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Interaction of mitochondrial porin with cytosolic proteins

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Summary. Intracellular phosphorylation is an important step in active uptake and utilization of carbohydrates. For example glucose and glycerol enter the liver cell along the extra intracellular gradient by facilitated diffusion through specific carriers and are concentrated inside the cell by phosphorylation via hexokinase or glycerol kinase. Depending on the function of the respective tissue the uptake of carbohydrates serves different metabolic purposes. In brain and kidney medulla cells which depend on carbohydrates, glucose and glycerol are taken up according to the energy demand. However, in tissues such as muscle which synthesize glycogen or like liver which additionally produce fat from glucose, the uptake of carbohydrates has to be regulated according to the availability of glucose and glycerol. How the reversible coupling of the kinases to the outer membrane pore and the mitochondrial ATP serves to fulfil these specific requirements will be explained as well as how this regulates the carbohydrate uptake in brain according to the activity of the oxidative phosphorylation and how this allows glucose uptake in liver and muscle to persist in the presence of high glucose 6-phosphate without activating the rate of glycolysis.
Key words. Mitochondria; outer membrane pore; hexokinase; glycerol kinase; metabolite exchange; energy metabolism.

The outer mitochondrial membrane contains a specific binding protein for hexokinase

In 1977, Felgner and Wilson[11] characterized a specific binding protein for hexokinase in the outer membrane of rat liver mitochondria. When we incorporated this isolated binding protein into bilayer membranes we observed the same properties concerning conductivity and voltage dependence[14] as those described for the outer membrane pore[9,42]. Vice versa, reconstitution of the pore protein in asolectin vesicles resulted in specific binding of hexokinase[14]. These observations suggested that the outer membrane pore and the hexokinase binding protein were the same proteins which was confirmed by Lindén et al.[29] who proved the identity of the two proteins by two-dimensional electrophoresis and peptide map.

Additional proof of the interaction between hexokinase and the outer membrane pore came from the observation that specific antibodies against porin suppressed the binding of hexokinase to liver mitochondria[7]. Furthermore, yeast hexokinase which was bound in vitro to the isolated outer membrane of yeast mitochondria became cross-linked to the pore protein by dithiobis-(succinimidylpropionate)[26]. One important reason for the specific binding of kinases may be that the exchange of charged metabolites such as ADP and ATP[4,42] across the outer membrane is exclusively possible by passing the pore.

Reversible binding of kinases to the outer membrane of liver mitochondria

Beside hexokinase, reversible binding of other ATP consuming enzymes to the mitochondrial surface in different tissues has been reported namely glycerol kinase[21,31,38,46,47] and ATP citrate lyase[20]. It could be demonstrated that glycerol kinase binds to the pore in experiments with the reconstituted, isolated protein[14]. Furthermore, specific antibodies against porin suppressed the binding of glycerol kinase to isolated mitochondria[38].

The existence of several enzymes in a free or mitochondrial bound state led Wilson to point out that the variations in the intracellular distribution of enzymes can be a factor in metabolic regulation and are therefore responsive to the cellular energy status. Enzymes like the kinases described above whose location, according to the metabolic state of the cell, can be either bound or free in solution were called 'ambiquitous' enzymes [61]. The view of ambiquitous enzyme behavior as a metabolic regulator is emphasized by the fact that it depends on a specific structural property of the low K_m hexokinases (isozymes I–III) while isozyme IV, glucokinase, which has a different molecular weight and a 10 times higher K_m for glucose is incapable of binding to the mitochondrial surface [57]. The corollary drawn by Srere [49] from this and related aspects was that enzymes are designed not only for their active sites, but also for specific intracellular organization.

Table 1. Unspecific and specific binding of hexokinase to phospholipid vesicles

	K_s (nM)	Cap_{max} (mU/mg phospl)
Asolectin	163.0	1.5
Asolectin + porin	45.0	0.9
Asolectin + cholesterin (20 mol%)	218.0	2.2
Asolectin + cholesterin + porin	102.0	2.2

Asolectin, asolectin + 20 mol% cholesterol and both lipid mixtures + 50 µg isolated pore protein per mg phospholipid were dissolved in 50 mM octylglycoside, 20 mM triethanolamine pH 8 and dialyzed against 100 mM triethanolamine/NaCl, 0.02% Naazide pH 8 to produce liposomes.
The liposomes were incubated for 20 min at room temperature with increasing concentrations of isolated hexokinase I in 100 mM triethanolamine pH 7.4, 1 mM $MgCl_2$. The samples were subsequently centrifuged for 10 min at 400,000 × g in a Beckman rotor TLA 100.2. Activity of hexokinase was determined in the supernates and pellets in the presence of 5% Triton. The half saturation constant (K_s) and maximal binding capacity (Cap_{max}) for hexokinase were determined by Eadie-Hofstee plots.

The nature of kinase binding to the outer mitochondrial membrane

The observation of tissue-specific and developmental stage-dependent specific binding of these kinases to the mitochondrial surface is quite intriguing, but incompletely understood. In brain about 80% of hexokinase is mitochondrial bound [58], whereas in liver the amount is considerably lower (10–20%) and depends on the metabolic state [1, 57]. A similar difference is observed for glycerol kinase between adult human brain and liver [38, 51], but it has also been noted in contrast to the adult, that 90% of human fetal liver glycerol kinase was mitochondrial bound [43]. These observations have been explained by different isozymes and regulation of binding by product concentration [58]. However, there is the definite possibility of tissue-specific and perhaps even developmental stage-specific porins. There is recent evidence on the basis of conductivity measurements that liver and brain porins are not the same [30]. It has been observed that the products glucose 6-phosphate of hexokinase [58] and glycerol-phosphate of glycerol kinase [21, 38, 47] solubilize the enzymes. Glucose 6-phosphate appears to induce a conformational change in a region of the enzyme that modulates interaction with the mitochondrial membrane [59]. This effect of glucose 6-phosphate is different from the inhibition of the activity of the free low K_m hexokinases which occurs at a 5 to 10 times lower concentration of this compound [58]. According to the studies of Wilson and coworkers, who intensely investigated the binding of isozyme I, the interactions between hexokinase and the outer mitochondrial membrane include two components: divalent cation-mediated interactions between negatively charged groups on the enzyme and membrane surface [12] as well as specific interactions with the pore protein. The NH_2-terminal M_r = 10,000 domain is involved in the latter interactions with the mitochondrial membrane. This domain includes a hydrophobic NH_2-terminal sequence which has been shown to be required for the binding of hexokinase I to mitochondria [40]. Eleven hydrophobic residues of this sequence were found to extend into the lipophilic core of artificial membranes and it has been speculated that this part of the molecule interacts with the pore protein in the core of the outer membrane [16].

Aside from providing a location for the specific binding protein for hexokinase the membrane phospholipids cannot be considered as neutral. Anionic phospholipids would increase the negative charge on the membrane and hence increase the repulsive force between the enzyme and the membrane. This may be especially important in the regulation of hexokinase isozyme II which at physiological pH has more negative charges compared to isozyme I. On the other hand, dilution of the charges on the membrane surface for instance by increase in cholesterol may cause the opposite effect namely better binding. We observed that the unspecific binding of hexokinase I to asolectin vesicles increased two-fold when the vesicles contained 20 mol% of cholesterol (table 1).

On the whole, interactions between hexokinase and the outer membrane include two components: one which is the specific interaction with the pore protein and is susceptible to glucose 6-phosphate, while the other is a more or less unspecific interaction with the lipid matrix of the membrane.

Metabolic regulation of the reversible binding of kinases to the outer membrane

Wilson and Felgner [60] pointed out that liver and muscle tissues which exhibit glycogen synthesis contain less and more variable activity of hexokinase in the mitochondrial fraction than brain and kidney medulla tissues [3] which depend mainly on blood glucose. Similar observations can be made concerning intracellular distribution of glycerol kinase when brain and liver are compared [38, 51]. It

may be inferred from this that tissues which do not depend on effective glucose and glycerol uptake by phosphorylation via hexokinase and glycerol kinase (because they have glycogen-stores and a potent gluconeogenesis) have variable activity of these kinases bound at the mitochondrial surface. The variation of bound activity implies a regulation of the binding. It was found that the negative charge at the surface of liver mitochondria definitely varies according to the extracellular level of free fatty acids[23, 62]. Correlated to this not only the activity of bound hexokinase was reduced in vitro and in vivo but also the total cellular activity of the enzyme[1]. In other words, the variation in the intracellular distribution of hexokinase is a factor in the regulation of catalytic activity. The desorption of hexokinase by increased negative charges is readily explained in terms of an increase of repulsive forces between the membrane surface and the more negatively charged hexokinase II which is the predominant isozyme in the liver.

It has been pointed out earlier in this chapter that a tissue-specific structure of the pore protein may be responsible for differences in kinase binding. Despite the possible existence of isoproteins in mitochondria of different tissues we may also consider changes of the pore structure by alterations of the membrane potential. We have recently discussed the existence of a capacitative coupling between inner and outer membrane in the contact sites[17, 24] which would result in an electric field larger than 30 mV across the outer membrane, with an outside negative polarity[2, 8]. As a consequence of this the pore protein would adopt a different structure which is characterized by decreased conductivity[42, 45] (see first section in this article). However, the pore proteins beyond the contact sites would have the structure which is present at low voltage and is characterized by anion selectivity and high conductance[9]. Based on these considerations one can expect the simultaneous existence of two different structures of the pore protein in the outer membrane. This may be reflected in a nonrandom distribution of hexokinase with preference in the contact sites which was seen by electron microscopy, using immuno-gold labelling, in liver and brain mitochondria[25, 57]. In agreement with the latter observation we were able to separate the contact sites (i.e. complexes between hexokinase and outer- and inner membrane components) in fragmented kidney and brain mitochondria from outer and inner membranes by immunoprecipitation with antibodies against hexokinase[2]. The nonrandom distribution of hexokinase can be explained by the observation of a higher affinity for the enzyme of the isolated contact fraction compared to the outer membrane (table 2). The data in table 2 additionally show that the binding properties of phosphorylating mitochondria (where the frequency of the contacts increases) almost resemble those of the isolated contacts. Supposing that the pore protein is mainly responsible for the hexokinase binding this would suggest two structures of the pore which differ in location and affinity for hexokinase. The intriguing aspect of this supposition is that the contact sites are dynamic structures[24] which were found to increase in correlation with the rate of oxidative phosphorylation. Thus, depending on the formation of contacts more and more kinases would associate at the mitochondrial surface and would become functionally coupled to the mitochondrial ATP.

Table 2. Half saturation constants for hexokinase I of outer membrane binding sites inside and beyond the contacts

Membrane fraction	K_s (nM HK I)	Binding capacity (mU/μg porin)
Contact sites	43.9 ± 7.2	0.44 ± 0.1
Outer membrane	131.0 ± 82.5	1.60 ± 0.3
Mitochondria phosphorylating	46.1 ± 17.1	1.10 ± 0.4

Contact sites and outer membrane were isolated as described recently[37]. The binding of isolated hexokinase isozyme I was performed in sucrose isolation medium in the presence of 5 mM $MgCl_2$ and glucose. The assay for phosphorylating liver mitochondria additionally contained 5 mM phosphate and succinate and 1 mM ADP. The probability that the different groups are statistically identical is P = 1%.

Function of the binding of kinases in metabolic regulation

Bessman and coworkers have produced rather clearcut evidence that the mitochondrial hexokinase of liver[15] and muscle[53] preferentially utilizes intramitochondrially generated ATP. Comparable results were reported for the mitochondrial bound glycerol kinase[21, 38]. These observations suggest that the location of kinases at the mitochondrial surface results in a discrete microcompartment in which energy-producing and energy-consuming reactions are coupled, with the net result of stimulation of either reaction. On the basis of localization studies[2, 25, 37] it appeared likely that the contacts are the sites of interaction between kinases and oxidative phosphorylation via the pore protein and the adeninnucleotide translocator as depicted in figure 1. Because the contacts are also the sites where the inner membrane potential might be transduced to the outer membrane, the latter may exert control over the coupling of kinases.

We have postulated that such microcompartmentation of the ATP and ADP would improve the exchange of mitochondrial energy[7, 8] for the following reasons: the ATP export is electrogenically driven by the inner membrane potential[22]. According to competitive in vitro measurements by Klingenberg et al.[22] the ATP/ADP translocator thus generates ATP/ADP ratios of 15. Comparable ratios are obtained from the cytosolic concentrations of free ATP and ADP in the steady state of active, intact cells (liver ATP/ADP = 10[48], muscle ATP/ADP = 100[18]). Considering these data one would expect the transport system close to equilibrium and to have low turnover rates. As a consequence of this and the fact that the ADP uptake system controls the oxidative phosphorylation to 40%[50] the mitochondrial activity would be reduced, which has never been observed in the active cell. In order

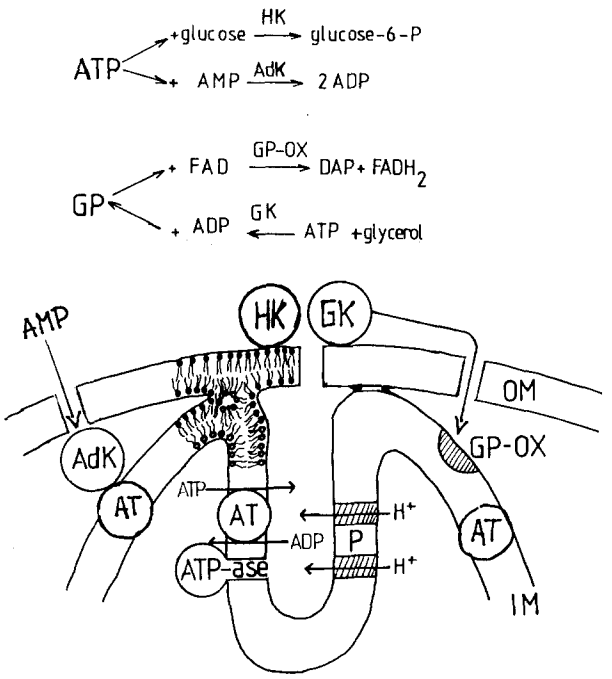

Figure 1. Model of the microcompartment at the mitochondrial periphery. The contacts between the two boundary membranes may be formed by semifusion between the two attached leaflets of the membranes in which the formation of hexagonal H_{II} cylinders by non-bilayer phospholipids is involved as proposed by Verkleij[52] (left side of the figure). Alternatively to the contact structure proposed by Verkleij we would assume a close attachment between the membranes performed by protein-protein interaction rather than a semifusion[8] (right side of the figure). The organization of kinases by the formation of contact sites separates enzymes which compete for ATP such as adenylate kinase (AdK) and hexokinase (HK). The latter kinases do not share the same ATP/ADP pool[7]. On the other hand it supports metabolite exchange between enzymes such as glycerol kinase (GK) and glycerol-phosphate oxidase (GP-OX). Isolated liver mitochondria can oxidize glycerol to dihydroxy acetone-phosphate because of the interplay between these two enzymes[38]. Abbreviations: AT = ATP/ADP-translocator, P = proton-pump, IM = inner membrane, OM = outer membrane, GP = glycerol phosphate, DAP = dihydroxy acetone-phosphate.

to explain this discrepancy we propose the existence of an effective mechanism to displace the adeninnucleotide translocator from energetic equilibrium despite a high cytosolic ATP/ADP ratio. Such a mechanism would be provided by the functional coupling of peripheral kinases. By the activity of the bound kinases the translocated ATP would be immediately converted to products which are not substrates of the translocator (fig. 1). With respect to the functional coupling of several kinases in the contact sites there is a second way of considering the regulatory role of this organization in energy metabolism. This way concerns the possibility of directing the load of energy (ATP) production either to the glycolysis or to the oxidative phosphorylation by binding or debinding of the energy-consuming kinases. The glycolytic rate would become reduced as a consequence of the kinases using intramitochondrial ATP. To base this view on some evidence we started to investigate the organization of kinases in tumor cells.

Physiological observations which suggest a metabolic function of the coupling between peripheral kinases and oxidative phosphorylation

In order to provide information about the physiological importance of the postulated mechanism I shall discuss three fields of investigation: inborn errors of metabolism, muscle transformation, and tumor metabolism.

Human glycerol kinase deficiency, an inherited disorder, has been discussed by McCabe as a model for observing the effects that disruption of the reversibly formed microcompartment might have on the functional integrity and energy economy of the cell[32]. The two organs which are consistently affected in patients with the infantile form of the glycerol kinase deficiency, the brain (developmental delay) and adrenal glands (functional insufficiency), are physiologically characterized by significant binding of glycerol kinase to the mitochondrial fraction[47,51]. The usual biochemical approaches, focussing on catalytic mutations, have not shown differences compared to the controls. Therefore, to understand the impact of mutations on the functionally intact system a variation of the porin-glycerol kinase binding was envisaged either because of alterations of the enzyme or a tissue-specific porin[31-33]. By *chronic low-frequency stimulation* a fast glycolytic muscle can be transformed into a slow oxidative one[39]. During this transformation the activity levels of the glycolytic enzymes decrease while those of the mitochondrial enzymes (citric acid cycle and cytochromes) increase. However, the experimental regime additionally induces hexokinase, mainly isozyme II[41,56]. This result that hexokinase is the only enzyme of the glycolytic pathway which is elevated by sustained contractile activity emphasizes the relation between this enzyme and the mitochondria. It furthermore points to the importance of glucose phosphorylation in the control of glucose utilization during high muscle activity. *The energy metabolism of tumor cell* is characterized by an imbalance between the two ATP supplying metabolic pathways, the glycolysis and the oxidative phosphorylation[55]. Consequently, when we analyzed the contribution of these two metabolic systems to cellular ATP in highly glycolytic HT 29 cells, we observed that the oxidative phosphorylation produced only 50% of the total ATP[10]. In contrast to this, in a low glycolytic subpopulation of these cells (obtained by adaptation to glucose-free medium) oxidative phosphorylation contributed more than 80% to the cellular ATP. As both populations of the HT 29 cells contained the same amount of well-coupled mitochondria, the only satisfying explanation for the difference in energy metabolism was to assume an insufficient mitochondrial ADP supply in the highly glycolytic cells. Supposing that the contact sites may provide an important structural basis in the ATP/ADP exchange, we looked for these structures at the mitochondrial periphery in freeze-fractured samples of the different HT 29 subpopulations. We found an almost complete absence

of contact sites in the highly glycolytic cells while their frequency was normal in the low glycolytic population [10]. In view of these findings the disregulation of tumor energy metabolism appeared to be a good example to emphasize the importance of the dynamic organization of kinases at the mitochondrial periphery in which contact site formation is involved. Irrespective of the amount of kinase activity bound to the mitochondrial surface the ADP supply of the oxidative phosphorylation seemed to depend on the organization of kinases in the contact sites. Considering the specific location of hexokinase in the contact sites, a plausible reason for this could be to provide a direct coupling of the enzyme via the pore protein to the ATP/ADP translocator.

Concerning this coupling between oxidative phosphorylation and hexokinase, somewhat contradictory results have been obtained in tumor mitochondria. While Krishan and Pedersen in hepatoma-[27] and Kurokawa et al. in Ehrlich ascites cells [38] reported that the bound enzyme was more effective in utilizing the internal ATP, we [10] in HT29 adenocarcinoma and Kabir and Nelson [35] in Novikoff tumor observed a complete uncoupling of the enzyme from the inner compartment although the enzyme was bound to the mitochondrial surface. The pore protein of tumor mitochondria has the same properties as other mitochondrial porins concerning conductivity in artificial membranes and binding of hexokinase [34]. The content of the pore protein is not significantly elevated in mitochondria of neoplastic cells [36] and therefore, cannot account for the frequently observed increased hexokinase binding. As calculated from data published by Nakashima et al. 50% of the hexokinase bound at the surface of tumor mitochondria could not be desorbed from the membrane by factors which affect the specific binding of the enzyme to the pore. This was glucose 6-phosphate which changes the conformation of the hexokinase [59] and DCCD (dicyclohexyl-carbodiimide) which alters the pore structure and suppresses hexokinase binding [34]. Thus, it must be assumed that the surface of tumor mitochondria exhibits an increase of unspecific binding sites for additional hexokinase. As I pointed out above, the composition of the lipid matrix of the membrane has an important effect on the association of hexokinase to the membrane surface. This aspect is emphasized by our observation that addition of 20 mol% cholesterol increases the hexokinase binding capacity of lipid vesicles irrespective of the presence of porin (table 1). The intriguing point of this observation is that the outer membrane of tumor mitochondria contains 3–4 times more cholesterol [13] which might explain the increased unspecific binding of hexokinase.

Conclusion

Although the reversible binding of hexokinase and glycerol kinase to the mitochondrial surface generally has the same effect on the kinase activity, it has specific metabolic functions in different tissues. As two very different examples I shall summarize the role in liver and brain metabolism.

In liver where hexokinase binding is regulated by metabolites (free fatty acids [1, 62]) and hormones (insulin increases the binding [6]) it has three functions; 1) it increases the response of the liver to rapid changes of blood glucose because it regulates the total cellular activity of low K_m kinases [1, 57]; 2) it rises glucose 6-phosphate levels which induce glycogen synthesis because the binding abolishes the control of glucose phosphorylation (i.e. uptake) by glucose 6-phosphate levels; 3) the binding reduces the glycolytic rate because the glucose phosphorylation at the expense of mitochondrial ATP saves 25% of the glycolytic ATP production. It goes without saying that this must have a great influence on the glycolytic flux. The latter would increase if the hexokinase became unspecifically bound such as in tumor mitochondria without being functionally coupled to the oxidative phosphorylation. The interesting aspect of unspecific hexokinase binding in tumor mitochondria is that the enzyme, in contrast to the free one, is no longer susceptible to

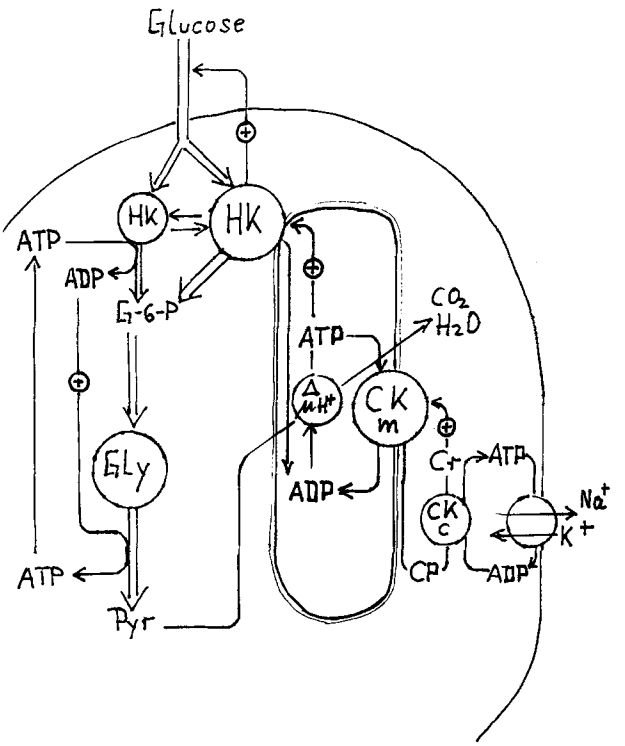

Figure 2. Scheme summarizing how the glucose uptake into brain cells via hexokinase activity is linked to the oxidative phosphorylation. Increased turnover of cytosolic ATP raises the level of creatine by the activity of cytosolic creatine kinase (CKc). The creatine (Cr) is rephosphorylated to phospho-creatine (CP) by the mitochondrial creatine kinase (CKm) resulting in (i) activation of the oxidative phosphorylation by elevated ADP supply and (ii) a correlated increase in contact sites and associated hexokinase (HK). The enzyme in the contacts becomes functionally coupled to the adenylate translocator in the inner membrane and therefore has a higher efficiency in glucose phosphorylation. Abbreviations: Gly = glycolysis, Pyr = pyruvate, G-6-P = glucose 6-phosphate, $\Delta\mu H^+$ = electrochemical potential as the driving force of the oxidative phosphorylation.

product inhibition by glucose 6-phosphate and thus, continues to phosphorylate glucose at the expense of glycolytic ATP.

The function of kinase binding in brain is mainly to control the glucose or glycerol uptake according to the mitochondrial activity by a mechanism which is schematically depicted in figure 2. The mitochondrial activity in brain is regulated by the creatine phosphate shuttle as proposed by several authors [5, 19, 44, 54]. In other words, the activity of the oxidative phosphorylation depends on the level of free creatine which reflects the ATP turnover in the extramitochondrial compartment. The creatine is rephosphorylated by the mitochondrial creatine kinase to phospho-creatine, and ADP and the latter activates the oxidative phosphorylation. Correlated to this activation, the frequency of contact sites increases [24]. As a consequence of that, more hexokinase and glycerol kinase at the surface of brain mitochondria become functionally coupled to the inner mitochondrial compartment because the affinity of the enzymes to the pore protein in the contact sites is higher compared to the pore beyond the contacts (table 2). The functional coupling of the kinases results in a higher efficiency [28, 32, 38, 57] of substrate phosphorylation and thus, increases the glucose and glycerol uptake of brain cells. By this mechanism it is possible to regulate the uptake of carbohydrates in brain cells through the activity of oxidative phosphorylation (fig. 2).

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