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BBA Report

Hexokinase bound to rat brain mitochondria uses externally added ATP more efficiently than internally generated ATP

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The rates of glucose phosphorylation by bound hexokinase were investigated in mitochondria isolated from rat brain. Initial rates obtained either with ATP generated from oxidative phosphorylation or with ATP added externally were compared. Our results show that the external ATP supports a 2–3-fold higher hexokinase activity than does ATP generated by oxidative phosphorylation under State 3 conditions. ATP formed by mitochondrial creatine kinase in the presence of creatine phosphate also supports higher initial rates of glucose phosphorylation than does oxidative phosphorylation. The data suggest that concentrations of ATP present in the cytosol of normal tissue will probably maintain higher rates of glucose phosphorylation than ATP being exported directly from the mitochondrial matrix at maximal State 3 rates.

Hexokinase is bound to the outer membrane of mitochondria in many tissues [1–7]. It has been postulated [8–10] that the bound enzyme has preferential access to matrix-generated ATP. In contrast, our previous studies [11] on tumor mitochondria showed that ATP added externally supports higher rates of glucose phosphorylation than ATP generated by oxidative phosphorylation under State 3 conditions. Our findings were recently confirmed for mitochondria from two additional tumor cells [12]. These authors [12] found that hexokinase activity in poorly differentiated, rapidly growing tumors is 6.5-fold higher when ATP is added externally than if supplied by oxidative phosphorylation. In well-differentiated, slowly growing tumors this value decreased to 1.8-fold. It was suggested [12] that the contribution of ATP by oxidative phosphorylation is increased in well-differentiated cells. This conclusion predicts that mitochondrial hexokinase in normal tissues should exhibit a relatively high requirement for ATP from oxidative phosphorylation. We have tested this idea using rat brain mitochondria. Our results show that external ATP supports a 2–3-fold greater hexokinase activity than does ATP generated within the matrix.

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ATP, ADP, AMP, diadenosine pentaphosphate, carboxyatractyloside and oligomycin were obtained from Sigma. Fluorodinitrobenzene was purchased from Fluka. All other reagents were highest purity grade commercially available. Male Sprague-Dawley rats (150–200 g) were used. All animals had free access to rat pellets and water ad libitum.

Non-synaptosomal brain mitochondria were prepared by a modification [13] of the method of Clark and Niklas [14]. Tumor mitochondria were isolated as described earlier [15].

Respiratory activity was measured polarographically [16] in a medium containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM KP_i and 50 mM Tris-HCl (pH 7.5), 10 mM succinate and 0.5 mM ADP [15]. Mitochondrial hexokinase was assayed in 1 ml of the same media supplemented with 1 mM glucose, 1 mM $NADP^+$, 20 units of glucose-6-*P*-dehydrogenase (yeast, Boehinger) and approx. 300 μg mitochondrial protein. The reaction was started by addition of ATP (1 mM) or ADP (0.5 mM), or, when the affects of increasing ADP was titrated, by glucose. $NADP^+$ reduction was measured at 340 nm. Adenylate kinase was determined spectrophotometrically according to Brdiczka et al. [17]. Mitochondrial protein concentration was determined by the method of Lowry et al. [18].

Mitochondria isolated from rat brain were well coupled and contained high levels of bound hexokinase.

The specific activity of hexokinase measured in four mitochondrial preparations was 596 ± 57 nmol/min per mg protein (means ± S.E.). To determine whether the bound enzyme uses mitochondrially-generated ATP more efficiently than cytosolic ATP, the activity of bound hexokinase was studied spectrophotometrically under State 3 conditions in the presence of ADP and succinate (i.e., endogenous ATP) or in the presence of added ATP (i.e., exogenous ATP). Table I shows that the initial rates of hexokinase is 2–3-fold greater with exogenous ATP than with endogenous ATP. Even when ATP was regenerated externally via the phospho*enol*-pyruvate/ pyruvate kinase system, the activity of hexokinase is about double that observed with ATP generated by mitochondria under State 3 conditions.

To check that the ADP used for generating mitochondrial ATP did not inhibit hexokinase, the assay was carried in the presence of 1 mM external ATP and increasing amounts of ADP (Fig. 1). The reaction was started by addition of glucose. Neither brain or hepatoma mitochondrial hexokinase activity was influenced by ADP (Fig. 1). ADP concentrations to 2 mM were without effect on brain mitochondrial hexokinase (not shown). Furthermore, reactions rates remained linear after approximately half of the ATP in the cuvette had been converted to ADP, indicating that increasing ADP did not affect hexokinase activity during the course of the assay.

Furthermore, hexokinase was not released from mitochondria during the course of the assay. This was tested by rapidly pelleting the mitochondria in a microfuge 2 min after the assay had been initiated in the cuvette. More than 90% of the activity remained bound after a 2 min incubation in the presence of 1 mM ATP, 0.5 mM ADP or a combination of the two (not shown).

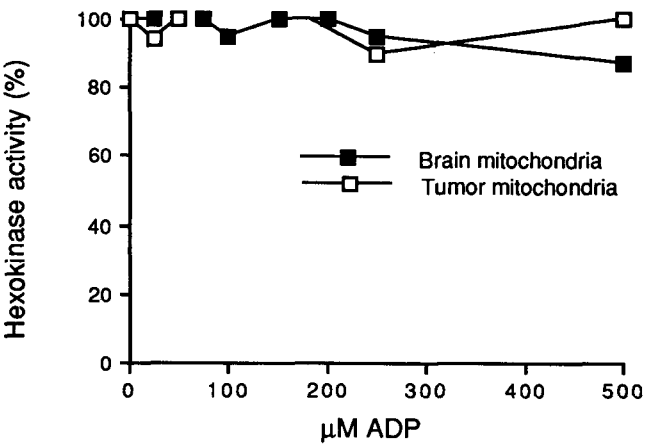

Fig. 1. ADP does not inhibit the ATP-supported hexokinase reaction in brain and hepatoma mitochondria. Rat brain and rat hepatoma [15] mitochondria were assayed for hexokinase activity in the presence of 1 mM ATP and increasing concentrations of ADP. The reaction was initiated by addition of 1 mM glucose. The conditions of the assay are given in the text. Hexokinase measured in the absence of ADP (100% activity) was 177 nmol/min per mg protein for brain and and 330 nmol/min per mg for tumor mitochondria.

Thus, we are measuring the activity only of the bound form of hexokinase.

Since ATP can be generated in tumor mitochondria either by oxidative phosphorylation or adenylate kinase [11], glucose phosphorylation was measured to study the relative contribution of these two ATP-generating systems in rat brain. Table I shows that the contribution by adenylate kinase is low (15%) in rat brain. This is most certainly due to the low adenylate kinase content in rat brain (50 nmol/min per mg) compared to rat liver mitochondria (800–1000 nmol/min per mg) [19,20] and Zajdela tumor mitochondria (350–500 nmol/min

TABLE I

External ATP, but not ATP generated from mitochondrial adenylate kinase, supports higher rates of hexokinase activity in isolated rat brain mitochondria than does ATP from oxidative phosphorylation

The activity of mitochondrial hexokinase was determined by a direct optical test system as described in the experimental procedures. ATP was generated via oxidative phosphorylation by addition of 0.5 mM ADP and 10 mM succinate. External ATP was generated with 1 mM phospho*enol*pyruvate (PEP) and 10 units of pyruvate kinase (PK), or by addition of 1 mM ATP. The following inhibitors were used: carboxyatractyloside (CAT), 25 μg/mg protein; oligomycin, 5 μg/mg protein; diadenosine pentaphosphate (DAPP, an inhibitor of adenylate kinase), 0.5 mM. The values are expressed as means ± S.E. for eight experiments.

Energy source:	Hexokinase activity					
	ADP + succinate		PEP/PK		ATP	
	nmol/min per mg	% inhibition	nmol/min per mg	% inhibition	nmol/min per mg	% inhibition
Control	75 ± 7	0	123 ± 11	0	191 ± 18	0
CAT	15 ± 1	80 ± 0.3	121 ± 11	2 ± 0.5	197 ± 18	1 ± 0.4
Oligomycin	14 ± 2	82 ± 1	121 ± 11	2 ± 1	196 ± 19	2 ± 1
DAPP	62 ± 6	17 ± 0.2	119 ± 11	3 ± 1	195 ± 18	2 ± 1
CAT + oligomycin	13 ± 1	83 ± 0.3	121 ± 11	1 ± 0.5	196 ± 18	2 ± 1
CAT + DAPP	0	100	121 ± 11	1 ± 1	197 ± 18	1 ± 0.5
DAPP + oligomycin	0	100	121 ± 12	2 ± 1	194 ± 18	3 ± 1

per mg) [11]. Metabolic inhibitors have almost no effect on hexokinase activity mediated by added ATP or by ATP generated with phospho*enol*pyruvate/pyruvate kinase (Table I). The latter ensures that ATP remains totally external (cytosolic) and does not equilibrate with the matrix pool.

In contrast, mitochondrial creatine kinase is present in significant amounts in brain [21]. Table II shows that in rat brain mitochondria creatine phosphate + ADP support a 3-fold higher hexokinase activity than ADP + succinate (oxidative phosphorylation). The data in Table II show that, in the presence of ADP + creatine phosphate, 60% of the ATP used for glucose phosphorylation comes from creatine kinase, 15% comes from oxidative phosphorylation, and 12% comes from adenylate kinase. The rates of hexokinase shown in Tables I and II varied with the mitochondrial preparation and the amount of bound hexokinase. However, the percent inhibition of hexokinase in the presence of different metabolic inhibitors remained almost the same.

In this report, we demonstrate that ATP added to a suspension of rat brain mitochondria supports a higher rate of hexokinase activity than does ATP generated from oxidative phosphorylation (Table I). Similar results were previously obtained by us with mitochondria isolated from a rapidly growing, poorly differentiated rat hepatoma [11], and this finding has been confirmed by Gauthier et al. [12] for two additional tumor cells, one slowly growing and well differentiated, and the other rapidly growing and poorly differentiated. Thus, it seems that in the presence of physiological levels of ATP, the bound hexokinase in most cells would use cytosolic ATP rather than ATP provided by oxidative phosphorylation.

Earlier reports [8–10] that bound hexokinase has preferential access to ATP supplied from oxidative phosphorylation were based upon experiments in which the measured hexokinase activity was low due either to the use of mitochondria from tissues which contain little or no hexokinase (liver) [8], or to the use of low, limiting concentrations of ADP which could produce ATP only through oxidative phosphorylation [7–10]. For example, although the apparent K_m for ADP in oxidative phosphorylation is about 30 μM [19], most earlier studies employed 10–30 μM ADP for measuring the contribution of oxidative phosphorylation to the hexokinase reaction [7–9]. Under these conditions, glucose phosphorylation is limited by ATP production (probably at the adenine nucleotide translocator), and ATP production is limited almost entirely to oxidative phosphorylation. Thus, the conclusion that matrix ATP is used preferentially by hexokinase was unavoidable.

We earlier [11] showed that increasing concentrations of ADP added to isolated hepatoma mitochondria decreased the relative contribution of matrix ATP (oxidative phosphorylation) to the hexokinase reaction and increased the contribution of ATP by adenylate kinase. This result was explained by differences in the K_{mADP} for oxidative phosphorylation (30 μM) and adenylate kinase (400 μM). In contrast, in the present study we show that adenylate kinase provides little ATP to hexokinase in brain compared to tumor [11]. This is due to the relatively low content of brain mitochondrial adenylate kinase. However, creatine kinase, which is abundant in brain mitochondria, contributes significant amounts of ATP to bound hexokinase in vitro. These studies indicate that in the presence of more physiological concentrations of ADP, non-matrix enzymes

TABLE II

External ATP or ATP generated from creatine kinase support higher rates of hexokinase activity in isolated rat brain mitochondria than does ATP from oxidative phosphorylation

The activity of mitochondrial hexokinase is expressed as nmol/min per mg protein. The reaction was initiated by addition of either 0.5 mM ADP + 10 mM succinate or 0.5 mM ADP + 1 mM creatine phosphate. The inhibitors were present in the following concentrations: carboxyatractyloside (CAT), 25 μg/mg protein; diadenosine pentaphosphate (DAPP), 0.5 mM and fluorodinitrobenzene (FDNB, an inhibitor of creatine kinase), 0.5 mM. The values are expressed as means ± S.E. for five experiments.

Energy source:	Hexokinase activity			
	ADP + succinate		ADP + creatine-PO_4	
	nmol/min per mg	% inhibition	nmol/min per mg	% inhibition
None	69 ± 10	0	212 ± 33	0
CAT	16 ± 3	77 ± 1	179 ± 26	15 ± 2
DAPP	57 ± 8	18 ± 2	187 ± 28	12 ± 1
CAT + DAPP	0	100	186 ± 28	12 ± 3
FDNB	65 ± 10	7 ± 1	88 ± 13	59 ± 0.5
CAT + FDNB	17 ± 3	76 ± 2	51 ± 8	76 ± 0.4
DAPP + FDNB	53 ± 8	23 ± 3	68 ± 10	68 ± 1
CAT + DAPP + FDNB	0	100	0	100

support higher hexokinase rates does oxidative phosphorylation, at least in vitro. However, such results do not necessarily mean that adenylate kinase and creatine kinase function in a similar fashion in vivo. The data are presented here only to point out possible complications in measuring 'channeling' in isolated mitochondria.

In summary, our data show that brain mitochondrial hexokinase catalyzes glucose phosphorylation at higher rates if saturating levels of ATP are supplied outside the mitochondria than if ATP is generated at maximal rates by oxidative phophorylation under State 3 conditions. Since similar results have also been obtained for both fast- and slow-growing tumor cells [11,12], this conclusion is probably valid for most tissues containing large amounts of bound hexokinase. Furthermore, since the levels of cytosolic ATP normally exceed the K_{mATP} for hexokinase, there appears to be little need for channeling ATP directly from the matrix to bound hexokinase. This is especially true for tumor cells which generate ATP via glycolysis [3].

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