INNER COMPARTMENT LOCALIZATION OF HEART MITOCHONDRIAL NUCLEOSIDE DIPHOSPHOKINASE

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Summary: Mitochondria isolated from rat heart contained nucleoside diphosphokinase (EC 2.7.4.6) at a specific activity of 30 mIU/mg protein, or about one half of liver mitochondrial activity, 60 mIU/mg. In contrast to liver mitochondria, no stimulation of O_2 uptake was observed when 150 μM GDP was added to heart mitochondria respiring in post-ADP State 4, and the transphosphorylation of [γ-^{32}Pi] from ATP into GTP was marginal. However, when heart mitochondria pretreated with oligomycin were solubilized with 0.03% Triton X-100, a five fold increase in the rate of GTP formation was observed. These results show that in heart mitochondria approximately 80% of the nucleoside diphosphokinase activity is localized within the inner compartment.

Mitochondrial nucleoside diphosphokinase has been purified and well characterized from both liver and heart sources (1-6). Studies carried out by Schnaitman and Greenawalt (7) indicated that in liver mitochondria most of the enzyme was localized in the soluble fraction between the inner and outer membranes. Since that report, nucleoside diphosphokinase has been considered a marker enzyme for the intermembrane space (8). Little attention has been paid to the submitochondrial compartmentation of the heart enzyme.

Investigations in this laboratory have focused on phosphotransferase enzymes of the myocardium which may regulate the levels of high energy compounds within the heart cytosol (9-11). Thus, experiments were initiated to investigate the possible involvement of mitochondrial nucleoside diphosphokinase in the channeling of high energy phosphate from the mitochondrion to the sarcoplasm. This hypothesis has been proposed by Lehninger (12), and documented by Pedersen (13) for the liver mitochondrial system. Data presented in this paper suggest that in contrast to liver, heart mitochondrial nucleoside diphosphokinase is principally localized within the inner compartment. The metabolic function of the enzyme is discussed from the standpoint of its integration with the Kreb's cycle at the level

of succinic thiokinase, as well as its potential function in the supply of GTP requisite for endogenous fatty acid activation.

MATERIALS AND METHODS

Materials: Nucleotides and Tris base were marketed by Sigma Chemical Co. Hexokinase and glucose 6-phosphate dehydrogenase were products of Boehringer Mannheim. The Enzyme Development Corp., New York, was the source of nagarse protease. [^{32}Pi] orthophosphate was purchased from ICN, Irvine, Calif. Thin layer polyethyleneimine-impregnated cellulose chromatography plates (Polygram Cel PEI) were prepared by Brinkmann Inst. Co. All other chemicals were of the highest purity available.

Preparation of Subcellular Fractions: Rat livers were homogenized in 70 mM sucrose, 210 mM mannitol, 2.1 mM HEPES buffer, and 0.5 g/l bovine serum albumin. Hearts were homogenized in 210 mM mannitol, 70 mM sucrose, and 0.1 mM ethylene glycol bis (β-aminoethyl ether)-N,N'- tetraacetic acid (EGTA). Homogenates were fractionated by differential centrifugation at g forces indicated in Table I.

Isolation of Mitochondria: Liver mitochondria were isolated by standard methods of differential centrifugation (15). Nagarse and non-nagarse treated heart mitochondria were isolated by methods previously detailed (16), where the purity and characteristics of these preparations have been reported.

Nucleoside Diphosphokinase Assay: Enzyme activity was assayed spectrophotometrically in the direction ADP + TTP → ATP + TDP. A hexokinase-glucose 6-phosphate dehydrogenase system was used to trap ATP formed (9). The increase in optical density caused by the reduction of NADP was recorded at 340 nm with a Gilford 2400 spectrophotometer. In addition to the normal components of the assay system, 280 mM KCN and 30 μM rotenone were included to inhibit electron transport, and 10 μl of 10% Triton X-100 was added to disrupt intact mitochondria.

Respiration Measurements: Rates of oxygen consumption of rotenone treated mitochondria were measured by standard techniques (9) in a 3.0 ml closed oxygraph chamber. The amounts of mitochondrial protein and the sequence of additions are indicated in the legends.

Chromatographic Isolation of GTP: GTP was separated from ATP and Pi by the method of Randerath and Randerath (17) using thin layer polyethyleneimine-impregnated cellulose plates, and 2.0 M sodium formate, pH 3.4, as the solvent. Non-radioactive standards of GTP and ATP, 10 nmoles each, were spotted as markers over the 5 μl samples. Following ascending chromatography, the samples were located by u.v.-light, cut, and quantitated by liquid scintillation.

RESULTS

The intracellular distribution of heart nucleoside diphosphokinase is quite similar to that of liver, Table I. Approximately 80% of the enzyme was found in the soluble cytosol, with about 2.5% associated with the mitochondrial fraction. It should be noted that the latter value represents a minimum estimate, for the yield of protein in this fraction was about 15 mgs per g tissue. Scarpa and Graziotti (18) have reported that the mitochondrial content of rat heart

TABLE I

DISTRIBUTION OF NUCLEOSIDE DIPHOSPHOKINASE IN VARIOUS SUBCELLULAR FRACTIONS OF RAT LIVER AND HEART

Cell Fraction	Percent Activity	
	Liver	Heart
Homogenate	100	100.0
480g Pellet	16	12.5
Mitochondria (12,000g)	4	2.5
175,000g Pellet	1	2.0
Soluble Supernatant	78	78.0
Recovery	98.6	95.2

Tissue subcellular fractions were separated by differential centrifugation (See Materials and Methods). Samples, 10 µl, were added to the 3.0 ml assay medium and preincubated for 2 min at 25°C. The control rate of NADP reduction was recorded for 2 min. The reaction was initiated by the addition of 0.1 ml of 30 mM TTP, and the rate of ATP production (9) was calculated from the increased slope. Total enzyme activity was calculated for each fraction and the results are expressed as percent of the activity in the initial homogenate.

approximates 75 mgs per g tissue. Thus, the content of mitochondrial enzyme may be 5-fold greater. The specific activity of the enzyme in intact mitochondria was 60 mIU/mg protein for rat liver, and 30 mIU/mg for nagarse prepared rat heart. It was considered that these differences could represent protease destruction or release of the heart enzyme. Heart mitochondria prepared in the absence of nagarse had a specific activity of 36 mIU/mg, a value which was corrected for non-mitochondrial protein contamination (16).

Pedersen (13) has shown that the liver enzyme may utilize ATP produced by oxidative phosphorylation to generate XTP and ADP; the latter recycling into the matrix for steady-state phosphorylation. By this path, the enzyme

might channel high energy phosphate from the mitochondrion in response to cytoplasmic metabolism. The data of Fig. 1 demonstrate that upon return to post-ADP State 4, the addition of GDP to mitochondria induced an acceleration of respiration for liver (Fig. 1A), but not for heart (Fig. 1B). Consistent with these data was the observation that under conditions of Fig. 1, the transphosphorylation of GDP to GTP by [γ-^{32}Pi] ATP was 125 nmoles/mg in 5 min for liver mitochondria, but merely 20 nmoles/mg in 5 min for heart.

The absence of GDP stimulated respiration could be accounted for either by unique compartmentation of the enzyme, or by a GDP specific inhibition of ADP transport which would prevent the recycling of ADP. However, the low rates of transphosphorylation under conditions of high levels of outer compartment ATP (data above), as well as the results of Fig. 2 strongly argue against the latter. At GDP concentrations ranging from 50 to 600 μM, the

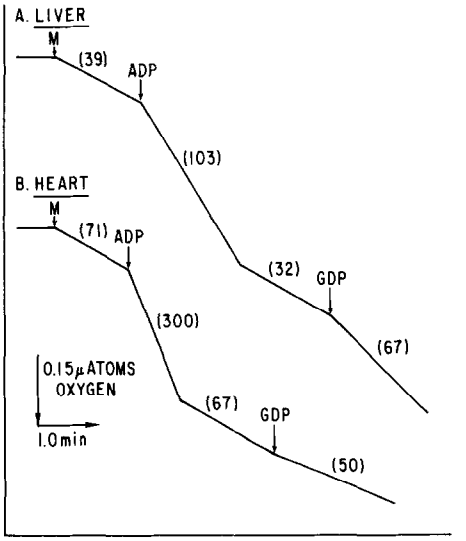

Fig. 1. Effects of nucleoside diphosphates on the respiratory rates of isolated mitochondria. A: Liver mitochondria, 3.15 mg, protein or B: Heart mitochondria, 1.0 mg, protein were incubated in 2.8 ml of mannitol-sucrose-Tris oxygraph medium (9) containing 5 mM succinate as substrate. The reaction was initiated by the addition of mitochondria (M). Where indicated, 500 nmoles of ADP and 500 nmoles of GDP were added to the chamber. The numbers on the traces refer to rates of oxygen consumption in nanogram atoms of oxygen per min per mg mitochondrial protein.

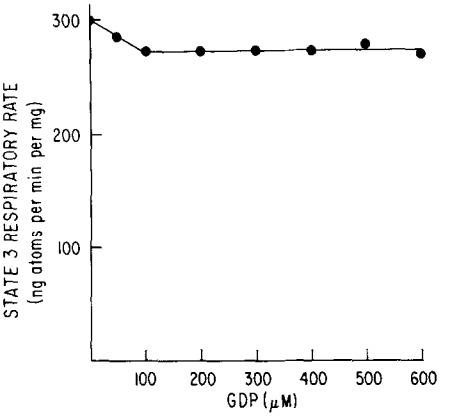

Fig. 2. Effect of GDP on heart mitochondrial State 3 respiratory rates. Heart mitochondria (1.0 mg) were incubated under conditions of Fig. 1. GDP at the indicated concentrations was added immediately prior to the initiation of State 3 respiration. State 3 was induced by pulsing the medium with 500 nmoles of ADP.

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rates of ADP induced State 3 respiration were only slightly lowered. Therefore, GDP did not inhibit the influx of exogeneous ADP.

The data of Fig. 3 confirm the inner compartment localization of the heart enzyme. Mitochondria were incubated in oxygraph medium containing [^{32}Pi]. Mitochondrial phosphorylation of ADP generated [γ-^{32}Pi] ATP; the reaction was complete within 30 s. Oligomycin (O) was used to block ATP'ase activity, insuring the maintenance of high levels of ATP during the subsequent incubation. The addition of GDP resulted in the slow incorporation of [^{32}Pi] into GTP. Following equilibration, Triton X-100 (T) was added to solubilize the membranes, and clarification of the mixture occurred in less than 30 s. Disruption of the inner membrane nucleotide permeability barrier resulted in a 5-fold increase in the rate of GTP production. In control experiments, Triton had no effect on the enzyme activity of osmotically lysed heart or liver mitochondria. Furthermore, the initial rise in GTP prior to the addition of Triton was still observed when atractyloside was added with oligomycin. When mitochondria were pretreated with both inhibitors in the absence of added ADP, no GTP was formed upon the addition of GDP even though matrix ATP was labelled and stable at a concentration

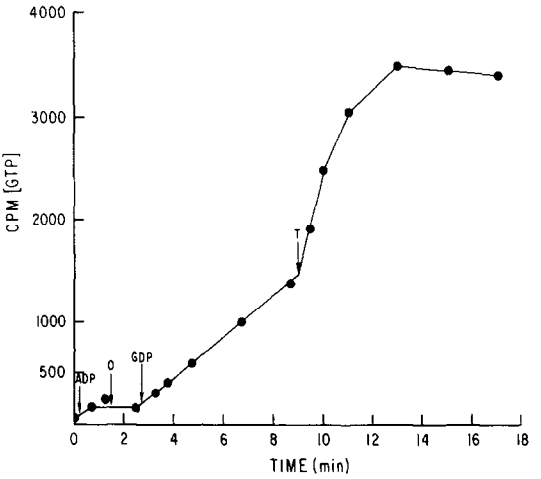

Fig. 3. Incorporation of [^{32}Pi] into GTP. Components of the 2.8 ml medium are described in Fig. 1, except for the inclusion of 100 µCi of [^{32}Pi]. Heart mitochondrial protein, 4 mg, was added to the mixture, incubated for 30 s, and a control sample (0.1 ml) removed. Within 15 s, 1000 nmoles of ADP was added to generate [γ-^{32}Pi] ATP. Following complete phosphorylation, in less than 1 min, 16 µg of oligomycin (O) was pulsed into the medium. One min later, 500 nmoles of GDP were added and equilibrated for 6 min. At the indicated point (T), Triton X-100 was added to a final concentration of 0.03%. Data points represent the removal of 0.1 ml samples, which were pipetted into 5.7 x 50 mm plastic centrifuge tubes containing 5 µl of 2.5 M $HClO_4$. The samples were extracted on ice for 15 min, neutralized with 5 µl of 2.5 M KOH, and centrifuged for 3.0 min. Aliquots, 5 µl, were spotted on PEI- cellulose sheets and chromatographed (See Materials and Methods).

approaching 10 mM. These latter experiments suggest that the GTP produced prior to Triton lysis was transphosphorylated by enzyme found in the outer compartment. The post-Triton rate was catalyzed by inner compartment enzyme, accounting for 80% of the total activity. Definitive submitochondrial localization of the enzyme is under current investigation.

DISCUSSION

New information concerning the enzymatic integration of heart mitochondrial metabolism has accrued from the results detailed in this report. The data in this communication document the inner compartment localization of nucleoside diphosphokinase and suggest several areas for future investigation. Since Sanadi et al. (19) examined the nucleotide specificity of succinic thiokinase (EC 6.2.1.5),

some mechanism for the regeneration of matrix GDP has been required. The participation of nucleoside diphosphokinase has been widely postulated although the outer compartment site of the enzyme raised questions as to the probability of its direct role.

Confirmation of the inner compartment kinase suggests at least two routes for the supply of nucleotide. In principle, matrix GDP required at this step may simply be reformed by the turnover of endogenous adenine nucleotides. On the other hand, ADP may enter the mitochondrion from the cytoplasm for trans-phosphorylation and export as ATP (Fig. 4A). This latter scheme suggests that levels of cytoplasmic adenine nucleotides, or the cellular energy charge (20), may modulate the Kreb's cycle via communication with nucleoside diphosphokinase (NDK) and succinic thiokinase (ST). Thus, this mechanism represents a plausible pathway for the coordination of mitochondrial metabolism with the beat-to-beat energy demands of the sarcoplasm. Detailed investigations of the regulatory properties of the enzyme are in progress.

The inner compartment enzyme may also play an important role in endogenous

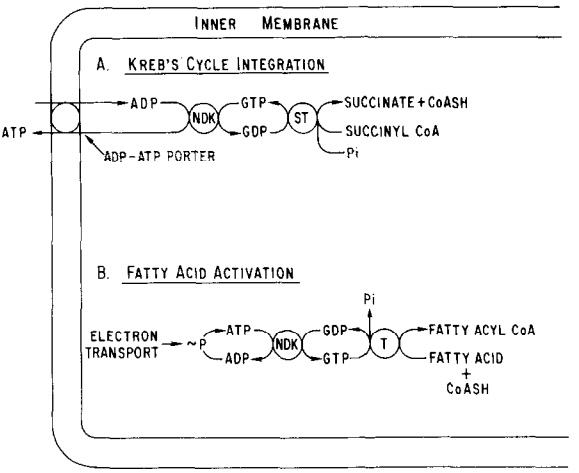

Fig. 4. Schematic representation of the metabolic functions of inner compartment nucleoside diphosphokinase. NDK = nucleoside diphosphokinase (EC 2.7.4.6). ST = succinic thiokinase (EC 6.2.1.5). T = GTP specific fatty acid thiokinase (EC 6.2.1.10).

fatty acid activation for β-oxidation (Fig. 4B). The activation of free fatty acids at the outer membrane utilizes ATP; the GTP requirement of matrix fatty acyl CoA synthetase (T) was reported by Rossi and Gibson (21). Thus, as visualized, nucleoside diphosphokinase could be an important link between substrate activation and mitochondrial electron transport.

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