Effect of Creatine Kinase Activity on Mitochondrial ADP/ATP Transport

EVIDENCE FOR A FUNCTIONAL INTERACTION*

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Mitochondrial adenine nucleotide translocation was measured in the presence of creatine or creatine phosphate to test the proposed functional couple between the nucleotide translocase and creatine kinase. Rat heart mitochondria were preloaded with the corresponding nucleotide which reacts with creatine kinase only after it is transported across the mitochondrial membrane; namely, radioactive ATP-preloaded mitochondria were assayed in the presence of 10 mM creatine plus ADP, and radioactive ADP-preloaded mitochondria in the presence of 10 mM creatine phosphate plus ATP. Results showed that forward creatine kinase reaction (in the direction of creatine phosphate synthesis) inhibits translocation of external ADP into mitochondrial matrix and backward reaction (cleavage of creatine phosphate to creatine) likewise inhibits translocation of ATP across the mitochondrial membrane. Control experiments showed that without the kinase activity when Mg2+ was omitted from the assay medium, the presence of creatine or creatine phosphate had no effect on ADP or ATP transport, respectively. Therefore, the observed inhibition of nucleotide exchange by these compounds is due to creatine kinase activity which upon reacting with the newly exported nucleotide can effectively compete for transport back into the mitochondrial matrix over nucleotides added in the assay medium. Kinetic analysis also indicated that the forward creatine kinase reaction inhibits external ADP uptake competitively. These results are interpreted to support the proposal that a functional interaction exists between the mitochondrial bound creatine kinase and the adenine nucleotide translocase.

The mechanism of energy transduction in heart muscle is believed to involve the interconversion of phosphate bond energy between ATP and creatine phosphate in such a way that the synthesis and cleavage of creatine phosphate occur preferentially at energy-consuming and -producing sites, respectively (1, 2). Furthermore, such preferential interactions are due to the close spatial proximity between various creatine kinase isoenzymes and ATP-producing and -consuming locations (1, 2). A critical aspect of this mechanism is the ability of mitochondria to synthesize creatine phosphate at a sufficient rate to meet changing metabolic demands. Two proposals which describe the mechanism of creatine phosphate synthesis are: (a) it is driven by a mass action effect due to the high extramitochondrial ATP/ADP ratio and (b) by a selective interaction with the mitochondrial adenine nucleotide translocator which allows matrix-derived nucleotides to have preferred access to creatine kinase over nucleotides present in the medium.

In supporting the "privileged access" of ATP to the enzyme, Saks et al. (3, 4) have shown that mitochondrial creatine kinase reacts slowly with externally added ATP, but rapidly utilizes ATP newly synthesized by oxidative phosphorylation. Yang et al. (5) reported a similar observation in studies examining the rate of isotope incorporation into creatine phosphate using [γ-32P]ATP. More recently, this difference in activity was attributed to matrix ATP having a lower K, value for creatine phosphate synthesis, suggesting that localized high concentrations are created through the action of the adenine nucleotide translocase (5) in the mitochondrial membrane. On the other hand, Borer (6) and Altschuld and Brierley (7) could not detect a specific coupling between the translocase and creatine kinase and concluded that there is no privileged access for ATP.

More recently, Moreadith and Jacobus (8) showed that ADP generated by creatine kinase can reverse the inhibition of respiration by atractyloside much more effectively than externally added ADP. These results are consistent with the findings by Gellerich and Saks (9) who showed that ADP generated by creatine kinase is less reactive with pyruvate kinase when the source of ATP is from oxidative phosphorylation compared to ADP generated from externally added hexokinase plus glucose. These authors also concluded that a functional interaction renders the translocase more effective in competing for the newly generated ADP which in turn can stimulate oxidative phosphorylation.

These findings, although consistent with the hypothesis of a functional coupling, fall short of directly demonstrating that adenine nucleotide transport per se is affected by creatine kinase activity. This issue is addressed in the current study. A preliminary report has been presented in an abstract (10).

EXPERIMENTAL PROCEDURES

Materials—ATP, ADP, AMP, diadenosine pentaphosphate, carboxyatractysolide, oligomycin, and bovine serum albumin (fraction V) were obtained from Sigma. Nagarse was from Enzyme Development Corp. [2,8-3H]ADP (56.4 Ci/mol) and [2-3H]ATP (16 Ci/mol) were purchased from New England Nuclear and Amersham Corp., respectively. Male Sprague-Dawley rats (275-325 g) were from Blue Spruce Farms, Inc., Herkimer, NY. All other reagents were highest purity grade commercially available.

Isolation of Mitochondria—Mitochondria were isolated from male Sprague-Dawley rats using a modification of the procedure described...
Mitochondrial ADP/ATP Transport and Creatine Kinase

Results

Results in Fig. 1A show that the rate of [$^3$H]ATP$_{in}$ (subscripts in and ex represent intramitochondrial and external nucleotides, respectively) exchanging for ADP$_{ex}$ is slowed by the presence of 10 mM creatine. A similar but less pronounced response is seen in Fig. 1B, in which the rate of [$^3$H]ADP$_{ex}$ exchanging for ATP$_{in}$ is slowed by the presence of 10 mM creatine phosphate. In both these experiments, creatine kinase activity should occur only after there was an exchange of adenosine nucleotides. These results therefore show that as measured by the back exchange reaction of the translocase, mitochondria are reacting with creatine kinase, with creatine phosphate, being converted to ADP and ATP, respectively, have prefered uptake over their unlabeled counterparts in the medium. The observation that the back reaction of creatine kinase affects transport rates is a new finding and underscores the degree to which the two proteins interact. Not shown here in these figures is the observation that increasing the external nucleotide levels decreased the extent of apparent inhibition of transport in the presence of creatine (see Fig. 4).

The experimental design of results shown in Fig. 2A is similar to that shown in Fig. 1A. In this experiment, the uptake of [$^3$H]ADP$_{ex}$ for ATP$_{in}$ is slowed by the presence of 10 mM creatine, suggesting that the occurrence of creatine kinase activity allows the newly formed ADP to have preferred uptake over medium [$^3$H]ADP. Results shown in Fig. 2B demonstrate that the rate of [$^3$H]ATP$_{ex}$ exchanging with ADP$_{in}$ is also decreased by the presence of 10 mM creatine phosphate. These results are consistent with the findings shown in Fig. 1 and again are strongly suggestive that the newly formed ATP molecule has preferred uptake into mitochondria over [$^3$H]ATP present in the medium.

A possible explanation of these results would be that under the experimental conditions used, creatine and creatine phosphate are simply acting as inhibitors of nucleotide exchange. This possibility is ruled out by results of control studies shown in Fig. 3, A and B, which demonstrate that in the absence of MgCl$_2$ and therefore creatine kinase activity, these compounds have essentially no effect on rates of nucleotide exchange. This observation thus strongly indicates that the observed inhibition of nucleotide exchange in the presence of creatine kinase activity is only apparent owing to the preferential reuptake of newly translocated matrix nucleotides. If creatine kinase activity induces competition between newly transported nucleotides from matrix and nucleotides present in the medium, then in the presence of creatine kinase activity, the nucleotide transport should be inhibited competitively. Results shown in Fig. 4 confirm this prediction by demonstrating that the forward reaction of creatine kinase shows only the apparent $K_m$ value for [$^3$H]ADP uptake, increasing it from 18 to 42 $\mu$M. The biphasic nature of the kinetic plot is consistent with previous studies on liver mitochondrial derived nucleotides for transport. A replot of these results expressed as the percentage of mitochondrial contribution versus external competing nucleotide level is shown in Fig. 4B, which indicates that a decline in ADP levels from 100 to 8 $\mu$M increases the magnitude of nucleotides originating from mitochondria being transported back into the matrix from 16 to 42%.

These results are remarkably consistent with a recent report by Erickson-Vitaen et al. (see Fig. 1 of Ref. 17) who quantitated this interaction by measuring the rate of labeled P$_i$ incorporation into ATP and creatine phosphate in isolated...
Mitochondrial ADP/ATP Transport and Creatine Kinase

FIG. 1. A, effect of the forward reaction of creatine kinase on \([^{3}H]ATP \rightarrow ADP\) exchange. Rat heart mitochondria were preloaded with \([^{3}H]ATP\) and assayed in the absence (■) and presence (○) of 10 mM creatine as described under "Experimental Procedures." The reaction was started by injecting 50 μl of 500 μM ADP (25 μM final concentration) and stopped at the indicated times by adding 10 μM carboxyatractyloside. The mitochondria were subsequently centrifuged, washed, and counted as described under "Experimental Procedures." B, effect of the back reaction of creatine kinase on \([^{3}H]ADP \rightarrow ATP\) exchange. Rat heart mitochondria were preloaded with \([^{3}H]ADP\) and assayed in the absence (■) and presence (○) of 10 mM creatine phosphate as described under "Experimental Procedures." The reaction was started by injecting 50 μl of 2.0 mM ATP (100 μM final concentration), stopped at the indicated times with carboxyatractyloside, centrifuged, washed, and counted as described above.

FIG. 2. A, effect of the forward reaction of creatine kinase on ATP\(_{in}\) \(\rightarrow [^{3}H]ADP\) exchange. Rat heart mitochondria were preloaded with unlabeled ATP and assayed in the absence (■) and presence (○) of 10 mM creatine as described under "Experimental Procedures." The reaction was started by injecting 50 μl of 168 μM \([^{3}H]ADP\) (5000 cpm/nmol, 8.4 μM final concentration) and stopped at the indicated times by adding 10 μM carboxyatractyloside, centrifuged, washed, and counted as described under "Experimental Procedures." B, effect of the back reaction of creatine kinase on ADP\(_{in}\) \(\rightarrow [^{3}H]ATP\) exchange. Rat heart mitochondria were preloaded with unlabeled ADP and assayed in the absence (■) and presence (○) of 10 mM creatine phosphate as described under "Experimental Procedures." The reaction was started by adding 50 μl of 1.0 mM \([^{3}H]ATP\) (3000 cpm/nmol, 50 μM final concentration) and stopped as indicated with 10 μM carboxyatractyloside, centrifuged, washed, and counted as described above.

Evidence presented thus far demonstrates that the forward and reverse reactions of creatine kinase can affect transport rates, thereby indicating that a possible functional couple exists in both directions. Should this be the case, it would be predicted that creatine phosphate synthesis should act to competitively stimulate oxidative phosphorylation. This is also predicted in view of the results shown in Fig. 4A. To confirm this, the effect of 10 mM creatine was examined on the initial rate of state 3 respiration at the same temperature that transport studies were performed (2°C). Results shown in Fig. 5 clearly demonstrate that, indeed, creatine phosphate synthesis acts to competitively activate oxygen consumption by lowering the apparent \(K_{m}\) value for ADP by a degree remarkably consistent with its effect on nucleotide exchange.

In conclusion, the current study has demonstrated that both the forward and reverse reactions of the creatine kinase can affect rates of nucleotide exchange by effective recycling of matrix nucleotides which compete with nucleotides present in the medium for transport back into the mitochondrial...
Mitochondrial ADP/ATP Transport and Creatine Kinase

FIG. 3. Lack of effect of creatine or creatine phosphate on adenine nucleotide transport. A, effect of creatine on ATP\textsubscript{in} \(\xrightarrow{\text{[H]ADP,}}\) exchange. Rat heart mitochondria were preloaded with unlabeled ATP and assayed as described under "Experimental Procedures" except that 3 mM EDTA was substituted for MgCl\textsubscript{2} in the assay medium. Mitochondria were assayed in the absence (■) and presence (○) of 10 mM creatine. The reaction was started by adding 50 µl of 200 µM [H]ADP (5000 cpm/nmol, 10 µM final concentration) and stopped at the indicated times with 10 µM carboxyatractylsodie, centrifuged, washed, and counted as described under "Experimental Procedures." B, effect of creatine phosphate on ADP\textsubscript{in} \(\xrightarrow{\text{[H]ATP,}}\) exchange. Rat heart mitochondria were preloaded with unlabeled ADP and assayed as described under "Experimental Procedures" except that 3 mM EDTA was substituted for MgCl\textsubscript{2} in the assay medium. Mitochondria were assayed in the absence (■) and presence (○) of 10 mM creatine phosphate. The reaction was started by adding 50 µl of 400 µM ATP (4000 cpm/nmol, 20 µM final concentration) and stopped at the indicated times with carboxyatractylsodie, centrifuged, washed, and counted as described above.

FIG. 4. Competitive nature of creatine kinase activity on adenine nucleotide transport. A, Lineweaver-Burk plot of the effect of the forward reaction of creatine kinase on [H]ADP uptake. Rat heart mitochondria were preloaded with unlabeled ATP and assayed in the presence (○) and absence (■) of 10 mM creatine as described under "Experimental Procedures." Initial velocity measurements were made using a syringe apparatus designed for the simultaneous addition of substrate or inhibitor (14). The reaction was started by adding [H]ADP (2000-5000 cpm/nmol) at a final concentration of 4, 27, 44, and 100 µM and stopped after 2 s by injecting 10 µM carboxyatractylsodie, centrifuged, washed, and counted as described under "Experimental Procedures." Results shown are the mean values of triplicate measurements. The precision of measurement was < ± 5% for all points. B, results shown are a replot of data in A expressed as the percentage that mitochondrial nucleotides contribute to nucleotide uptake versus external competing ADP levels.

matrix. This selective interaction can thus act to augment rates of oxidative phosphorylation (Fig. 5), which in turn acts to drive effective creatine phosphate synthesis (1).

DISCUSSION

Studies examining the interaction between the translocase and creatine kinase have largely focused on how mitochondrial ATP affects the rate of creatine phosphate synthesis (3–5). The current study, on the other hand, is the first to examine this interaction from the viewpoint of nucleotide transport. A functional interaction between the adenine nucleotide translocase and mitochondrial creatine kinase could conceivably comprise several partial reactions, not all of which need to occur to facilitate metabolic flux. For example, for the forward reaction of creatine kinase, intramitochondrial ATP could have preferred access to creatine kinase, but the newly
generated ADP molecule may or may not have preferred access for transport back into mitochondria. Alternatively, both the nucleotide substrate and product could have preferred access to the transporter. A similar situation could exist for the reverse reaction of creatine kinase; namely, matrix ADP could have preferred access to the enzyme, whereas the newly generated ATP molecule may or may not have preferred access for transport.

Most studies to date have focused on whether mitochondrial generated ATP has preferred access to creatine kinase, leaving unresolved the status of the newly generated ADP molecule, and have not explored a possible interaction involving the reverse reaction of creatine kinase. The experimental protocol used in the current study examines the degree to which matrix nucleotides are actually recycled. The experimental design used here provides a minimal estimate as to the degree to which mitochondrial nucleotides acting as preferred substrates interact with the enzyme. Evidence presented here fully supported the previous findings that both the nucleotide substrate and product of creatine phosphate synthesis are preferentially delivered to (3-5) and removed from (8, 9) the enzyme, respectively, by the action of the translocase. This finding is important since it may largely account for why the mitochondrial isoenzyme, when bound to the inner membrane, is more resistant to product inhibition compared to in the unbound state and the other isoenzyme forms (4, 18). Results shown in Fig. 1B and 2B demonstrate that this two-way interaction is also seen for the reverse reaction of creatine kinase; namely, newly transported matrix ADP preferentially reacts with the enzyme, and the resulting ATP is preferentially translocated across the membrane over medium ATP.

Recently, Erickson-Viitanen et al. (19) have reported that the interaction between the translocase and creatine kinase is largely dependent on the integrity of the outer mitochondrial membrane and can be abolished by disrupting the outer membrane with digitonin or by preparing mitochondria from trypsin-treated homogenates. In the current study, mitochondria were also prepared from protease-treated homogenates, yet the interaction remained intact. This discrepancy may be attributable to differences in exposure time to the protease, specificity of protease, and species sensitivity. (Mitochondria used here were prepared from rat heart homogenates exposed to Nagarse for 8 min, whereas Erickson-Viitanen et al. (19) prepared mitochondria from rabbit heart homogenates exposed to trypsin for 35 min.) In addition, they concluded that a functional interaction results from a “restricted diffusion” barrier established by the outer membrane. This conclusion is plausible but unlikely in view of previous findings demonstrating that the outer membrane is freely permeable to molecules with a molecular weight less than 5000 (20). A more likely explanation is that treatment of mitochondria with digitonin induces subtle conformational changes in the inner membrane, perhaps due to the disruption of the outer membrane, which abolishes a functional coupling.

The exchange of adenine nucleotides across the inner membrane is an electrogenic process (21) in which the preferential transport of ADP versus ATP is greatly affected by the energy state of mitochondria (13, 14, 22-24). Results reported here were performed under conditions of high energy state, and thus it might well be expected that a variation in the mitochondrial energy state could affect the degree to which an interaction occurs. This is especially true in view of the results reported here and by others (8, 9) that this interaction is a two-way phenomenon.

A functional coupling between the translocase and creatine kinase may also have unexpected thermodynamic consequences concerning the efficiency of energy transduction from substrate oxidation by the electron transport chain and creatine phosphate synthesis. It is known that the exchange of ATP for ADP is a fully electrogenic process (21). Since the orientation of the membrane potential is negative inside, electrogenic nucleotide exchange will act to lower the membrane potential (i.e., the proton motive force) and thereby the driving force for ATP synthesis. The energy requirements to maintain electrogenic transport are not established and depend on the ratio of H⁺ efflux/ATP synthesized, but data from Mitchell (25) would indicate that up to 50% of the energy liberated by substrate oxidation is required. Since creatine phosphate synthesis generates one proton for each molecule of creatine phosphate formed, this should act to increase the ΔpH across the inner membrane and therefore “recover” some of the energy necessary to drive electrogenic nucleotide exchange.

The dynamics of energy transduction in heart are such that upon inhibition of oxidative phosphorylation, creatine phosphate levels decline prior to a decline in the levels of ATP. This observation has been used to support the hypothesis that creatine phosphate, not ATP, is the primary molecular species responsible for cellular energy transduction (1). This model is fully consistent with the observation that the end product of oxidative phosphorylation in muscle is creatine phosphate and that this reaction is facilitated by a functional interaction between the translocase and creatine kinase. Curiously, however, one would expect that a functional interaction should result in mitochondrial ATP levels, which represent approximately 15% of total cellular levels, declining prior to a decline in creatine phosphate levels upon inhibition of oxidative phosphorylation. This effect should be transient and would be quickly followed by a decline in creatine phosphate levels. Using a newly developed 31P NMR oxygen-gating technique, which can resolve changes in metabolite levels on a much faster time frame than previous techniques, we have observed that approximately 1 s following anoxic perfusion of isolated working rat hearts, cellular ATP levels decline by 10-15% prior to a decline in creatine phosphate levels (26).
REFERENCES