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EVIDENCE AGAINST DIRECT TRANSFER OF THE ADENINE NUCLEOTIDES BY THE HEART MITOCHONDRIAL CREATINE KINASE-ADENINE NUCLEOTIDE TRANSLOCASE COMPLEX

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Summary: Adenine nucleotide translocase inhibitors, atractyloside and carboxyatractyloside, added to respiration inhibited rat heart mitochondria had no significant effects on the apparent Km's for ATP and ADP and Vmax's of the creatine kinase reaction. The results suggest that there is no sequential, mandatory, direct transfer of the nucleotides between the translocase and the creatine kinase active site. The reaction was run in both the forward and reverse directions in media containing either 0.25 M sucrose or 0.13 M KCl. The apparent Km's, however, were found to be 2-3 times higher in the KCl medium than in sucrose, suggesting the use of the more physiological medium for meaningful kinetic studies.

Previous studies have shown that there is some form of functional coupling between the adenine nucleotide translocase and mitochondrial creatine kinase (1-6). Functional coupling, in the forward direction, is defined kinetically as a decrease in the apparent K_m for ATP (4,7). In the reverse reaction, ADP transfer from the enzyme to the translocase is enhanced (6,8). In either case there are mechanistic implications with respect to the dynamics of the creatine kinase-translocase interaction. One possible mode of action is that the translocase is merely in close proximity to creatine kinase. Therefore, an unstirred region of high nucleotide concentration is created. This model would imply no obligatory coupling, but solely a favorable supply situation. Another possibility would be that ATP must first interact with the translocase prior to transfer to the creatine kinase nucleotide binding site. For ADP, the nucleotide must bind to the translocase prior to transport into the matrix or release into the medium. In either case, nucleotide binding to the translocase would be an obligatory intermediate step in the coupled reaction. This model implies a direct coupling of the active sites of the two

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proteins which should be affected by use of inhibitors of the translocase, actractyloside and carboxyatractyloside. Thus, if the latter model were valid these inhibitors would change the binding capabilities of nucleotides to the translocase, and thereby the kinetic parameters of the creatine kinase reaction. The experiments in this report were designed to differentiate between these two models.

EXPERIMENTAL METHODS

Materials: All enzymes, nucleotides, phosphoenolpyruvate, inhibitors and Tris buffer were obtained from the Sigma Chemical Co. All other reagents were of the highest purity commercially available. Deionized water was used to prepare all solutions.

Mitochondrial Isolation: Mitochondria were isolated from the hearts of Sprague-Dawley female retired breeder rats by differential centrifugation in a sucrose-mannitol solution containing 0.1 mM EGTA, according to the method of Pande and Blanchaer (9). All centrifugations were performed at 2°C in a Beckman J-21B refrigerated high-speed centrifuge. All solutions and instruments were kept on ice. Sigma bacterial protease Type VII was substituted for Nagarse in all isolations. The mitochondrial fractions demonstrated respiratory control ratios of 3-6 and ADP/O ratios of over 1.50 with succinate as the substrate and 20μM rotenone to inhibit endogenous respiration. Protein assays were performed according to the method of Bradford (10) with nitrogen-standardized bovine serum albumin as the primary standard.

Creatine Kinase Assay: The enzymatic activity of mitochondrial creatine kinase was assayed in the reverse direction, ADP+PCr$\rightarrow$ATP+Cr, by using a standard hexokinase-glucose-6-phosphate dehydrogenase regenerating system to monitor the rate of ATP production (2,11). The activity of the forward reaction, was followed by using a pyruvate kinase-lactic dehydrogenase system to monitor the rate of ADP production via the oxidation of NADH (4). To correct for contaminating ATPase activity, the rates of ADP production in the absence of creatine were also determined. All assay media contained either 0.25 M sucrose or 0.13 M KCl to minimize swelling and thus preserve mitochondrial integrity. The media were titrated to pH 7.4 at 30° C. All mitochondria were treated with 20μM rotenone and 2μg/mg oligomycin. Concentrations of 5 nmoles carboxyatractyloside and 20 nmoles atractyloside per mg protein were added in accordance with values reported to completely inhibit adenine nucleotide translocation (6).

RESULTS

Mitochondrial creatine kinase activity was assayed using both the forward and reverse reactions to determine the effects, if any, of adenine nucleotide transport inhibitors on the dissociation constants for ATP and ADP, and on the rates of the reactions. In addition, assays were performed in two different media, 0.25 M sucrose and 0.13 M KCl, to determine if they also effected the reaction. Figure 1 presents, in double reciprocal form, the results of the kinetic data for reactions assayed in the sucrose medium. Figure 1A presents

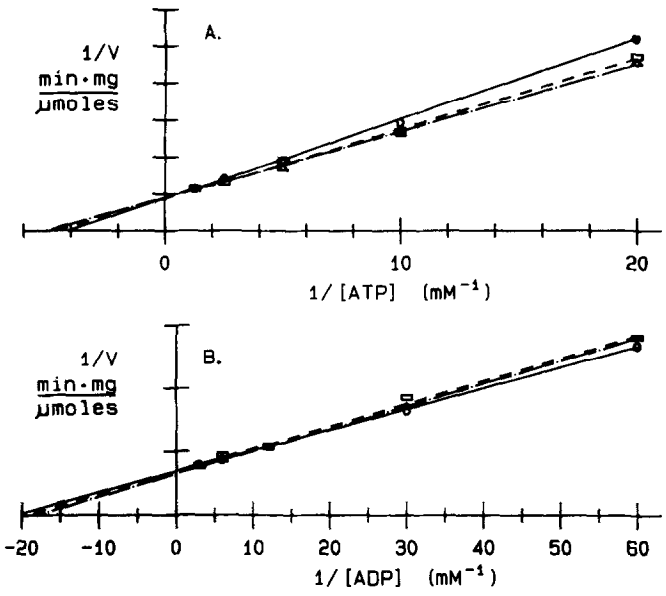

Figure 1: Lineweaver-Burk plots of the kinetic data for the forward (A) and reverse (B) mitochondrial creatine kinase reactions assayed in a 0.25 M sucrose medium, pH 7.4 at 30°C. The assay mix for the forward reaction contained 10mM Tris A, 3.0mM phosphoenolpyruvate, 0.12mM NADH, 3.3mM $MgCl_2$, 4IU/ml pyruvate kinase, 8 IU/ml lactic dehydrogenase, and 20mM creatine. The assay mix for the reverse reaction contained 10mM Tris Cl, 0.15mM NADP, 3mM Mg Cl_2, 3.3mM glucose, 1mM AMP, 0.10 IU/ml hexokinase, 0.07 IU/ml glucose-6-phosphate dehydrogenase, and 8.3mM phosphocreatine. Rat heart mitochondria were inhibited with 2.0 μg/mg oligomycin and 20μM rotenone. The symbol O represents the averaged data points (N=10) and the solid line is the regression line for the uninhibited system while the symbols △ and □ and the lines ----- and —·— represent the 20 nmoles/mg atractyloside and 5nmoles/mg carboxyatractyloside conditions, respectively. The intersections of the lines on the x-axis equal $-1/K_m$ and the intersections on the y-axis equal $1/V_{max}$.

the data for the forward reaction with ATP concentrations ranging from 50-800μM. The three lines represent the control condition and when atractyloside or carboxyatractyloside were present. Figure 1B corresponds to the reverse reaction for a concentration range of 16.7-333.3μM ADP, under the three experimental conditions. In both plots, the abscissa intercepts represent the apparent K_m for nucleotide binding. Table I lists the values for the K_m's as well as the V_{max}'s for the three experimental conditions. From these data it becomes apparent that blocking nucleotide interaction with the translocase has no significant effects upon the kinetics of the mitochondrial creatine kinase reactions.

Figure 2 represents the kinetic data for the creatine kinase reaction assayed in KCl medium, a condition considered to be more physiological. The

TABLE I

KINETIC CONSTANTS FOR MITOCHONDRIAL CREATINE KINASE REACTION ASSAYED IN 0.25 M SUCROSE MEDIUM

	Cr + ATP → PCr + ADP			PCr + ADP → Cr + ATP		
Conditions	Km ATP	Vmax	R^2	Km ADP	Vmax	R^2
Control	242.7	1.13	.9991	47.5	1.44	.9996
+Atractyloside	198.4	1.11	.9972	55.3	1.54	.9996
+Carboxyatractyloside	216.0	1.14	.9976	52.0	1.45	.9962

Data were derived from Figure 1: Km values are μM.
Vmax values are μmoles/min/mg protein.

apparent K_m and V_{max} data for these experiments are listed in Table II. Table II again shows that there is no significant difference between the inhibited and uninhibited systems. However, when these data are compared to the values reported in Table I, one notes significant differences in the K_m's from medium to medium. The values obtained in KCl medium are consistently 2-3 times higher that the corresponding values in sucrose. These data are consistent with the observations of Nihei, et al.(12) concerning anion inhibition of the

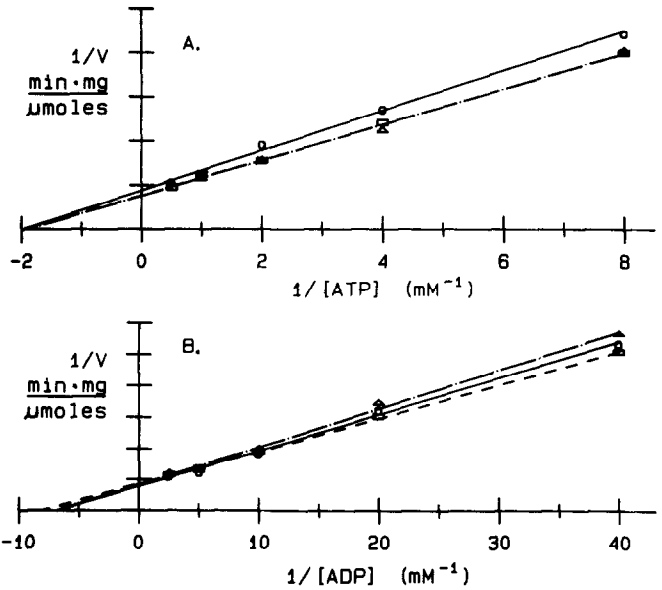

Figure 2: Lineweaver-Burk plots of the kinetic data for the forward (A) and reverse (B) mitochondrial creatine kinase reactions assayed in a 0.13 M KCl medium, pH 7.4 at 30°C. All other conditions of the assays and graphic symbols are identical to those described for Figure 1. The data points are again averaged (N=6).

TABLE II

KINETIC CONSTANTS FOR MITOCHONDRIAL CREATINE KINASE REACTION ASSAYED IN 0.13 M KCl MEDIUM

	Cr + ATP → PCr + ADP			PCr + ADP → Cr + ATP		
Conditions	Km ATP	Vmax	R^2	Km ADP	Vmax	R^2
Control	509.9	1.13	.9995	147.5	1.28	.9966
+Atractyloside	527.8	1.31	.9956	146.6	1.20	.9954
+Carboxyatractyloside	519.5	1.30	.9987	123.4	1.17	.9995

Data were derived from Figure 2; units are same as Table I.

creatine kinase reaction. This finding necessitated that the concentration ranges for ADP and ATP be raised to 25-400μM and 125-2000μM respectively to obtain valid kinetic data in the KCl medium.

DISCUSSION

The experiments presented in this report were designed to explore in greater depth the mechanistic implications of the functional coupling of the mitochondrial creatine kinase-adenine nucleotide translocase complex. Atractyloside was used because it competitively displaces ATP or ADP from the translocase, while carboxyatractyloside was used as a non-competitive inhibitor. If there were sequential, direct, nucleotide transfer, atractyloside would alter the kinetics by significantly raising the apparent K_m values. This would be expected because of its action as a competitive inhibitor of the translocase. Carboxyatractyloside, on the other hand, would have lowered the V_{max} of the reaction, if effective, since it operates as a non-competitive inhibitor. The results of our experiment show no significant difference in the dissociation constants for ATP or ADP or in the V_{max} when the translocase nucleotide binding site is blocked.

Since neither of these inhibitors displayed any effect on the kinetic parameters of the creatine kinase reaction, the data seems to rule out the idea that for maximal enzymatic activity the nucleotide must first bind to the translocase before interacting with creatine kinase. More likely, the data suggests that the translocase is creating a region of higher nucleotide

concentration to which creatine kinase has preferred access because of its position on the outer aspect of the inner mitochondrial membrane. This would also account for the lowering of the apparent K_m of ADP for oxidative phosphorylation when the creatine kinase reaction is generating ADP, as opposed to when ADP is supplied exogenously (13).

It must be kept in mind, however, that our results pertain to a rather abnormal situation. We have used rotenone and oligomycin to block electron transport and ATPase activity. Thus, we do not know the situation when mitochondria are in an "energized" state. Hackenbrock and others have demonstrated considerable morphological changes as a function of respiratory state (14). Vignais et al. (15) have also shown effects of membrane energization on the kinetics of ADP translocation. Thus, under more in vivo conditions, a different set of interactions could exist which would influences the dynamics of the creatine kinase-translocase complex.

Another interesting aspect of the experimental results is the rise in the apparent K_m's when KCl medium is used versus sucrose. This poses the question as to which is the preferred medium for kinetic studies of mitochondrial processes. The normal cell cytoplasm contains nearly 150mM K^+ and a comparatively lower amount of sugars. This would suggest that results obtained in KCl medium more properly reflect the physiological state in vivo and is an important consideration for further studies on the dynamics of the creatine kinase-translocase system.

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