of 27.0% was found and at 360 μM ATP a contribution of 6.9% was found; values not much higher than those shown in Fig. 1 for these same ATP concentrations when only labeled phosphate was the source of isotope. These are the starred values shown (Fig. 1). Thus, the specific activities of de novo synthesized and exogenous ATP pools appear to be adequately represented by the measured inorganic phosphate and total ATP specific activities, respectively.

**Effect of Source of ATP Generation on Mitochondrial Contribution to Phosphorylated Products**—Further insight on the nature of the compartmentation of mitochondrial creatine phosphokinase was gained when the incorporation of labeled phosphate into creatine phosphate formed by mitochondrial enzyme was contrasted with the labeling of glucose 6-phosphate formed by added yeast hexokinase. Glucose 6-phosphate from the "external" kinase would presumably reflect the outside ATP environment in terms of specific
activity. Relatively large quantities of the yeast enzyme were added so that the effect of possible compartmentation of native heart mitochondrial hexokinase (46) would be minimal.

Fig. 3 shows the specific activity versus time profiles of ATP, glucose 6-phosphate, and creatine phosphate. Clearly, creatine phosphate and glucose 6-phosphate were not formed from the same pool of ATP. Calculations using Equation 9 show that creatine phosphate appeared to have been formed from mitochondrial ATP to the extent of 24.3 ± 4.3%, whereas for glucose 6-phosphate synthesized the value was -2.92 ± 1.5% (means ± S.E., n = 3). The fact that the "contribution" to glucose 6-phosphate synthesis is a small negative number indicates that the specific activity of glucose 6-phosphate was actually slightly less than the value used for the average ATP specific activity. The magnitude of this negative effect is in agreement with the extent to which actual medium ATP specific activities were decreased relative to total ATP specific activities (Fig. 2) and represents the degree to which our method of analysis underestimates the effect.

The importance of the source of ATP generation on apparent compartmentation can also be observed through comparison of respiring versus inhibited mitochondrial incubations where an external ATP regenerating system is present. This approach was used previously by Saks and co-workers (47) in support of a close interaction between oxidative phosphorylation and creatine phosphate synthesis. Fig. 4 shows the quantities of nucleotide and creatine phosphate formed by rat skeletal muscle mitochondria under conditions of respiration (Fig. 4A), atracyloside inhibition (Fig. 4B), and atracyloside inhibition plus an external regenerating system (Fig. 4C) containing 20 IU/ml of pyruvate kinase and 2.4 mM P-enolpyruvate. Although this regenerating system was able to maintain ATP and ADP levels essentially identical with those present with respiration, creatine phosphate was synthesized at 68% of the rate found with respiring mitochondria. Similar results were obtained with rabbit heart mitochondria (not shown).

In terms of contribution as measured by isotope incorporation, we compared the labeling of nascent creatine phosphate and the γ-phosphate of ATP under the three conditions described above in the presence of 80 μM ATP containing [γ-32P]ATP. Two of the three conditions (B and C above) thus serve as controls. Table II shows that inhibited mitochondria with or without the added regeneration system incorporated label into creatine phosphate as expected for a single ATP source, whereas in the presence of respiration a deficit of label in creatine phosphate was observed. The discrepancy between the extent of compartmentation as measured by the isotope technique versus that observed when the velocities of creatine phosphate synthesis were compared (Fig. 4) was ultimately related to additional inhibition of creatine phosphate synthesis by the concentration of P-enolpyruvate used in the regenerating system. The observed Kₐ values for ATP and creatine phosphokinase under various conditions are reported in the accompanying paper (57). It is apparent, however, that measurement of mitochondrial contributions to creatine phosphate synthesis by the use of isotopes does not represent an artifact caused by changing ATP specific activities per se, but reflects the close interaction between oxidatively formed ATP and the native creatine phosphokinase.

Considering this apparent close interaction, it was of interest to know whether or not creatine phosphate could be formed from matrix nucleotide directly. The active site of creatine phosphokinase is believed to be on the cytosolic side of the inner mitochondrial membrane (38). Recently, Iyengar and Iyengar (48) suggested that the creatine phosphokinase molecule may span the inner mitochondrial membrane to interact with matrix ATP directly. We therefore determined the ability of matrix nucleotide to form creatine phosphate when nucleotide transport was blocked by atracyloside. Because the quantities of creatine phosphate formed directly from the endogenous mitochondrial nucleotide were expected to be very small, the rabbit heart mitochondria were first incubated with 15 μCi of 32P/mg of protein in the basic incubation medium (see "Experimental Procedures") for 4 min prior to addition of creatine. This loading or priming period allowed isotopic equilibration of the mitochondrial nucleotide so that very small amounts of creatine phosphate could be detected.

As shown in Table III, in the presence of atracyloside, creatine phosphate was not formed even after 60 s of incubation.
Compartmentation of Mitochondrial Creatine Phosphokinase

TABLE III
Creatine phosphate (CP) synthesis from endogenous mitochondrial nucleotide—effect of atractyloside

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>Atractyloside</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 s</td>
<td>30 s</td>
</tr>
<tr>
<td>Nanomoles ATP/mg</td>
<td>7.36</td>
<td>7.64</td>
</tr>
<tr>
<td>Nanomoles CP/mg (measured)</td>
<td>2.67</td>
<td>19.21</td>
</tr>
<tr>
<td>Nanomoles CP/mg (calculated)</td>
<td>2.37</td>
<td>18.77</td>
</tr>
<tr>
<td>Counts in CP (cpm/mg)</td>
<td>13,612</td>
<td>108,186</td>
</tr>
<tr>
<td>Counts in P (cpm/nmol)</td>
<td>5,593</td>
<td>5,696</td>
</tr>
<tr>
<td>Counts in ATP (cpm/nmol)</td>
<td>5,741</td>
<td>5,763</td>
</tr>
</tbody>
</table>

* Rabbit heart mitochondria were preincubated with or without 10 μM atractyloside for 4 min with 15 μCi/mg of 32P. Creatine was then added to a final concentration of 20 mM.

† Measured with the automated phosphate analyzer as described under “Experimental Procedures.”

Calculated from the counts/min of 32P, incorporated into creatine phosphate divided by the specific activity of ATP (γ-phosphate).

TABLE IV
Respiratory activity of rabbit heart mitochondria with various substrates

The activity was determined polarographically as described under “Experimental Procedures.” State 3 was initiated by the addition of 174 nmol of ADP in the case of α-ketoglutarate and succinate and 70 nmol of ADP in the case of ascorbate plus TMPD. Values represent mean ± S.E. for at least duplicate determinations using 2 different preparations of mitochondria.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>State 3 V, Respiratory control index</th>
<th>ADP/O ratio</th>
<th>32P into ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 s</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>305 ± 14</td>
<td>2.96 ± 0.06</td>
<td>3.37 ± 0.15</td>
</tr>
<tr>
<td>Succinate plus rotenone</td>
<td>394 ± 8</td>
<td>2.40 ± 0.16</td>
<td>2.18 ± 0.09</td>
</tr>
<tr>
<td>Ascorbate plus TMPD plus rotenone plus antimycin A</td>
<td>926 ± 46</td>
<td>1.66 ± 0.05</td>
<td>1.05 ± 0.15</td>
</tr>
</tbody>
</table>

Incorporation of 32P into ATP is expressed relative to the specific activity of endogenous phosphate. Incubation conditions of Yang (21) except 10 mM Hepes, 2.5–5 mM potassium phosphate, 20 mM creatine, and 0.035–0.7 mM ATP, pH 7.2. MgCl2 concentration was 1 mM when nucleotide was ≤0.2 mM and 2 mM when nucleotide was >0.2 mM.

Compartmentation of Mitochondrial Creatine Phosphokinase: Nature of Coupling to Mitochondrial ATP Synthesis—The observed compartmentation effect described above could reflect a higher concentration of ATP within the intermembrane space surrounding the bound creatine phosphokinase. Alternatively, a functional or physical coupling of creatine phosphokinase and the nucleotide translocase (13, 20) could, via a “proximity effect,” lead to compartmentation. Considering such an interaction, it seemed possible that a second level of coupling might be superimposed upon the functional association of creatine phosphokinase and nucleotide transport, that of site-directed creatine phosphate syntheses. In other words, ATP produced at certain levels of the respiratory chain of electron transport could be specifically directed into the production of creatine phosphate in an extension of the “cafeteria principle” (49).

The results of experiments under three substrate-inhibitor combinations which direct ATP synthesis to different levels of the electron transport chain are shown in Tables IV and V.

TABLE V
Mitochondrial sites of ATP synthesis and creatine phosphokinase compartmentation in muscle mitochondria

Incubations were carried out as described under “Experimental Procedures” (see also Table IV) utilizing 0.5–1.0 mg of mitochondrial protein. A mixture of 32P, and creatine was added to start incubations. Apparent mitochondrial contributions were calculated as described under “Analysis of Data.” Values represent averages of two mitochondrial preparations for each condition.

<table>
<thead>
<tr>
<th>Source of mitochondria</th>
<th>Substrate (s)</th>
<th>ATP fraction</th>
<th>Apparent mitochondrial contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit ventricle</td>
<td>α-Ketoglutarate</td>
<td>57</td>
<td>381</td>
</tr>
<tr>
<td>Douch method</td>
<td>Succinate</td>
<td>73</td>
<td>379</td>
</tr>
<tr>
<td>Ascorbate/TMPD</td>
<td>Succinate</td>
<td>66</td>
<td>347</td>
</tr>
<tr>
<td>Rat skeletal muscle</td>
<td>α-Ketoglutarate</td>
<td>37</td>
<td>413</td>
</tr>
<tr>
<td>polytron method</td>
<td>Succinate</td>
<td>62</td>
<td>366</td>
</tr>
<tr>
<td>Ascorbate/TMPD</td>
<td>Succinate</td>
<td>62</td>
<td>386</td>
</tr>
</tbody>
</table>

The three conditions were: (a) 5.0 mM α-ketoglutarate as substrate (sites I, II, and III), (b) 5.0 mM succinate plus 1 mM rotenone to inhibit oxalacetate formation (50) (sites II and III), (c) 5.0 mM ascorbate plus 0.75 mM TMPD in the presence of 1 μM rotenone and 0.5 μM antimycin A for the assay of oxidative phosphorylation at the cytochrome oxidase region (51). Table IV shows the oxygen consumption and ADP/O ratio for the three conditions, which are in good agreement with previous findings (52–54). Incorporation of 32P into the total ATP fraction was similar for the three conditions, indicating that the rates of ATP synthesis were similar as predicted from state 3 oxygen consumption and ADP/O ratios. Furthermore, the apparent mitochondrial contributions to creatine phosphate synthesis at equivalent levels of total ATP were the same for all 3 conditions (Table V) in both rabbit heart and rat skeletal muscle mitochondria. This finding is consistent with the idea that all sources of ATP provided by the mitochondria through oxidation are able to interact with creatine phosphokinase to the same extent prior to total ATP mixing. As site III ATP synthesis is the common parameter in the 3 conditions tested, it might be presumed that the data were indicative of a site III-directed creatine phosphate synthesis. However, if sites I- and II-produced ATP molecules were not able to interact directly with the bound creatine phosphokinase before mixing, the degree of compartmentation expected when these two noncompartmented influences were removed (e.g. with ascorbate plus TMPD) would presumably be greater. This does not appear to be the case (Table V). The degree of structural organization of ATP synthesizing...
molecules, translocase molecules, and creatine phosphokinase molecules appears to be such that all newly synthesized and exported ATP molecules have the same ability to interact directly with creatine phosphokinase.

**DISCUSSION**

We have systematically studied the direct mitochondrial contribution to creatine phosphate formation. The ability of newly synthesized versus exogenous ATP pools to contribute to creatine phosphate synthesis is compared through the use of different isotopically labeled precursors. The finding that, at high concentrations of ATP, the specific activity of \(^{32}\)P creatine phosphate was roughly one-half that of the \(^{32}\)P-phosphate of ATP, while at lower concentrations it was greater than one-half (Table I), suggested that the isotope incorporation pattern depended on total nucleotide concentration in a manner consistent with the dependence of creatine phosphate synthesis on total ATP previously reported (20). However, a quantitative statement regarding presumed direct contribution of mitochondrial ATP to creatine phosphate synthesized represented consideration of the two possible sources of ATP. The relationship derived was based on consideration of the linear synthesis of creatine phosphate, constancy of total ATP levels (Table I), rapidity of phosphate transport (40, 42), and the assumption that matrix contribution to total ATP specific activity was negligible. The latter was directly tested and shown to be correct (Fig. 2).

Further verification of our method of analysis is apparent from studies carried out with \([\gamma-^{32}\text{P}]\text{ATP}\). In this reaction system, no assumptions as to equilibration and export of a labeled, newly synthesized ATP pool are necessary; the mitochondrial ATP pool is available from "time zero" and even labeled, newly synthesized ATP pool are necessary; the mitochondrial ATP pool is available from "time zero" and even labeled, newly synthesized ATP has a specific activity less than 5% of that of the originally labeled ATP. Virtually identical results were obtained with this isotope as with labeled phosphate. In addition, the expected pattern of creatine phosphate labeling can be observed when either no ATP regeneration or exogenous ATP regeneration was present (Table II).

Finally, that creatine phosphokinase has a special connection to mitochondrial ATP synthesis was apparent when creatine phosphate labeling was compared to that of glucose 6-phosphate formed from added yeast hexokinase (Fig. 3). As both products' specific activities were determined in the same manner, a systematic error in the determination seems unlikely. Thus, our method of analysis appears both valid and indicative of a microenvironment surrounding the bound creatine phosphokinase such that newly synthesized ATP has a special ability to interact with the active site of creatine phosphokinase.

This method of analysis when applied to experiments over a wide range of total ATP concentrations results in a "concentration-effect" curve in which the competition between de novo synthesized and pre-existing ATP pools is demonstrated (Fig. 1). That the range of significant contributions is observed only at low levels of nucleotide (0.2 mM) is critical. Previous reports concerning the use of isotope tracers have led to contradictory statements concerning such a creatine phosphokinase compartment (21-24, 55). These reports concern the presence (21) or lack of (23, 24, 55) apparent mitochondrial ATP contribution to creatine phosphate synthesis at levels of ATP of 0.50-3.0 mM, and furthermore differ as to what was believed to indicate compartmentation. For instance, beef heart mitochondria respiring in the presence of \(\beta\)-hydroxybutyrate and 3.0 mM ATP formed creatine phosphate only slowly and yielded identical specific activity values for ATP and creatine phosphate when \(^{32}\)P incorporation was studied at 1-min intervals (24). However, the specific activity of ATP and creatine phosphate was only 10% of that of inorganic phosphate precursor after 5 min of incubation at 25°C. Because mitochondrial contributions to creatine phosphate synthesis require consideration of the specific activities of both ATP and P\(_{i}\) pools (Equation 9), contributions calculated from the data of Ref. 24 (Fig. 9) are less than 10% even though the nascent creatine phosphate specific activity exceeds that of the average ATP. When the more rapidly respiring rabbit heart mitochondrial system was studied with the use of a lower temperature (25°C), 3.0 mM ATP, and \(\beta\)-hydroxybutyrate, creatine phosphate specific activity was only slightly greater than one-half of that of ATP as expected from Fig. 1 (23).

Recently, Lipskaya et al. (55) reported two experiments conducted with beef heart mitochondria respiring in the presence of 0.5-0.6 mM ATP and \(^{32}\)P, using two-dimensional thin layer chromatography to separate labeled compounds and spectrophotometric measurements of creatine phosphate and ATP content. Although early time points are absent, one of these experiments yielded specific activity versus time plots ATP in creatine phosphate specific activity "lagged behind" that of the ATP as shown above in Table I, while in the second experiment, ATP and creatine phosphate specific activities were essentially identical initially, with creatine phosphate specific activity exceeding that of ATP after 2 min (see Fig. 2, Ref. 55). With rabbit heart mitochondria, we have carried out incubations from 10 s up to 5 min at a similar concentration of ATP; creatine phosphate ultimately approached but never exceeded the specific activity of ATP. Obviously, differences in the method of quantitation of specific activity data make it difficult to compare directly the data from different laboratories, aside from the difference in the source of mitochondria. One of the advantages of a chromatographic system in the case of specific activity determination is the simultaneous measurement of radioactive counts and contents for the peaks of interest (38), such that possible artifacts due to differential recovery of counts/min versus content is avoided.

As Fig. 1 shows that the observable compartmentation effect depends on the total ATP concentration present in the reaction mixture, the presumed compartment is not a closed system, but is subject to pressure or competition by the external ATP concentration. The proximity to mitochondrial creatine phosphokinase of mitochondrially synthesized ATP compared to extramitochondrial ATP was suggested (11) and was verified kinetically (18) to support a functional coupling (13, 19, 20). This analysis was interpreted to indicate a direct relation between the translocase and creatine phosphokinase. Our data do not support such a conclusion for they show this compartment to be ATP concentration-dependent. A plot of ATP concentration versus "coupling coefficient" for rat heart mitochondria (Fig. 5 in Ref. 20) showed a sharp increase in the theoretical and experimentally determined coefficient at ATP levels below 0.6 mM (compare with Fig. 1 above). Whether this shift to the right in the "concentration-effect" curve is species-dependent is unknown.

A key requirement for a coupling to the translocase is that the active site of creatine phosphokinase be located on the cytosolic side of the translocase. Although this feature is largely accepted (17, 38), the discussion in the recent publication of Lyengar and Lyengar (48) indicated that some controversy still existed concerning the ability of bound enzyme to utilize matrix nucleotide directly. This was not found in rabbit heart mitochondria as shown in Table III. Furthermore, the use of various substrate-inhibitor combinations in conjunction with measurements of the velocity of creatine phosphate formation and labeled phosphate incorporation indicate
Compartmentation of Mitochondrial Creatine Phosphokinase

a generalized ability for newly synthesized ATP to interact with creatine phosphokinase within the intermembrane space (Tables IV and V).

We might then ask, “What is the physiological significance of our experimental findings since the effect of compartmentation would appear small in the face of millimolar quantities of ATP often assumed to bathe the mitochondria in vivo?” We believe that in vivo mitochondria never see such levels of ATP. In vivo ATP is most likely compartmented or generally bound to many enzyme sites and therefore not easily diffusible, as we have pointed out for the creatine-phosphorylcreatine shuttle mechanism of energy transfer (2, 3).

There is increasing evidence to indicate that the millimolar levels of nucleotide measured in a muscle cell extract do not represent the nucleotide available for interaction with enzymes. Gudbjarnason et al. (57) were among the first who suggested that the adenine nucleotides were largely compartmented within the cytoplasm after changes in the level of nucleotide and creatine phosphate at or near mitochondria and myofibrils. We have reported that the incorporation of $^{31}P$ into the $\beta$- and $\gamma$-phosphate groups of ATP is much slower than the incorporation of phosphorus into 3-phosphoglycerate, glucose 6-phosphate, and fructose 6-phosphate in hemidiaphragms (2, Table III). Thus, the actual levels of ATP in the vicinity of mitochondria, available to compete with de novo synthesized nucleotide for active sites on at least the kinases for creatine and glucose remains unknown—they are certainly not the same as are measured as total ATP in the tissue. We hope that a clear demonstration of the kinds of conditions under which compartmentation and enzyme localization is observed (58) will stimulate further research into the conditions occurring in the living cell.

Further studies on the possible nature of the mitochondrial creatine phosphokinase compartment are described in the accompanying paper (57).

REFERENCES

Compartmentation of Mitochondrial Creatine Phosphokinase