INTRACELLULAR AND SUBMITOCHONDRIAL LOCALIZATION OF PIG HEART HEXOKINASE

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1. Introduction

In the cardiac muscle, during aerobiosis, the regulation of energetic metabolism is mainly linked to the availability of ADP which stimulates oxidative phosphorylation.

Several mitochondrial enzymes provide ADP, among them creatine kinase and adenylate kinase which have been studied in our laboratory, seem to have a very important role in ADP supply and in energy transfer. Bessman [1] has suggested that hexokinase could be an important parameter in the regulation of oxidative phosphorylation because of its acceptor effect, especially if it is associated with mitochondria.

In the present paper, the subcellular distribution of hexokinase in pig heart muscle was studied by fractional extraction and differential centrifugation. It was proved that a significant fraction is firmly bound to mitochondria.

The intramitochondrial localization has also been determined. During the purification of submitochondrial fractions, the specific activity of hexokinase increased by 15-fold in purified outer membranes and did not increase in other fractions. The De Duve's plot [2] and comparisons with specific activities of other marker enzymes prove the association of hexokinase with outer membranes. Kinetic parameters of this mitochondrial hexokinase were determined.

2. Materials and methods

Pig heart mitochondria were prepared in 0.25 M sucrose, 10 mM phosphate (K), pH 7.4, washed and tested as previously [3]. The main steps of the preparation are summarized under table 2.

Isolation and purification of mitochondrial membranes and measurements of activities of marker enzymes were preformed according to Maïsterrena et al. [4].

Fractional extraction of pig heart muscle was conducted as described by Arnold and Pette [5] : pig heart muscle was minced with a meat grinder; the mince was suspended in a ten-fold volume (w/v) of 0.3 M sucrose 10 mM triethanolamine hydrochloride, 2 mM EDTA, 1 mM β-mercaptoethanol, pH 7.2. The suspension was stirred for 15 min and centrifuged for 30 min at 105 000 *g*. The supernatant was recovered (S_1) and the pellet was resuspended with a loosely fitted pestle in a volume of fresh medium equal to that of the supernatant. The suspension was stirred and centrifuged as described above (S_2). The pellet was resuspended, stirred, and centrifuged as above (S_3). The third pellet was then suspended in the same volume of 0.1 M phosphate buffer, 1 mM β-mercaptoethanol, pH 7.2, stirred for 15 min and centrifuged at 105 000 *g*, 30 min. The supernatant (S_4) was collected and the pellet was desintegrated in the same volume of phosphate buffer for 2 × 60 sec. with a Virtis homogenizer. The homogenate was centrifuged as before, and the supernatant was collected (S_5).

Protein was estimated by the method of Lowry [6].

The activity of hexokinase (EC 2.7.1.1.) was determined at 30°C by measuring the reduction of $NADP^+$ at 340 nm in the presence of excess glucose 6-phosphate dehydrogenase.

The reaction medium (3 ml final vol.) contained 10

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mM glucose, 5 mM $MgSO_4$, 0.2 mM $NADP^+$, 2 μg oligomycin, 1.4 units glucose 6-phosphate dehydrogenase, 5 mM ATP, 50 mM Tris-HCl pH 7.5 and protein equivalent to about 0.01 hexokinase unit.

For K_M and V_M determination of mitochondrial hexokinase, the amount of glucose 6-phosphate formed during one minute in one ml final volume was determined according to Hohorst [7] in the following medium: 50 mM Tris-HCl, pH 7.5, 2 μg oligomycin, ATP, Mg^{2+} and glucose as described in the test, 30°C.

Malate dehydrogenase (EC 1.1.1.37), lactate dehydrogenase (EC 1.1.1.27.) and creatine kinase (EC 2.7. 3.2.) were tested as previously described [8–10].

Adenylate kinase (EC 2. 7. 4. 3.) activity was measured by a modification of the method of Oliver [11].

3. Results and discussion

3.1. *Subcellular distribution of hexokinase*

Fractional extraction of minced pig heart muscle as described in Materials and methods, allowed to achieve separation of mitochondrial from cytoplasmic enzymes. As shown in table 1, a cytoplasmic enzyme, like lactate dehydrogenase was extracted at 95 per cent by magnetic stirring of the mince in slightly hypertonic sucrose (thrice-supernants S_1, S_2 and S_3); this procedure also extracted 70% creatine kinase and 74% malate dehydrogenase, both enzymes located in mitochondrial enzymes was initiated by stirring in 0.1 M phosphate, followed by disintegration of tissue in a Virtis homogenizer; table 1 shows that these treatments resulted in solubilizing the remaining following activities (supernants S_4 and S_5): 40% hexokinase, 26% malate dehydrogenase 30% creatine kinase and about 9% adenylate kinase.

In the course of the routine mitochondria preparation by differential centrifugation of pig heart muscle homogenate (table 2) the 1600 *g* supernatant contained 65% adenylate kinase, 68% creatine kinase, 25% hexokinase and 78% lactate dehydrogenase. The 1600 *g* pellet retained part of hexokinase activity.

When mitochondria were sedimented at 15 000 *g* from the 1600 *g* supernant they retained half the hexokinase activity of this supernatant and only a little fraction of creatine kinase and adenylate kinase activities. A significant percentage of hexokinase (10% of total homogenate) was located in purified mitochondria, 4–5 times greater than that of creatine kinase and adenylate kinase. These activities could not be due to cytosolic contamination as shown by the negligible

Table 1
Compared fractional extraction of several enzymes from pig heart

	Percent of activity extracted in each supernatant				
	Sucrose			Phosphate	
	S_1	S_2	S_3	S_4	S_5
Hexokinase	35	16.4	8.4	10	30
Adenylate kinase	80	6.6	2.9	3.2	6.2
Creatine kinase	56.7	8.4	3.3	10.1	21.7
Malate dehydrogenase	61	8.9	3.7	6.1	20.4
Lactate dehydrogenase	81.5	10.5	2.8	1.5	3.6

S_1, S_2, S_3, S_4, S_5 as described in Materials and methods.

Table 2
Compared subcellular distribution of several enzymes in pig heart[a]

	Pre-mitochondrial supernatant (1600 *g*)	Crude mitochondria (15 000 *g* pellet)	Washed mitochondria
Hexokinase	25	12	9.6
Adenylate kinase	65	4.7	2.5
Creatine kinase	68	3.5	2
Lactate dehydrogenase	78	1.5	0.2

Activities are expressed as percents of total activity in homogenate.

[a] Pig heart was homogeneized in a three-fold volume (w/v) of 0.25 M sucrose, 10 mM potassium phosphate, pH 7.6. The homogenate was centrifuged at 1600 *g*, 10 min. The pellet was discarded and the supernatant (pre-mitochondrial supernatant) was centrifuged at 15000 *g* 20 min. The crude mitochondrial pellet was resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 and centrifuged again at 15000 *g*, 20 min. Washed mitochondria were resuspended in the same medium at 30 mg protein per ml.

activity of lactate dehydrogenase, a typically cytoplasmic enzyme.

Therefore both technical approaches gave the same results: 30% (fractional extraction) and 10% (differential centrifugation) of the hexokinase activity was linked to mitochondria. Percentages obtained by differential centrifugation were lower because after purification, the recovery of mitochondria cannot be quantitative. Moreover if purified mitochondria were incubated in 0.02 M potassium phosphate, pH 7.2, 10°C, 15 min, 75% adenylate kinase and 50% creatine kinase leaked out from the intermembrane space while no hexokinase was released.

It can be concluded that pig heart mitochondria contained a very significant percentage of hexokinase like brain mitochondria [12–13]. Previously it had been shown that skeletal frog muscle contained particulate hexokinase [14] and that pig heart hexokinase was partly associated with some particulate fraction of the whole homogenate [15].

In contrast to the mitochondrial hexokinase from rat brain [12,13,16] or from chicken skeletal muscle [17], in our experiments hexokinase from pig heart mitochondria did not exhibit any latency; indeed additions of Triton X-100 or Lubrol WX (final conc. 0.03%) did not effect the enzyme activity.

In table 3, we see that the whole cardiac muscle contained 50 times more creatine kinase and 7 times more adenylate kinase than hexokinase: however in mitochondria the specific activity of hexokinase and adenylate kinase were similar and about a fifth of that of creatine kinase.

The total hexokinase activity in pig heart muscle appeared similar to that of rat heart [18].

3.2. *Intramitochondrial distribution of hexokinase*

Since in mitochondria, hexokinase did not appear freely soluble in the intermembrane space like adenylate kinase, it was important to determine its location.

During the fractionation and purification of pig heart mitochondrial membranes [4], it was found that hexokinase activity was associated with outer membranes. Fig.1 shows that the specific activity increased in parallel with the purification of outer membranes; on the contrary, hexokinase specific

Table 3
Compared specific activities of three mitochondrial kinases

	Activity in homogenate μmoles substrate per min and per g wet weight	Activity in mitochondria μmoles substrate per min and per mg protein
Adenylate kinase	52 ± 13 (8)[a]	0.14 ± 0.01 (9)
Creatine kinase	355 ± 101 (4)	0.77 ± 0.12 (13)
Hexokinase	7.24 ± 1.83 (6)	0.15 ± 0.08 (10)

[a] Numbers in brackets indicate number of assays.

activity decreased as inner membranes were purified.

In fig.2, the De Duve's plot applied to the first steps of purification of outer membranes, shows that the relative specific activity of hexokinase was much higher in outer membranes than in other fractions and appeared as a specific enzyme of outer membranes. In contrast, although the total activity of inner membrane plus matrix crude fraction was large, a possible localization of the enzyme also in inner membrane was ruled out since during purification of inner membranes, the specific activity decreased. Table 4 gives the distribution of hexokinase in submitochondrial fractions as compared to that of typical marker enzymes. We see that during the purification procedure of pig heart mitochondrial membranes, hexokinase activity increased in parallel with monoamine oxidase and rotenone insensitive NADH-cytochrome *c* reductase which are specific

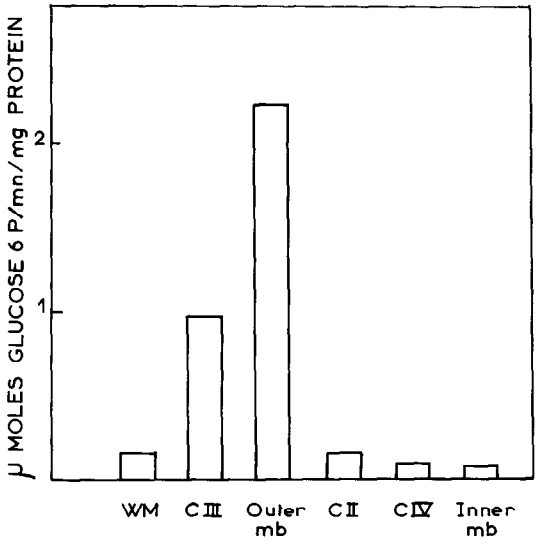

Fig.1. Distribution of hexokinase during purification of outer mitochondrial membrane from pig heart. Averages of 8 determinations using 4 different tissue fractionations. According to procedure [4]: WM = whole mitochondria. CIII = curde outer membranes. Outer mb = purified outer membranes. CII = crude mitoplasts (inner membranes + matrix). CIV = crude inner membranes. Inner mb = purified inner membranes.

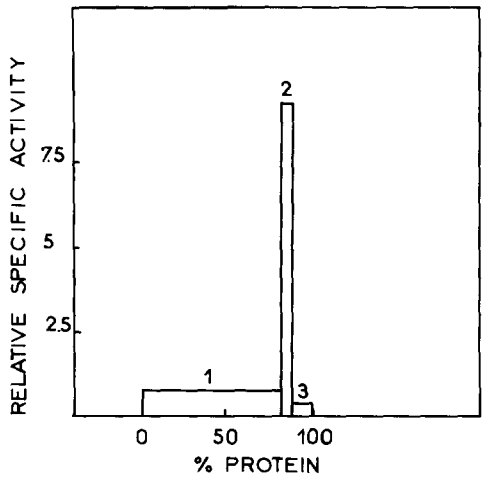

Fig.2. De Duve's plots [2] applied to the first steps of purification of outer membranes from pig heart mitochondria. 1 - Mitoplasts (Inner membrane + matrix), 2 - crude outer membranes, 3 - soluble fractions.

Table 4
Specific activity of hexokinase and marker enzymes of mitochondrial membranes from pig heart mitochondria

	Whole mitochondria	Purified outer membranes	Purified inner membranes	Ratio outer membranes over whole mitochondria	Ratio inner membranes over whole mitochondria
Hexokinase	0.15	2.2	0.06	14.7	0.4
Monoamine oxidase	0.97	12.7	0.3	13.1	0.34
Rotenone-insensitive NADH-cytochrome *c* reductase	0.075	0.38	0.04	5.1	0.52
Cytochrome oxidase	1.10	0.22	2.93	0.22	2.65

Specific activities as μmoles substrate/min/mg protein except for monoamine oxidase, nmoles benzylamine/min/mg protein.

markers of outer membranes. The purification ratio was even better than for monoamine oxidase activity and was very different from that of inner membrane markers (cytochrome oxidase). Our purification procedure appears more efficient than that applied to rat brain mitochondrial membranes [12, 13].

3.3. *Kinetic parameters of mitochondrial hexokinase*

Kinetic measurements were performed on whole mitochondria as described under Materials and methods.

When kinetic constants relative to ATP were estimated, glucose was 1 mM, and ATP varied from 0.4 to 2 mM; for glucose determinations, ATP was held at 5 mM and glucose concentrations varied from 0.05 to 0.2 mM. In all cases the ATP/Mg^{2+} ratio was 1.

The Lineweaver and Burk plots were perfectly linear.

As shown in table 5, pig heart mitochondrial hexokinase has a high affinity for glucose: app. K_M = 0.075 mM ± 0.026; the affinity for ATP is lower: app. K_M = 0.63 mM ± 0.12, but this value is probably overestimated because of other systems which bind ATP in whole mitochondria. However these values are close to those estimated for heart hexokinases purified from rat, ox and pig (whole heart) [18–20].

Table 5
Apparent kinetic constants of mitochondrial hexokinase from pig heart

app. K_M ATP mM	app. K_M glucose mM	V_m
0.63 ± 0.12	0.075 ± 0.026	0.146 ± 0.026

Mean of three different experiments. V_m was expressed as μmoles of glucose 6-phosphate formed per min per mg protein.

4. Conclusions

Our results confirm that in pig heart, hexokinase is essentially particulate (cf. [21]); more over they prove that contrarily to other glycolytic enzymes, a significant fraction is bound to mitochondria, while an important part of the activity sedimented at 1600 *g*, perhaps associated with structural proteins as for aldolase [5].

Studies on submitochondrial localization show that hexokinase is bound to the outer membrane of pig heart mitochondria as observed in rat brain [12–13]. This conclusion is in agreement with observations suggesting that in brain or ascites mitochondria hexokinase would be located before the 'atractylate barrier' since its activity towards added ATP was not altered by atractylate [22,23]. In rat liver mitochondria, hexokinase exhibited a better affinity for ATP internally generated by oxidative phosphorylation than for externally added ATP [24].

This association of hexokinase with mitochondrial membranes may serve to control glucose phosphorylation, especially in muscle and brain; the localization close to the ATP-generating system would efficiently provide ATP, and thus, glucose 6-phosphate in sufficient amounts to ensure metabolism, but high concentrations of glucose 6-phosphate or ATP, solubilize

hexokinase [15,16,23,25,26] and modify its kinetic properties [27,28].

Therefore the localization of hexokinase on outer mitochondrial membrane as proved here could be an important parameter in the regulation of cellular energetic metabolism, at the level of glycolysis by catalyzing the first energy-dependent step, and at the level of oxidative phosphorylation by regenerating the phosphate acceptor ADP.

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