Biochimica et Biophysica Acta, 975 (1989) 231–238
Elsevier

BBABIO 43029

Study on ATP-generating system and related hexokinase activity in mitochondria isolated from undifferentiated or differentiated HT29 adenocarcinoma cells

Thierry Gauthier, Colette Denis-Pouxviel, Hervé Paris and Jean-Claude Murat

INSERM U 317, Institut de Physiologie, Université Paul Sabatier, Toulouse (France)

(Received 16 March 1989)

Key words: Hexokinase; Tumor cell mitochondria; Glycolysis; Adenylate kinase; Oxidative phosphorylation; (HT29 cell)

The functional properties of mitochondria bound hexokinase are compared in two subpopulations of the HT29 human colon cancer cell-line: (1) the HT29 Glc^+ cells, cultured in the presence of glucose, which are poorly differentiated and highly glycolytic and (2) the HT29 Glc^- cells, adapted to grow in a glucose-free medium, which are 'enterocyte-like' differentiated and less glycolytic when given glucose (Zweibaum et al. (1985) J. Cell Physiol. 122, 21–28). The activities of hexokinase, phosphofructokinase-1 and pyruvate kinase are found to be twice as high in Glc^+ cells when compared to Glc^- cells. Besides, the respiration rate is decreased in Glc^+ cells compared to Glc^- cells. These results correlate with the higher glycolytic rate in Glc^+ cells. In many tissues, it has been shown that the binding of hexokinase to the mitochondrial outer membrane allows a preferential utilization of the ATP generated by oxidative phosphorylation which, in turn, is activated by immediate restitution of ADP. In highly glycolytic cancer cells, although a large fraction of hexokinase is bound to the mitochondria, the existence of such a channeling of nucleotides is still poorly documented. The rates of glucose phosphorylation by bound hexokinase were investigated in mitochondria isolated from both Glc^+ and Glc^- cells either with exogenous ATP or with ATP generated by mitochondria supplied with ADP and succinate (endogenous ATP). Diadenosine pentaphosphate (Ado_2P_5), oligomycin and carboxyatractyloside (CAT) were used in combination or separately as metabolic inhibitors of adenylate kinase, ATP synthase and ATP/ADP translocator, respectively. Exogenous ATP appears to be 6.5-times more efficient than endogenous ATP in supporting hexokinase activity in the mitochondria from Glc^+ cells and only 1.8-times more efficient in mitochondria from Glc^- cells. The rate of oxidative phosphorylation being higher in mitochondria from Glc^- cells, hexokinase activity is higher in this model when ATP is generated by respiration. Furthermore, in Glc^+ mitochondria, the adenylate kinase reaction appears to be an important source of endogenous ATP for bound hexokinase, while, in Glc^- mitochondria, hexokinase activity is almost totally dependent on the ATP generated by oxidative phosphorylation. This result might be explained by our previous finding that mitochondria from Glc^+ cells lack contact sites between outer and inner membrane, whereas numerous contacts were observed in mitochondria from Glc^- cells (Denis-Pouxviel et al. (1987) Biochim. Biophys. Acta 902, 335–348).

Introduction

In many tissues, a wide portion of hexokinase is found in the particulate fraction of the cell (Ref. 1, for review see Refs. 2 and 3), bound to the outer mitochondrial membrane at the level of the pore protein [4–9]. It was first observed in liver [6] and later in brain [4] and muscle [10] that this bound form has preferential access to the ATP generated by oxidative phosphorylation. Acting as an acceptor for high-energy phosphate, glucose and hexokinase increase in return the efficiency of oxidative metabolism by immediate restitution of ADP [3]. The existence of such a channeling of nucleotides between hexokinase and oxidative phosphorylation is supported, on a structural basis, by the observation that close contacts exist between the outer and inner limiting mitochondrial membranes. Such contacts were first described by Hackenbrock and Miller [11] and their existence was further evidenced by the

Abbreviations: Ado_2P_5, diadenosine pentaphosphate; CAT, carboxyatractyloside; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

Correspondence: J.-C. Murat, Institut de Physiologie, Université Paul Sabatier, 2 rue François Magendie, 31400 Toulouse, France.

analysis of freeze-fractured mitochondria [12,13] and by the biochemical characterization of subfractionated mitochondrial membranes [14]. It was postulated by Brdiczka [15] that the contact sites create a functional micro-compartment, permitting a direct exchange of nucleotides between hexokinase and the site of oxidative phosphorylation.

However, most of these studies were carried out with normal cells and the existence or significance of such a coupling in highly glycolytic cancer cells is still an open question. Cancer cells have been known for a long time to be highly glycolytic even in aerobic conditions [16] and this metabolic peculiarity has been often correlated with an increase of bound hexokinase activity [17–19]. This has led Pedersen and co-workers [18] to postulate that the preferential access of bound hexokinase to the ATP generated by oxidative phosphorylation could increase glucose 6-phosphate and pyruvate production, exceeding the capacity of oxidation in mitochondria. Nevertheless, it has been recently demonstrated by Nelson and Kabir that oxidative phosphorylation is not the preferential source of ATP for the bound hexokinase in Zajdela hepatoma cells [19].

In a previous paper [20], we have investigated the mitochondrial metabolism of cultured HT29 cells. These cells, derived from a human colon cancer [21], can be obtained under two different phenotypes according to culture conditions. When cultured in standard medium containing glucose, HT29 cells (referred to as Glc^+ cells) are undifferentiated and highly glycolytic. Long-term culture in a glucose-free medium results in an 'enterocyte-like' differentiation of the cells (referred as differentiated or Glc^- cells) which slowly utilize glucose when this sugar is added for a short time in the medium [22]. We have found that 75% of hexokinase is bound to the particulate fraction in both HT29 cell types (see below). Analysis of freeze-fractured cells and biochemical characterization of mitochondria treated with digitonin has revealed that contacts between mitochondrial membranes are almost absent in the undifferentiated cells. We have suggested that this defect could hinder the channeling of nucleotides between hexokinase and oxidative phosphorylation. By contrast, numerous contact sites have been observed in the differentiated Glc^- cells and this has been correlated with the low rate of lactate production [20].

The experiments presented in this paper have been undertaken in order to determine whether the glycolytic abnormality of HT29 cells could be related to a functional defect at the level of the mitochondrial bound hexokinase. For that purpose, we have compared the undifferentiated HT29 Glc^+ cells and the differentiated HT29 Glc^- cells, the latter being considered the closest to normal cells. We have studied the activity of three key enzymes of the glycolytic pathway and focused on the functional relationship between hexokinase and the ATP-producing system in isolated mitochondria from the two cell subpopulations. The rates of glucose phosphorylation by bound hexokinase were investigated either with exogenous ATP or with ATP generated by mitochondria supplied with ADP and succinate (endogenous ATP). Ado_2P_5, oligomycin and CAT have been used in combination or separately as metabolic inhibitors of adenylate kinase, ATP synthase and ATP/ADP translocator, respectively. We conclude with some findings on the origin of ATP which is used by bound hexokinase in mitochondria from undifferentiated or differentiated HT29 cells.

Materials and Methods

Dulbecco's modified Eagle medium was from Eurobio (Paris, France). Fetal calf-serum was from Boehringer (Mannheim, F.R.G.). All other drugs and chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) or Serva (Heidelberg, F.R.G.) and were of the highest purity grade.

Cell culture. The HT29 cell-line was established in permanent culture from a human colon adenocarcinoma [21]. The differentiated HT29 subpopulation [22] was obtained from Dr. A. Zweibaum (Villejuif, France). The cells were routinely cultured in plastic Petri dishes 60 mm in diameter, with Dulbecco's modified Eagle medium containing either 25 mM D-glucose (undifferentiated Glc^+ cells) or no glucose (differentiated Glc^- cells) and supplemented with 10% dialyzed fetal-calf serum. The medium was changed every day for Glc^- cells and every two days for Glc^+ cells. 24 h before the experiments, the differentiated cells were given a medium containing 25 mM glucose.

Isolation of mitochondria. Cells in the stationary phase, i.e., at day 12 for the standard cells and at day 22 for the Glc^- cells, were harvested with 2 ml of Ca^{2+} and Mg^{2+}-free 0.13 M phosphate buffer (pH 7.4) containing 0.6 mM EDTA and were centrifuged at $500 \times g$ for 3 min. The cell pellets collected from 30 dishes (about 150 mg of cell proteins) were pooled and resuspended in 10 ml of ice-cold isolation medium containing 0.21 M mannitol/0.07 M sucrose/2 mM Hepes (pH 7.4)/1 mM EGTA. The suspension was homogenized with a glass Potter and mixed with 250 mg of DEAE-cellulose in 5 ml of isolation medium, as proposed by Lawrence and Davies [23]. Nagarse was then added at a final concentration of 5 μg/ml of cell protein [24]. The mixed solution was stirred on ice for 7 min, then the proteinase action was stopped by adding 15 ml of isolation medium containing 0.1% bovine serum albumin. The suspension was centrifuged 10 min at $700 \times g$. The supernatant was collected and centrifuged 10 min at $7800 \times g$. The sediment was resuspended in 3 ml of the isolation medium containing 0.1% bovine serum albumin and centrifuged 4 min at $700 \times g$. The resulting

supernatant was centrifuged 10 min at 11 300 × *g* and the final pellet was resuspended in 1 ml of the isolation medium without EGTA containing 0.1% bovine serum albumin and 5 mM $MgCl_2$ in order to prevent solubilization of the bound hexokinase [10]. Isolated mitochondria from the two cell subpopulations remained well coupled for at least 2 h. The integrity of isolated mitochondria was controlled by measuring the activity of soluble enzymes (adenylate kinase and glutamate dehydrogenase) by reference to the activity of membrane-integrated succinate dehydrogenase in the cell homogenate and the isolated mitochondrial fraction. In this respect, the method and results were identical to those reported in a previous paper [20].

Oxygen consumption. Mitochondrial respiration was determined with a Clark-type oxygen electrode at room temperature according to Estabroock [25] using an isolation medium containing 0.21 M mannitol/70 mM sucrose/15 mM Hepes (pH 7.4)/0.1% bovine serum albumin/8 mM $MgCl_2$/4 mM KH_2PO_4/2 μM rotenone and 5 mM succinate as substrate.

Assays. Hexokinase activity (EC 2.7.1.30) was determined according to Bücher et al. [26]. Phosphofructokinase-1 (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) were estimated as in Ref. 27. The assays of adenylate kinase (EC 2.7.4.2), succinate dehydrogenase (EC 1.3.99.19) and glutamate dehydrogenase (EC 1.4.1.3) were performed as in Ref. 28. 1 milliunit (mU) is defined as the amount of enzyme converting 1 nmol of substrate per minute at 30 °C. Protein content was determined by the method of Bradford [29] using bovine serum albumin as standard.

Analysis of the data and statistics. All data were analyzed with an Apple IIe computer using the Biodata Handling programs written by Barlow [30]. Kinetic curves were fitted to a hyperbola (correlation coefficient > 0.97). The expression 'K_m for ADP' actually refers to the concentration of ADP which, in our system, corresponds to the half-velocity of the hexokinase reaction; therefore, K_m for ADP is somewhat equivalent to EC_{50}. Unless otherwise indicated, a paired *t* test was used for comparing the values, significance being admitted for $P < 0.05$.

Results

Activity of key enzymes of glycolysis in standard (Glc$^+$) and differentiated (Glc$^-$) HT 29 cells. The activity of three key enzymes of the glycolytic pathway was measured in homogenates from the two cell subpopulations. As seen in Table I, the activities of hexokinase, phosphofructokinase-1 and pyruvate kinase are twice as high in the HT 29 Glc$^+$ cells compared to the Glc$^-$ cells. This could account for the higher lactate production in Glc$^+$ cells compared to the Glc$^-$ cells when glucose is added for a short time [20]. In both models, about 75% of the total hexokinase activity is found to be in the bound form. Besides, the hexokinase/succinate dehydrogenase activity ratio is found to be similar in the cellular homogenate and in the isolated mitochondria (2.54 ± 0.41 vs. 2.13 ± 0.38 in Glc$^+$ cells and 0.77 ± 0.15 vs. 0.66 ± 0.10 in Glc$^-$ cells). This favors the mitochondrial location of the hexokinase, as also described in other models [4–9]. The difference in the values observed between Glc$^+$ and Glc$^-$ cells is mainly due to higher succinate dehydrogenase activity (199 ± 18 vs. 129 ± 20 mU/mg protein) and lower hexokinase activity (132 ± 16 vs. 276 ± 48 mU/mg protein) in Glc$^-$ mitochondria. In electron microscope studies (data not shown), the number of mitochondria per cells was found to be almost identical. Besides, it was previously calculated that mitochondrial protein amounts to 29% of the cellular protein in Glc$^+$ cells vs. 21% in Glc$^-$ cells [20].

Respiration rate of isolated mitochondria. We have studied (Table II) the oxygen consumption of isolated mitochondria in the presence of ADP and succinate.

TABLE I

Activity of key enzymes of the glycolytic pathway in HT29 Glc$^+$ and Glc$^-$ cells

The enzyme activities were measured in cellular homogenates as described in Materials and Methods. Phosphofructokinase-1 activity was determined in the presence of saturating concentrations of Fructose 2,6-bisphosphate (3 μM). The percentage of bound hexokinase was determined by digitonin treatment of cell homogenates as described by Denis-Pouxviel et al. [20]. Values are the means of at least four determinations ± S.E.

mU/mg protein	Glc$^+$ cells		Glc$^-$ cells
Hexokinase	54.4 ± 13.3	$P < 0.05$	28.8 ± 7.2
Phosphofructokinase-1	216 ± 11	$P < 0.05$	135 ± 4
Pyruvate kinase	3692 ± 458	$P < 0.05$	1966 ± 94
Bound hexokinase (%)	72 ± 8		76 ± 7

TABLE II

Oxygen consumption and respiratory indices

The oxygen consumption was measured with isolated mitochondria as described in Materials and Methods. ADP/O is the ratio of nmol ADP added in the medium over the corresponding nmol of oxygen consumed by mitochondria. The Respiratory Control Ratio (RCR) is obtained by dividing the rate of O_2 consumption in state 3 by the rate of O_2 consumption in state 4. Results are the means ± S.E. from four separate experiments (n.s. = not significantly different)

	Glc$^+$ mitochondria		Glc$^-$ mitochondria
(nmol O_2/min per mg):			
ADP 500 μM	44.7 ± 5.0	$P < 0.05$	68.0 ± 6.8
ADP + CCCP 5 μM	112.4 ± 16.0	$P < 0.05$	174.2 ± 15.1
ADP/O ratio	1.47 ± 0.009	n.s.	1.57 ± 0.14
RCR	3.53 ± 0.31	n.s.	4.28 ± 1.22
K_m ADP (μM)	16.8 ± 8.8	n.s.	12.9 ± 4.6

Oxygen consumption is found to be completely inhibited by adding 5 μM CAT (which irreversibly inhibits the ADP/ATP translocator) or 10 μg per ml oligomycin (which blocks ATP synthase) and to be greatly increased with 5 μM CCCP (uncoupler), indicating that isolated mitochondria were originally well-coupled. The affinity of the respiratory chain for ADP is not significantly different in the two types of mitochondria. Furthermore, the ADP/O ratio and the respiratory control ratio (RCR) are also approximately similar. However, the respiration rates measured in the presence of 500 μM ADP or in the presence of 5 μM CCCP are 1.5-times higher in the Glc^- mitochondria than in the Glc^+ mitochondria. These results suggest that the difference in respiration rates between the two types of cell could be due to the difference in the ability to oxidize succinate and not in the ability to phosphorylate ADP. This is supported by the observed decrease in succinate dehydrogenase activity (see below). From the respiration rates and related ADP/O ratio, it can be calculated that Glc^- mitochondria might produce ATP 1.6-times faster than Glc^+ mitochondria [213.5 $\pm$ 21.3 vs. 131.4 $\pm$ 14.7 nmol ATP/min per mg protein).

Kinetic parameters of bound hexokinase in isolated mitochondria from the two HT29 subpopulations. The activity of bound hexokinase was studied in mitochondria freshly isolated from the two cell subpopulations either in the presence of added ATP (exogenous ATP) and CAT or in the presence of ADP and succinate (endogenous ATP, i.e., generated from ADP by the mitochondria). In contrast to our previous report [20], new buffering conditions (addition of 15 mM Hepes in the incubation medium) allowed us to detect an hexokinase activity with exogenous ATP in mitochondria from differentiated Glc^- cells.

Since some metabolites, mainly ATP and glucose 6-phosphate were shown to be able to solubilize the bound enzyme, we have studied in preliminary experiments (unpublished results) the effect of numerous agents on the hexokinase/mitochondria adsorption/desorption equilibrium. We have found that concentrations up to 1 mM ATP were unable to solubilize the bound enzyme under our experimental conditions. Moreover, 5 mM Mg^{2+} or phosphate was found to prevent the solubilization caused by 100 μM glucose 6-phosphate. As also shown by Salotra and Singh [31], it is likely that the concentrations of Mg^{2+} and phosphate (8 and 4 mM, respectively) we actually used in our present experiments are high enough to prevent any solubilization of the bound hexokinase by the increasing concentrations of nucleotides.

As shown in Fig. 1, the activity of hexokinase is higher with exogenous ATP than with endogenous ATP in both models. In Glc^- mitochondria, the difference of activity with the two sources of ATP is less important

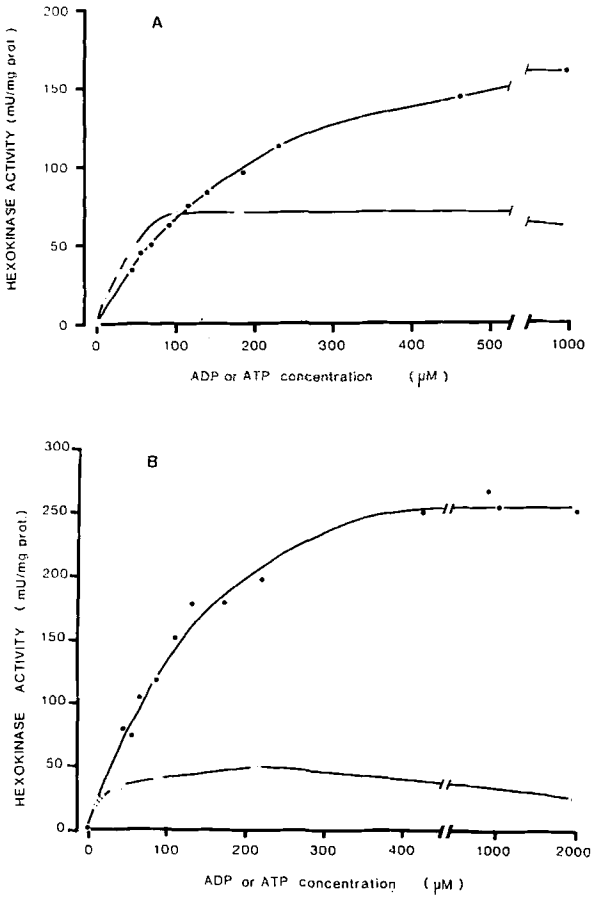

Fig. 1. Activity of bound hexokinase in the presence of ATP or ADP. The activity of bound hexokinase was determined by a direct optical test system. Isolated mitochondria (40–60 μg protein) from Glc^- (A) or Glc^+ (B) cells were incubated at 30 °C in 1 ml of isolation medium containing 0.21 M mannitol/70 mM sucrose/15 mM Hepes (pH 7.4), 0.1% bovine serum albumin/8 mM $MgCl_2$/4 mM KH_2PO_4/2 mM glucose/0.6 mM NAD/13 μM rotenone/1.6 U glucose-6-phosphate dehydrogenase and 30 μM CAT when ATP was used. Reaction was started by addition of ATP (●) or 10 mM succinate and ADP (○). Results from one representative experiment are presented.

than in Glc^+ mitochondria. The kinetic curves obtained with ATP are hyperbolic in the two models. The curves obtained with ADP show a plateau, which could result from the combination of an activatory effect of increasing amount of ATP generated by the mitochondria with the known inhibitory effect of high concentration of ADP on hexokinase activity [32].

The kinetic curves obtained from four separate experiments were fitted to a hyperbola and kinetic constants (K_m and V_{max}) were determined. V_{max} values in the presence of ADP were calculated from activities measured in the low-ADP concentration range. This leads to an overestimation of the velocity which can be actually obtained in the cuvette. However, it should reflect maximum velocities obtainable in intact cell where ADP does not accumulate but rapidly exchanges with ATP in the mitochondria.

TABLE III

Kinetic constants for mitochondrial hexokinase with ATP or ADP + succinate

The activity of bound hexokinase was determined in mitochondria isolated from Glc^+ and Glc^- cells, with increasing concentrations of ATP or ADP. ATP is the direct source for hexokinase (exogenous ATP), while ADP (+succinate) is the source of endogenous ATP formed by mitochondria. The kinetic curves were fitted to a hyperbola and kinetic constants were calculated. V_{max} values are the means ± S.E. from four experiments.

	K_m (μM)		V_{max} (mU/mg protein)		V_{max} ATP: V_{max} ADP ratio
	ATP	ADP	ATP	ADP	
Glc^+ cells	235 ± 69	65 ± 26	371 ± 88	57 ± 10	6.5 ± 0.6
	$P < 0.01$		$P < 0.01$		
Glc^- cells	171 ± 27	56 ± 24	209 ± 68	133 ± 66	1.8 ± 0.7
	$P < 0.01$		$P < 0.02$		

As shown in Table III, the apparent K_m for ADP is significantly lower in both models than the K_m for preformed ATP. This suggests that the endogenous ATP has greater affinity for hexokinase than the exogenous ATP.

In the Glc^+ model, exogenous ATP elicits a 6.5-times higher hexokinase activity (V_{max} value) than does ADP. By contrast, in the Glc^- model, glucose phosphorylation rate is only 1.8-times higher with exogenous ATP than with ATP generated by the mitochondria. This suggests that ATP generated by the mitochondria is rate-limiting for glucose phosphorylation in both cases, as also shown in rat liver mitochondria by Gots et al. [33], but in a more pronounced way in Glc^+ than in Glc^- cells.

Effect of inhibitors of ATP synthesis on bound hexokinase activity. Under our experimental conditions, ATP can be generated from ADP within the mitochondria either by oxidative phosphorylation or by adenylate kinase reaction. In order to study the relative contribution of these two ATP-generating systems, the glucose phosphorylation rate was studied in the presence of succinate and increasing concentrations of ADP, either with Ado_2P_5, which inhibits adenylate kinase or with oligomycin or CAT. Simultaneous addition of Ado_2P_5 and CAT was found to totally inhibit the hexokinase activity, showing that adenylate kinase and oxidative phosphorylation were the only generators of mitochondrial ATP (data not shown). The effect of 10 μM antimycin which stops the electron flow was also investigated and was found to be similar to that of oligomycin or CAT (data not shown).

In the mitochondria from differentiated Glc^- cells (Fig. 2A), Ado_2P_5 appears to be a weak inhibitor of the hexokinase activity at any ADP concentration, indicating that adenylate kinase is not an important source of ATP for glucose phosphorylation. By contrast, oligomycin or CAT are found to be very efficient inhibitors of the hexokinase reaction, especially at low ADP concentrations (< 25 μM).

In the mitochondria from undifferentiated Glc^+ cells (Fig. 2B), inhibition of adenylate kinase by Ado_2P_5 is without effect on hexokinase activity at 20 μM ADP, suggesting that hexokinase uses ATP generated by oxidative phosphorylation. This is confirmed by the

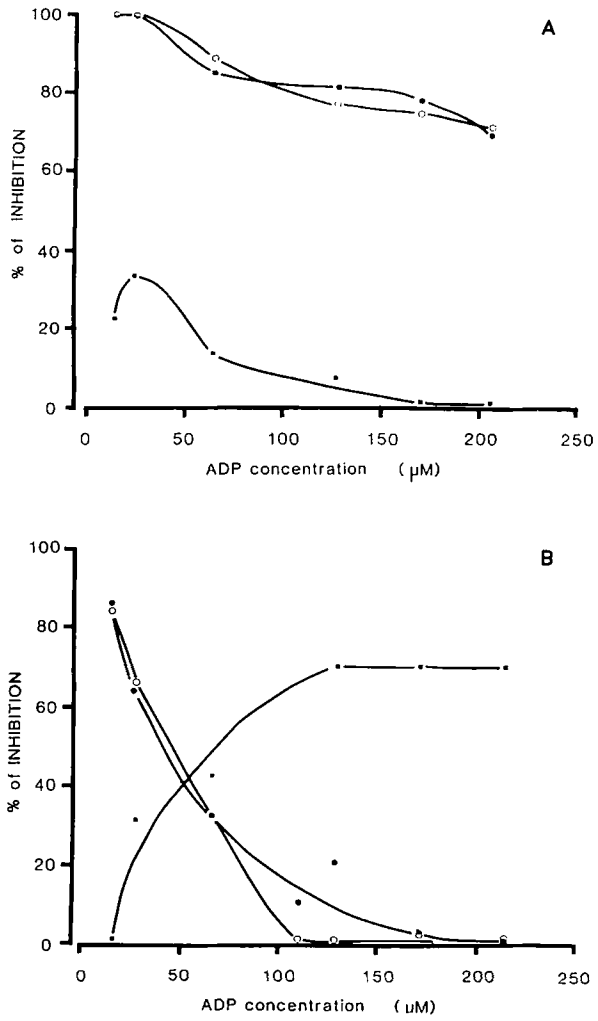

Fig. 2. Effects of inhibitors of ATP production on bound hexokinase activity. Bound hexokinase activity was determined in mitochondria isolated from Glc^- cells (A) or Glc^+ cells (B), with increasing concentrations of ADP, in the presence of 50 μg/ml oligomycin (●) or 30 μM CAT (○) or 0.25 mM Ado_2P_5 (■). One representative experiment is presented and results are expressed as a percent of inhibition of the activity measured in the absence of inhibitor.

TABLE IV

Characterization of the ADP/ATP translocator and ATP synthase

The activity of bound hexokinase was determined in mitochondria from Glc^+ and Glc^- cells, in the presence of 0.25 mM Ado_2P_5. Reaction was started by addition of 200 μM ADP+10 mM succinate. The effect of increasing concentrations of oligomycin or CAT was studied. Reported values represent the inhibitor concentration which totally abolishes glucose phosphorylation, and the respective calculated K_i (inhibition of the ATP supplying system). Values are the means ± S.E. from three separate experiments (n.s. = not significantly different)

	Glc^+ cells		Glc^- cells
CAT concentration for 100% inhibition (nmol/mg protein)	0.86 ± 0.21	n.s.	0.71 ± 0.08
K_i CAT (nM)	20.2 ± 5.6	n.s.	28.1 ± 6.6
Oligomycin concentration for 100% inhibition (μg/mg protein)	0.31 ± 0.06	n.s.	0.21 ± 0.07
K_i oligomycin (ng/ml)	5.0 ± 1.2	n.s.	6.5 ± 2.0

almost total inhibition of hexokinase reaction by oligomycin or CAT. By contrast, at ADP concentrations above 100 μM, Ado_2P_5 inhibits the reaction by 70%, showing in this case the importance of the adenylate kinase in supplying ATP to bound hexokinase.

Fig. 3 shows the bound hexokinase activity as a function of ADP concentration, in the presence or in the absence of Ado_2P_5. In mitochondria from Glc^+ cells (Fig. 3B), the hexokinase reaction is dependent only on respiratory ATP (in the presence of Ado_2P_5 and rapidly reaches a plateau. This is not the case in mitochondria from Glc^- cells (Fig. 3A) where almost similar rates of glucose phosphorylation are observed whether adenylate kinase is inhibited or not.

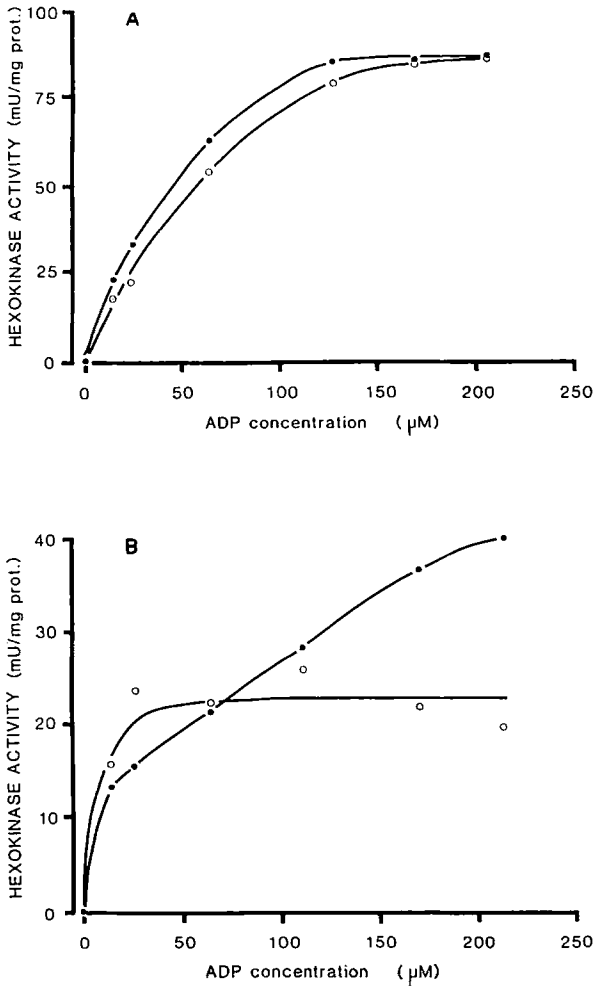

Fig. 3. Activity of bound hexokinase in the presence of ADP or ADP with Ado_2P_5. Hexokinase activity was determined in mitochondria from Glc^- cells (A) or Glc^+ cells (B), with increasing concentrations of ADP alone (●) or ADP+0.25 mM Ado_2P_5 (○). Results from one representative experiment are presented.

The glucose phosphorylation rate was also determined with 210 μM ADP alone or in combination with Ado_2P_5. It appears that the inhibition of adenylate kinase greatly decreases hexokinase activity in Glc^+ mitochondria (19.4 ± 5.0 vs. 45.4 ± 9.2, $P < 0.01$), while this is not the case in Glc^- mitochondria (41.2 ± 10.6 vs. 55.8 ± 16.7).

Characterization of the translocator and of the ATP synthase. In order to compare the ATP-delivering system in both models, the adenylate kinase activity was inhibited by Ado_2P_5 and the hexokinase activity was determined in the presence of ADP, i.e., with ATP generated by oxidative phosphorylation as sole substrate. Increasing concentrations of CAT or oligomycin were then added to block progressively ATP/ADP translocation or ATP synthesis. The CAT and oligomycin concentrations which completely abolish glucose phosphorylation and the respective affinity constants of

TABLE V

Kinetic parameters of adenylate kinase

Mitochondria freshly isolated from Glc^+ and Glc^- cells were treated with 0.2% Triton X-100 in order to release adenylate kinase. The enzyme activity was measured by a direct optical test system at 30 °C in 1 ml of 50 mM triethanolamine buffer (pH 7.6) containing 5 mM EDTA/8 mM $MgSO_4$/0.6 mM NAD/2 mM glucose/2 U hexokinase/3.2 U glucose-6-phosphate dehydrogenase/50 ng oligomycin. ADP was added at concentrations ranging from 20 to 1000 μM. Reaction was started by addition of mitochondria (about 5 μg protein). V_{max} and K_m values (means ± S.E.) were calculated from three separate experiments. (n.s. = not significantly different)

	Glc^+ cells		Glc^- cells
K_m ADP (μM)	120.6 ± 21.3	n.s.	132.0 ± 19.6
V_{max} (mU/mg protein)	518 ± 114	$P < 0.01$	924 ± 130

the inhibitors were determined (Table IV). There is no significant difference in the value of these parameters when comparing Glc^+ and Glc^- mitochondria, suggesting that translocator and ATP synthase are equally efficient in both cell types.

Study of adenylate kinase. Adenylate kinase appears to be an important ATP-generating system in mitochondria from Glc^+ cells under our experimental conditions; therefore, we have investigated the kinetic parameters of this enzyme. The activity of adenylate kinase being determined in the direction of ATP synthesis (Table V), the K_m for ADP is found to be identical in both models. The maximum velocity is even higher in the Glc^- mitochondria model. Therefore, the greatest contribution of adenylate kinase in the ATP production, observed in Glc^+ mitochondria, cannot be attributed to differences in the kinetic behavior of this enzyme. It might be better explained by a modification of the mitochondrial membrane structure (lack of contact sites, see discussion below) which facilitates the supply of ATP by adenylate kinase reaction and alters the channeling of nucleotides between the bound hexokinase and the translocator.

Discussion

Glycolytic enzymes

The activities of hexokinase, phosphofructokinase-1 and pyruvate kinase are twice as high in Glc^+ cells compared to Glc^- cells. If we postulate that Glc^- cells are the closest to normal cells [22], such a result is in accordance with observations that the activity of key enzymes of the glycolytic pathway in several types of tumor cells is very high when compared to the normal corresponding tissues (for a review see Refs. 35 and 35).

Thus, in the undifferentiated cells, the increase in glycolytic enzyme activities and the low mitochondrial respiration both contribute to increase the glycolytic flux.

Mitochondria-bound hexokinase

In isolated mitochondria from both Glc^+ and Glc^- cells, hexokinase has more affinity for ATP provided by the mitochondria (endogenous ATP) than for ATP added in the medium. However, at high nucleotide concentrations (> 100 μM), exogenous ATP is 6.5-times more efficient than endogenous ATP in Glc^+ cells and 1.8-times more in Glc^- cells. Although twice as much hexokinase is present in Glc^+ cells, the activity with endogenous ATP is similar in both models, suggesting that in Glc^+ cells smaller amounts of the enzyme have access to this ATP.

When the experimental models are supplied with ADP, the oxidative phosphorylation becomes rate-limiting as also described in liver and brain mitochondria [36]. This effect is more pronounced in Glc^+ cells. It does not seem to be related either to differences in the ATP synthase or ATP/ADP translocator efficiency (see Table IV), nor to insufficient rate of mitochondrial ATP synthesis which is, on a molar basis, higher than hexokinase activity in both models.

Origin of the mitochondrial ATP

When ATP is provided only by respiration (i.e., in the presence of Ado_2P_5), hexokinase activity is found to be proportional to the rate of mitochondrial ATP synthesis. The higher rate of oxidative phosphorylation observed in Glc^- mitochondria could be related to a better efficiency in oxidizing succinate, since no difference with Glc^+ mitochondria was found with the ATP-delivering system.

In mitochondria from Glc^- cells, the hexokinase reaction appears to rely mainly on the ATP generated by the oxidative phosphorylation. Adenylate kinase does not appear to be able to provide ATP to hexokinase. The fact that adenylate kinase has the same K_m ADP in the two types of mitochondria and seems to be even more active in the Glc^- cells cannot account for this result. It could be better explained by the presence of a micro-compartment which, by enclosing the adenylate kinase in the intermembrane space, hinders this enzyme in providing ATP to hexokinase.

In mitochondria from Glc^+ cells, it appears that, at high concentrations of ADP, about 55% of ATP used to phosphorylate glucose is provided by the adenylate kinase reaction. This is in total agreement with the results obtained by Nelson and Kabir [19] with Zadjela hepatoma cells. These authors have found that adenylate kinase provides 50% of the ATP used by hexokinase in well-coupled mitochondria. The affinity of the respiratory chain for ADP (K_m = about 15 μM, table II) is in accordance with the data given by Barbour et al. [37]. This affinity is higher than that of adenylate kinase for this nucleotide (K_m = about 120 μM, Table V). Therefore, in Glc^+ mitochondria, low concentrations of ADP can promote the respiration, as also shown by Arora and Pedersen [38] in hepatoma mitochondria using low concentrations of nucleotides (30 μM), whereas high concentrations of ADP activate the adenylate kinase reaction. This is confirmed by the total inhibition of the hexokinase activity by CAT or oligomycin at low concentrations of ADP and by abolition of this inhibition at about 200 μM ADP. Under blockade of ATP synthase or ADP/ATP translocator, the total ATP provided to the hexokinase comes from the adenylate kinase. This enzyme appears active enough to support the hexokinase reaction. The absence of a micro-compartment could explain why ATP generated by the adenylate kinase reaction is free to reach the active site of hexokinase.

The physiological importance of contact sites between the outer and inner mitochondrial membranes to

form such a micro-compartment is now well-documented. Contact sites are known to play a role in the import of mitochondrial proteins [39]. Besides, it has been recently shown that porin (hexokinase-binding protein) which is synthesized in the cytosol, requires the participation of the inner membrane in tight contact or even fused with the outer membrane, to be integrated in the outer membrane [40]. An important role of the contact sites in the functional coupling between oxidative phosphorylation and ATP-consuming enzymes was also postulated [3]. This could be the case for creatine kinase which appears to be coupled to the ADP/ATP translocator [41].

Brdiczka et al. [14] have reported that, in rat liver mitochondria, the affinity of the enzyme for the porin is greater at the level of contact sites. In the differentiated Glc^- cells, such a morphological arrangement could facilitate coupling between the bound hexokinase and the translocator and could hinder a supply of ATP coming from the adenylate kinase. In such cases, ATP produced by oxidative phosphorylation is not in competition with the ATP produced by the adenylate kinase reaction. The situation is different in the Glc^+ mitochondria model where the absence of contacts sites [20] could allow the adenylate kinase reaction to play a major role in forming ATP from ADP.

Acknowledgements

We wish to thank Ms. Colette Larrey and Véronique Trocheris for their skillful technical assistance in carrying out cell cultures.

References

1 Crane, R.K. and Sols, A. (1953) J. Biol. Chem. 203, 273–292.
2 Wilson, J.E. (1980) Curr. Top. Cell. Regul. 16, 1–54.
3 Bessman, S.P. and Geiger, P.J. (1980) Curr. Top. Cell. Regul. 16, 55–86.
4 Inui, M. and Ishibashi, S. (1979) J. Biochem. 85, 1151–1157.
5 Pedersen, P.L. (1978) Prog. Exp. Tumor Res. 22, 190–274.
6 Gots, R.E. and Bessman, S.. (1974) Arch. Biochem. Biophys. 163, 7–14.
8 Parry, D.M. and Pedersen, P.L. (1984) J. Biol. Chem. 259, 8917–8923.
9 Fiek, C., Benz, R., Roos, N. and Brdiczka, D. (1982) Biochim. Biophys. Acta 688, 429–44.
10 Vitanen, P.V., Geiger, P.J., Vitanen-Erickson, S. and Bessman, S.P. (1984) J. Biol. Chem. 295, 9697–9698.
11 Hackenbrock, C.R. and Miller, K.J. (1975) J. Cell Biol. 65, 615–630.
12 Van Venetië, R. and Verkleij, A.T. (1982) Biochim. Biophys. Acta 692, 397–405.
13 Knoll, G. and Brdiczka, D. (1983) Biochim. Biophys. Acta 733, 102–110.
14 Ohlendieck, K., Riesinger, I., Adams, V., Krause, J. and Brdiczka, D. (1986) Biochim. Biophys. Acta 860, 672–689.
15 Brdiczka, D., Knoll, Riesinger, I., Weiler, U., Klug, G., Benz, R. and Krause, J. (1986) in Myocardial and Skeletal Muscle Bioenergetics (Braubar, N., ed.), pp. 55–70, Plenum Press, New York.
16 Warburg, O., Posener, K. and Nagelin, E. (1924) Biochem. J. 152, 309–344.
17 Rose, I.A. and Warms, J. (1967) J. Biol. Chem. 242, 1635–1645.
18 Bustamante, E. and Pedersen, P.L. (1977) Proc. Natl. Acad. Sci. USA 74, 3735–3739.
19 Nelson, B.D. and Kabir, F. (1985) Biochim. Biophys. Acta 841, 195–200.
20 Denis-Pouxviel, C., Riesinger, I., Bülher, C., Brdiczka, D. and Murat, J.C. (1987) Biochim. Biophys. Acta 902, 335–348.
21 Fogh, J., Fogh, J.M. and Orfeo, T. (1977) J. Natn. Cancer Inst. 59, 221–226.
22 Zweibaum, A., Pinto, M., Chevalier, G., Dussault, E., Triadou, N., Lacroix, B., Haffen, K., Brun, J.L. and Rousset, M. (1985) J. Cell Physiol. 122, 21–28.
23 Lawrence, C.B. and Davies, N.T. (1986) Biochim. Biophys. Acta 848, 35–40.
24 Aubert-Foucher, E., Font, B. and Gautheron, D.C. (1985) Molec. Cell. Biochem. 67, 111–118.
25 Estabroock, R.W. (1967) Methods Enzymol. 10, 41–47.
26 Bücher, T., Luh, W. and Pette, D. (1964) in Hoppe-Seyler-Thierfelder, Handbuch der Physiologisch-Chemischen Analyse, Vol. 6A, pp. 293–339, Springer Verlag, Berlin.
27 Denis, C., Cortinovis, C., Terrain, B., Viallard, V., Paris, H. and Murat, J.C. (1984) Int. J. Biochem. 16, 87–91.
28 Brdiczka, D., Pette, D., Brunner, G. and Miller, F. (1968) Eur. J. Biochem. 5, 249–304.
29 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
30 Barlow, R.B. (1983) Biodata Handling with Microcomputers, Elsevier, Amsterdam, The Netherlands.
31 Salotra, P.T. and Singh, V.N. (1982) Arch. Biochem. Biophys. 216, 758–764.
32 Grossbard, L. and Schimke, R.L. (1966) J. Biol. Chem. 241, 3546–3560.
33 Gots, R.E., Gorin, F.A. and Bessman, S.P. (1972) Biochem. Biophys. Res. Commun. 49, 1249–1255.
34 Pitot, H.C. (1981) in Neoplasms. Comparative pathology of growth in animals, plants and man (Kaiser, H.E., ed.), pp. 351–359, Williams and Wilkins, Baltimore, London.
35 Weber, G. (1982) Colonic Carcinogenesis, Falk Symposium No. 31 (Malt, R.A. and Williamson, R.C., eds.), pp. 369–381, MTP Press, Lancaster, BO.
36 Kottke, M., Adams, V., Riesinger, I., Bremm, G., Bosch, W., Brdiczka, D., Sandri, G. and Panfili, E. (1988) Biochim. Biophys. Acta, in press.
37 Barbour, R.L., Ribaudo, J. and Chan, S.H.P. (1984) J. Biol. Chem. 259, 8246–8251.
38 Arora, K.K. and Pedersen, P.L. (1988) J. Biol. Chem. 263, 17422–17428.
39 Hay, R., Böhni, P. and Gasser, S. (1984) Biochim. Biophys. Acta 779, 65–87.
40 Ono, H. and Tuboi, S. (1987) Eur. J. Biochem. 168, 509–514.
41 Brooks, S.P.J. and Suelter, C.H. (1987) Arch. Biochem. Biophys. 257, 144–153.