

Review

Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function

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Summary

The first step in metabolism of glucose (Glc) is usually phosphorylation, catalyzed by hexokinase. However, the Glc-6-P produced can then enter one or more of several alternative pathways. Selective expression of isozymic forms of hexokinase, differing in catalytic and regulatory properties as well as subcellular localization, is likely to be an important factor in determining the pattern of Glc metabolism in mammalian tissues/cells. Despite their overall structural similarity, the Type I, Type II and Type III isozymes differ in important respects. All three isozymes are inhibited by the product, Glc-6-P, but with the Type I isozyme, this inhibition is antagonized by P_i , whereas with the Type II and Type III isozymes, P_i actually causes additional inhibition. Reciprocal changes in intracellular levels of Glc-6-P and P_i are closely associated with cellular energy status, and it is proposed that the response of the Type I isozyme to these effectors adapts it for catabolic function, introducing Glc into glycolytic metabolism for energy production. In contrast, the Type II, and probably the Type III, isozymes are suggested to serve primarily anabolic functions, e.g. to provide Glc-6-P for glycogen synthesis or metabolism *via* the pentose phosphate pathway for lipid synthesis. Type I hexokinase binds to mitochondria through interaction with porin, the protein that forms channels through which

metabolites traverse the outer mitochondrial membrane. Several experimental approaches have led to the conclusion that the Type I isozyme, bound to actively phosphorylating mitochondria, selectively uses intramitochondrial ATP as substrate. Such interactions are thought to facilitate coordination of the introduction of Glc into glycolysis, *via* the hexokinase reaction, with the terminal oxidative stages of Glc metabolism occurring in the mitochondria, thus ensuring an overall rate of Glc metabolism commensurate with cellular energy demands and avoiding excessive production of lactate. The Type II isozyme also binds to mitochondria. Whether such coupling occurs with mitochondrially bound Type II hexokinase in normal tissues, and how it might be related to the proposed anabolic role of this isozyme, remain to be determined. The Type III isozyme lacks the hydrophobic N-terminal sequence known to be critical for binding of the Type I and Type II isozymes to mitochondria. Immunolocalization studies have indicated that, in many cell types, the Type III has a perinuclear localization, the possible metabolic consequences of which remain unclear.

Key words: hexokinase, isozyme, subcellular localization, mitochondria, mammalian.

Introduction

Glucose (Glc) is of central metabolic importance in virtually all organisms, from microbes to humans. Glycolytic metabolism of Glc is a major pathway for the generation of energy (ATP), and glycolytic intermediates also serve as precursors for biosynthesis of other cellular constituents. Metabolism of Glc through the pentose phosphate pathway generates NADPH and precursors required for a variety of anabolic pathways. Alternatively, Glc may be converted to its polymeric forms (glycogen, starch), which are the storage forms of this carbohydrate in many organisms. In mammals, the relative importance of these and other pathways depends on the particular tissue, or cell type within a tissue, but the

potential for metabolism of Glc *via* alternative pathways exists in most mammalian cells.

The initial step in metabolism of Glc through most common pathways is phosphorylation to form glucose-6-phosphate (Glc-6-P) (Fig. 1), the reaction catalyzed by hexokinase. It is evident that indiscriminate metabolism of Glc-6-P through the various potential pathways would not be in the cell's best interest. So what regulates the formation of Glc-6-P and its direction to the metabolic fate that is appropriate for the physiological status of the cell at the time? Are there any differences in the way in which mammalian cells generate Glc-6-P intended for glycolytic metabolism compared with

Table 1. *Kinetic and regulatory parameters of isozymes of mammalian hexokinase*^a

Isozyme	K_m (mmol l ⁻¹)		K_i (mmol l ⁻¹) for Glc-6-P	Effect of P _i
	for Glc	for ATP		
Type I	0.03	0.5	0.02	Low concentrations antagonize inhibition by Glc-6-P; high concentrations inhibit
Type II	0.3	0.7	0.02	Inhibitory at all concentrations
Type III	0.003 ^b	1.0	0.1	Inhibitory at all concentrations

Glc, glucose; Glc-6-P, glucose-6-phosphatase.

^aA range of values have been reported by various investigators (for a compilation, see Wilson, 1985); the values shown here are representative.

^bSubstrate inhibition is seen at higher Glc concentrations (approx. 1 mmol l⁻¹).

Glc-6-P destined for metabolism *via* the pentose phosphate pathway? How are cells adapted to favor metabolism of Glc *via* particular pathways that are related to the role that cell plays in the overall metabolism of the organism? While the answers to such questions are far from clear, selective expression of isozymic forms of HK is likely to be an important factor in determining the pattern of Glc metabolism in mammalian cells/tissues.

Isozymes of mammalian hexokinase

Four distinct isozymes of hexokinase were separated by ion exchange chromatography (González et al., 1964) or electrophoresis (Katzen and Schimke, 1965) of extracts from various mammalian tissues. Generally these are referred to as the Type I, Type II, Type III and Type IV isozymes, with the latter commonly called 'glucokinase'. Relatively recent reviews (Wilson, 1995; Cárdenas et al., 1998) include extensive discussion of the mammalian isozymes and, more recently, an excellent review on the Type IV isozyme has been provided by Postic et al. (2001). Since most of our own work has dealt with the Type I, Type II and Type III isozymes, these will be the primary focus of the present discussion.

The Types I–III isozymes are 100 kDa molecules thought to have evolved by duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase. Thus, these isozymes display internal sequence repetition, and the N- and C-terminal halves have extensive sequence similarity, both to each other and to other members of the hexokinase family, which includes the 50 kDa mammalian Type IV isozyme and 50 kDa hexokinases found in other organisms (Bork et al., 1993; Wilson, 1995; Cárdenas et al., 1998).

Susceptibility to relatively potent inhibition by the product, Glc-6-P, is generally considered to be an important regulatory feature of the mammalian Type I–III isozymes, and is also found with 50 kDa hexokinases from lower organisms such as starfish and the parasite *Schistosoma mansoni* (White and Wilson, 1989; Tielens et al., 1994). It is thus likely that sensitivity to inhibition by Glc-6-P evolved prior to the gene duplication and fusion event that gave rise to the 100 kDa mammalian isozymes. Duplication and fusion of a gene encoding a 50 kDa Glc-6-P-sensitive hexokinase should give

rise to a gene encoding a 100 kDa hexokinase with both N- and C-terminal halves having catalytic activity susceptible to product inhibition. The Type II isozyme has such characteristics (Ardehali et al., 1996; Tsai and Wilson, 1996), and on that basis has been suggested to be most closely related to the ancestral 100 kDa hexokinase produced by the gene duplication and fusion event. In contrast, it is clear that catalytic function resides solely in the C-terminal half of the Type I and Type III isozymes (White and Wilson, 1989; Baijal and Wilson, 1992; Tsai and Wilson, 1995, 1997). Thus, the Type I and Type III isozymes likely resulted from further duplication of the gene encoding an ancestral 100 kDa hexokinase, with subsequent mutations leading to functional differentiation of the N-terminal halves to serve noncatalytic (regulatory) functions.

If the capability for phosphorylation of Glc were the sole *raison d'être* for hexokinase, then it would seem that a single hexokinase would be sufficient. Yet, it is intriguing that isozymes of hexokinase exist even in 'simpler' organisms such as yeast (Rodríguez et al., 2001). Surely there is more to this than simply making Glc-6-P! Thus, it is reasonable to conclude that the Types I–IV isozymes of hexokinase play distinct roles in Glc metabolism in mammalian tissues. In the case of the Type IV isozyme, that role has been rather well defined (Postic et al., 2001). What about the Type I, Type II and Type III isozymes?

As noted previously (Wilson, 1997), one could imagine at least three good (and not mutually exclusive) reasons for the existence of isozymes: (1) the isozymes may differ in their catalytic and/or regulatory properties, suiting them for particular metabolic roles; (2) differences in transcriptional regulation of the isozymes may permit their selective expression in particular tissues, with distinct responses to altered metabolic status, e.g. hormonal effects or chronic changes in physiological activity (Hofmann and Pette, 1994); (3) differences in subcellular location of the isozymes may result in compartmentation of Glc metabolism, with 'channeling' of Glc-6-P to particular metabolic pathways (Ureta, 1978; Ovádi and Srere, 2000).

Kinetic and regulatory properties of the Types I–III isozymes are compared in Table 1. The usual caveats apply to extrapolation of kinetic parameters determined *in vitro* to conditions *in situ* and, as will become evident later, this may

be particularly the case with the K_m of the Type I (and possibly the Type II) isozyme for ATP. Radojković and Ureta (1987) previously noted that the Type III isozyme is distinguished by having the highest apparent affinity for Glc – but also the lowest apparent affinity for the other substrate, ATP – as well as being least sensitive to inhibition by the product, Glc-6-P. The Type III isozyme is also unique in showing substrate inhibition at higher Glc levels (>approximately 1 mmol l^{-1}), with this inhibition being antagonized by ATP. Based on the results of Radojković and Ureta (1987), and assuming intracellular [ATP] in the $1\text{--}3 \text{ mmol l}^{-1}$ range, one might anticipate that any inhibitory effects would be relatively modest at the concentrations of Glc likely to exist in most cells. Hence, the physiological significance of these unique regulatory features of the Type III isozyme remains unclear.

In contrast, we believe that the distinct response of these isozymes to P_i provides a useful clue to their physiological roles (Wilson, 1985, 1995). Specifically, only the Type I isozyme shows inhibition by Glc-6-P that is antagonized by P_i . With the Type II and Type III isozymes, P_i is itself inhibitory and does not antagonize but rather, adds to any inhibition by Glc-6-P (Wilson, 1995, and references therein; Tsai and Wilson, 1995, 1996, 1997). Increase in cellular [P_i] (due to increased hydrolysis of high energy phosphate compounds) and decrease in cellular [Glc-6-P] (due to increased flux through an activated phosphofructokinase reaction) are typically seen during periods of increased energy demand and associated increase in glycolytic metabolism, e.g. in brain (Lowry et al., 1964). The resulting increase in the [P_i]/[Glc-6-P] ratio would increase Type I hexokinase activity, leading to the suggestion (Wilson, 1985, 1995) that the Type I isozyme functions primarily in a catabolic role, introducing Glc into glycolytic metabolism with the primary purpose of generating energy (ATP). The ubiquitous expression of the Type I isozyme is consistent with this view, given the importance of glycolysis in virtually all mammalian tissues. Moreover, the Type I isozyme is expressed at particularly high levels in brain, a tissue well known for its virtually total reliance on glycolytic metabolism of Glc to sustain a high rate of energy metabolism (Clarke and Sokoloff, 1998). In contrast, the Type II isozyme is much more limited in its expression, primarily being found in insulin-sensitive tissues such as skeletal muscle and adipose tissue. We have previously argued (Wilson, 1985, 1995) that the response of the Type II isozyme to these same ligands, Glc-6-P and P_i , would better suit it for an anabolic role, e.g. providing Glc-6-P for resynthesis of glycogen during recovery of skeletal muscle after contraction. More recent work is also consistent with an anabolic role for the Type II isozyme as a source of Glc-6-P for metabolism *via* the pentose phosphate pathway, providing NADPH required for lipid synthesis in liver (Sebastian et al., 2000) or lactating mammary gland (Kaselonis et al., 1999). By this same reasoning, the similarity in response of the Type II and Type III isozymes to Glc-6-P and P_i (Table 1) suggests that the latter isozyme may also have primarily an anabolic role, but presently there is no basis for

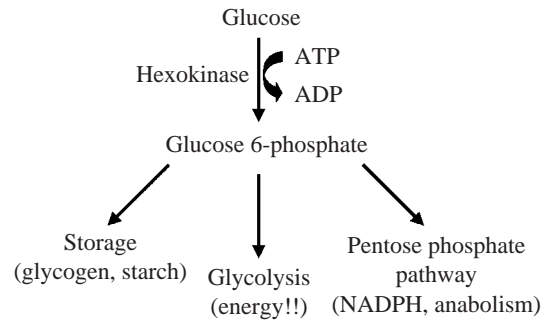


Fig. 1. Phosphorylation, catalyzed by hexokinase, is the initial step in common pathways of Glc metabolism.

associating the Type III isozyme with a particular anabolic pathway (or pathways).

Promoter regions governing the transcription of the Type I (White et al., 1996; Liu and Wilson, 1997), Type II (Mathupala et al., 1995; Osawa et al., 1996; Heikkinen et al., 2000) and Type III (Sebastian et al., 1999, 2001) isozymes have been isolated and characterized to some extent. It is clear that these are distinct in character, and responsive to quite different transcription factors. Thus the existence of multiple genes encoding the isozymes of hexokinase does offer the flexibility in expression, in different tissues or in different physiological states, perceived as a benefit of isozymic forms (Wilson, 1997).

Subcellular location and hexokinase function

Type I isozyme

As evident from Fig. 1, hexokinase serves as the gateway through which Glc enters alternative metabolic pathways. Nonetheless, it is probably safe to say that hexokinase is most commonly thought of as a glycolytic enzyme, and glycolytic metabolism is generally considered a cytoplasmic process. It was thus remarkable that, unlike other glycolytic enzymes, hexokinase activity (now known to be the Type I isozyme) was found predominantly in 'particulate fractions' of brain homogenates (Crane and Sols, 1953). Subsequent work (Johnson, 1960; Rose and Warms, 1967; see also Wilson, 1995) demonstrated that the particulate hexokinase was associated with mitochondria, and more specifically, with the outer mitochondrial membrane (Rose and Warms, 1967; Kropp and Wilson, 1970). Binding is critically dependent on a hydrophobic N-terminal sequence (Polakis and Wilson, 1985) that selectively targets the Type I isozyme to mitochondria (Gelb et al., 1992; Sui and Wilson, 1997) and is inserted into the hydrophobic core of the outer mitochondrial membrane in the course of binding (Xie and Wilson, 1988). Porin (also called 'VDAC,' the acronym for 'voltage dependent anion channel') forms the channel through which metabolites traverse the outer mitochondrial membrane, and was identified as the outer mitochondrial membrane protein that interacts with hexokinase (Felgner et al., 1979; Lindén et al., 1982; Fiek et al., 1982). Moreover, there is evidence to support the view that

binding of hexokinase occurs preferentially to porin that is located in contact sites, regions in which there is intimate contact between the inner and outer mitochondrial membranes (Dorbani et al., 1987; Kottke et al., 1988; BeltrandelRio and Wilson, 1992a).

The classic studies of Johnson (1960) and of Rose and Warms (1967) were soon followed by reports of mitochondrially bound hexokinase from other normal tissues, in addition to brain, as well as from various tumor cells (for references, see Wilson, 1985, 1995). The potential physiological significance of this association of a 'glycolytic' enzyme with mitochondria, the primary site for oxidative metabolism, has been the subject of much speculation. From early on, and particularly after recognition that the 'hexokinase binding protein' of the outer mitochondrial membrane was identical to porin, and thus that hexokinase might be positioned near the point at which ATP would exit the mitochondria (and ADP re-enter the mitochondria), speculation was largely focused on the possibility that this proximity might foster intimate metabolic interaction between intramitochondrial oxidative phosphorylation as a source of substrate ATP and Glc phosphorylation by the mitochondrially bound hexokinase. This had, in fact, been considered by Rose and Warms (1967), but could not be supported by their experimental results. However, subsequent work by others (again, for references see Wilson, 1985, 1995), using mitochondrially bound hexokinase from various sources, produced evidence in support of the view that mitochondrially bound hexokinase had 'preferential' or 'privileged' access to intramitochondrially generated ATP.

Work in our laboratory has provided a firm basis for the view that hexokinase bound to actively phosphorylating brain mitochondria is indeed tightly coupled to an intramitochondrial compartment of substrate ATP, generated by oxidative phosphorylation. The experimental support for this conclusion has been described in a series of publications (BeltrandelRio and Wilson, 1991, 1992a,b; de Cerqueira Cesar and Wilson, 1995, 1998, 2002; Hashimoto and Wilson, 2000). A complete review of this work is not possible within the constraints of the present context, but we will highlight some of the principal findings to illustrate the variety of experimental approaches, all leading to the same conclusion, that have been utilized in these studies.

Initial studies (BeltrandelRio and Wilson, 1991, 1992a,b) were done using spectrophotometric methods in which ATP production by various intramitochondrial processes (oxidative phosphorylation, adenylate kinase reaction, creatine kinase reaction) and Glc phosphorylation by hexokinase were linked to NADPH production, monitored at 340 nm, by the use of appropriate coupling enzymes. The basic idea was that by comparing the rate of Glc phosphorylation with the rate of ATP production from various sources, one might deduce the relative importance of various intramitochondrial ATP-generating processes as a source of substrate ATP for hexokinase. And the conclusion was that, when oxidative phosphorylation was occurring, neither adenylate kinase nor creatine kinase was a significant source of substrate ATP. Moreover, only a fraction of the ATP produced by oxidative phosphorylation was

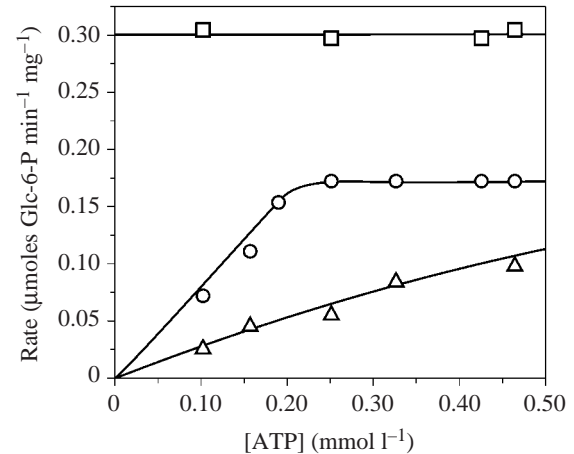


Fig. 2. Glucose (Glc) phosphorylation to glucose-6-phosphate (Glc-6-P) by mitochondrially bound hexokinase with exogenous ATP or with ATP generated by oxidative phosphorylation. The rate of Glc phosphorylation was determined at equivalent [ATP], either added exogenously in the absence of oxidative phosphorylation (triangles) or generated by oxidative phosphorylation (circles). From either source, the [ATP] was subsaturating, with the rate of Glc phosphorylation well below that seen when the ATP levels were acutely raised by addition of saturating levels of exogenous ATP (squares). Reprinted with permission from BeltrandelRio and Wilson (1991).

utilized by hexokinase, with the result that [ATP] in the extramitochondrial medium continued to increase as oxidative phosphorylation continued. The rate of Glc phosphorylation did not steadily increase in response to continued increase in extramitochondrial [ATP], however, despite the fact that the latter was comparable to the K_m for ATP seen with added extramitochondrial ATP in the absence of oxidative phosphorylation, i.e. the hexokinase would not have been 'saturated' with extramitochondrial ATP as substrate (Fig. 2). This strongly suggested that it was not extramitochondrial ATP that was being used as substrate.

Further support for this was provided by results such as those shown in Fig. 3. Here, Glc phosphorylation was monitored after initiation of oxidative phosphorylation by addition of ADP, with increasing concentrations of extramitochondrial ATP present from the start. As expected from classical Michaelis-Menten kinetics, the initial rate of Glc phosphorylation increased with increasing extramitochondrial [ATP]. However, with time, a steady state rate of Glc phosphorylation was attained that was independent of the amount of residual extramitochondrial ATP present. These results were interpreted as indicating that, while the mitochondrially bound hexokinase initially used extramitochondrial ATP, initiation of oxidative phosphorylation led to a switch in substrate preference, with hexokinase becoming dependent on an intramitochondrial compartment of ATP in which [ATP] was determined by the rate of oxidative phosphorylation and independent of the extramitochondrial [ATP].

Although ATP production began almost immediately after

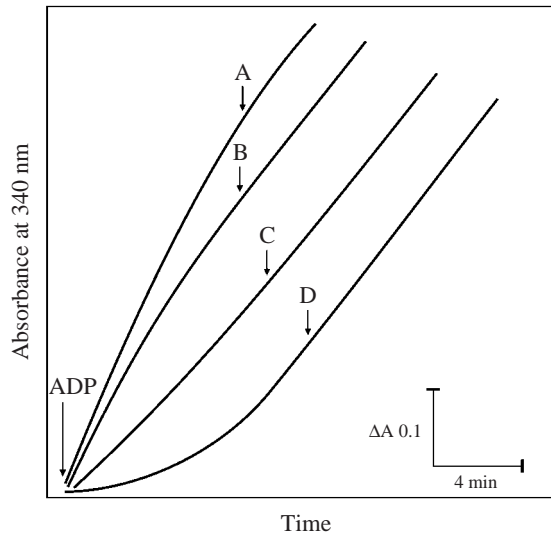


Fig. 3. Glucose (Glc) phosphorylation by mitochondrially bound hexokinase, with ATP generated by oxidative phosphorylation in the presence of increasing concentrations of exogenous ATP. ATP production by oxidative phosphorylation was initiated by addition of ADP at the indicated time. Glc phosphorylation was coupled to NADPH production, monitored by absorbance at 340 nm (A), in the presence of excess glucose-6-phosphate (Glc-6-P) dehydrogenase. The concentrations of exogenous ATP, present at the time of ADP addition, were 1.1, 0.66, 0.22 and 0 mmol l⁻¹ for curves A–D, respectively. Note that the steady state attained was independent of the original extramitochondrial [ATP]. Reprinted with permission from BeltrandelRio and Wilson (1992b).

initiation of oxidative phosphorylation by addition of ADP, there was a marked lag in initiation of Glc phosphorylation by the mitochondrially bound hexokinase (Fig. 3D) before attainment of a steady state rate that persisted for an extended period. This initial lag period was interpreted as the time required to fill an intramitochondrial compartment with ATP generated by oxidative phosphorylation and from which the mitochondrially bound hexokinase drew its substrate ATP. Inhibition of electron transport by addition of KCN resulted in apparent release of ATP, presumably from the intramitochondrial compartment, and the properties of this 'compartment' (kinetics of filling, linkage to intramitochondrial ATP production, etc.) were in satisfying agreement with expectations based on this interpretation (BeltrandelRio and Wilson, 1991, 1992a,b). Unfortunately, agreement with expectations is not an infallible guide to true fact, and the 'apparent release of ATP' was subsequently found to be an artifact (Laterveer et al., 1993), the source of which is still not understood and which could not be reproduced in later work (de Cerqueira Cesar and Wilson, 1998). Despite this discouraging, and embarrassing, setback, the basic concept of coupling mitochondrially bound hexokinase to an intramitochondrial compartment of ATP was supported by other evidence and has withstood subsequent experimental tests.

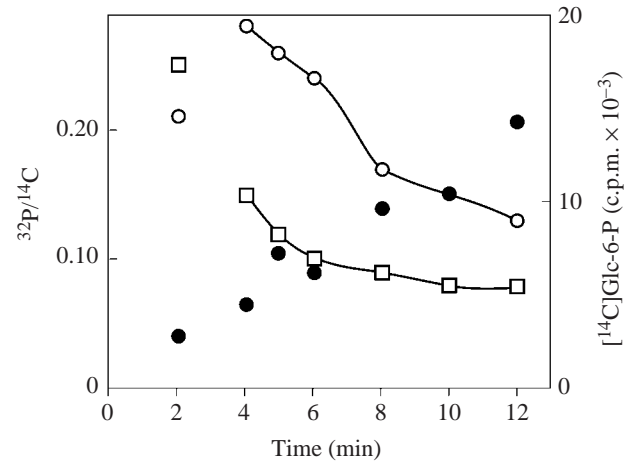


Fig. 4. Effect of adding excess unlabeled P_i on the ³²P/¹⁴C ratio of glucose-6-phosphate (Glc-6-P) formed by mitochondrially bound hexokinase or nonmitochondrially bound yeast hexokinase. Oxidative phosphorylation was initiated with ³²P_i present as substrate for oxidative phosphorylation, and [¹⁴C]Glc as substrate for hexokinase. At 3 min, excess ³¹P_i was added, reducing the specific activity of ATP subsequently produced by oxidative phosphorylation. This resulted in a precipitous decrease in the ³²P/¹⁴C ratio of Glc-6-P formed by yeast hexokinase (squares) using extramitochondrial ATP as substrate, but a much slower decrease in the ³²P/¹⁴C ratio of Glc-6-P produced by mitochondrially bound hexokinase (open circles). Filled circles, total Glc-6-P produced by mitochondrially bound hexokinase. Reprinted with permission from de Cerqueira Cesar and Wilson (1995).

A double isotopic labeling method was developed as an alternative to the spectrophotometric procedures (de Cerqueira Cesar and Wilson, 1995). ¹⁴C-Labeled Glc was used as a substrate for hexokinase, and ³²P_i supplied as substrate for ATP synthesis by oxidative phosphorylation. The ³²P/¹⁴C ratio in Glc-6-P thus provided a measure of the specific activity of substrate ATP used by hexokinase. The ³²P/¹⁴C ratio of Glc-6-P produced by mitochondrially bound hexokinase was compared with that produced by yeast hexokinase, which does not bind to mitochondria and thus necessarily utilizes extramitochondrial ATP as substrate. The kinetics of labeling of the ATP pools used as substrate by mitochondrially bound and yeast hexokinases were strikingly different, again consistent with the view that the mitochondrially bound hexokinase was not utilizing extramitochondrial ATP as substrate, but rather, drawing on an intramitochondrial compartment of ATP furnished by oxidative phosphorylation. For example (Fig. 4), addition of excess ³¹P_i, thereby markedly decreasing the specific activity of the ³²P-ATP synthesized by oxidative phosphorylation, resulted in a rapid decrease in the ³²P/¹⁴C ratio of Glc-6-P produced by yeast hexokinase, using extramitochondrial ATP. In contrast, there was a lag and subsequently somewhat slower decrease in the ³²P/¹⁴C ratio of Glc-6-P produced by mitochondrially bound hexokinase. The latter observations again were consistent with the view that the mitochondrial hexokinase was utilizing an intramitochondrial

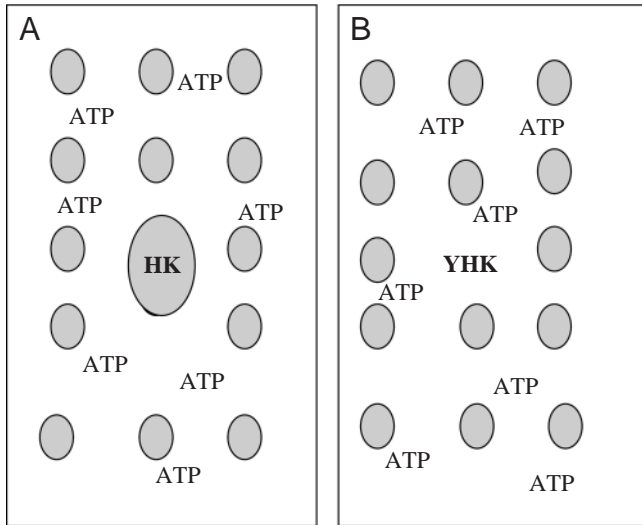


Fig. 5. Schematic representation of the experimental strategy for comparing utilization of extramitochondrial ATP by mitochondrially bound hexokinase or nonbound yeast hexokinase. (A) Mitochondrially bound hexokinase (HK) is represented at the center of the panel, with additional mitochondria, containing little or no bound hexokinase, shown in more peripheral regions. For the latter, rat brain mitochondria that had been depleted of hexokinase by treatment with glucose-6-phosphate, which causes release of the mitochondrially bound hexokinase, were used in earlier experiments. Later experiments, however, used rat liver mitochondria which, as isolated, do not contain bound hexokinase. Extramitochondrial ATP is distributed throughout the extramitochondrial space. (B) Analogous situation, but with an equivalent amount of nonmitochondrially bound yeast hexokinase (YHK) in place of the mitochondrially bound hexokinase. The basic strategy is to determine the rate of glucose phosphorylation by a fixed amount of bound or nonbound hexokinase as the rate of extramitochondrial ATP production is increased by addition of increasing numbers of mitochondria devoid of bound hexokinase. Reprinted with permission from de Cerqueira Cesar and Wilson (2002).

compartment of ATP that was not freely equilibrated with extramitochondrial ATP.

Still another experimental approach was based on a comparison of the mitochondrially bound hexokinase and the non-bound yeast enzyme (de Cerqueira Cesar and Wilson, 1998, 2002). The underlying logic is illustrated in Fig. 5. A fixed amount of brain mitochondria, with bound hexokinase, is mixed with increasing amounts of rat liver mitochondria, which contain no bound hexokinase. Both brain and liver mitochondria are actively phosphorylating and thus there is an increasing rate of ATP production in the system as the amount of liver mitochondria is increased. By design, the concentration of extramitochondrial ATP is kept subsaturating, i.e. $\approx K_m$ of hexokinase with extramitochondrial ATP as substrate (in the absence of oxidative phosphorylation). If the mitochondrially bound hexokinase is using extramitochondrial ATP as substrate, a progressive increase in the rate of Glc phosphorylation is expected as the rate of ATP production is

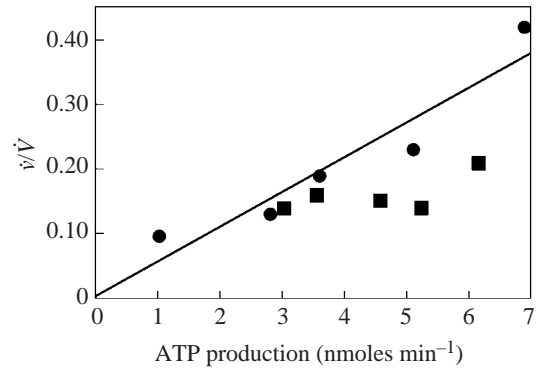


Fig. 6. Rate of glucose (Glc) phosphorylation by mitochondrially bound and nonbound hexokinase, with increasing rates of ATP production from oxidative phosphorylation. The rate of Glc phosphorylation (\dot{v}) is expressed relative to the maximal rate of phosphorylation (\bar{V}), the latter determined with saturating levels of exogenous ATP in the absence of oxidative phosphorylation. The rate of Glc-6-P production by nonmitochondrially bound yeast hexokinase (circles) is closely correlated with the rate of ATP production. In contrast, the rate of Glc phosphorylation by mitochondrially bound hexokinase (squares) is insensitive to increasing levels of extramitochondrial ATP produced by non-hexokinase-bearing mitochondria, consistent with the view that the mitochondrially bound enzyme is restricted to intramitochondrial ATP, produced by the mitochondria to which the enzyme is bound, as substrate. Reprinted with permission from de Cerqueira Cesar and Wilson (1998).

increased by addition of increasing amounts of liver mitochondria. In fact, this is not what is observed; rather, the rate of Glc phosphorylation by the mitochondrially bound hexokinase is not significantly affected by increase in the rate of ATP production (Fig. 6). In contrast, the expected increase in the rate of Glc phosphorylation is seen if the mitochondrial hexokinase is replaced by an equivalent amount of yeast hexokinase. These results are thus again consistent with the view that mitochondrially bound hexokinase is using intramitochondrial ATP, intrinsic to the mitochondria with which the hexokinase is associated but independent of any increases in extramitochondrial ATP emanating from the hexokinase-free liver mitochondria.

Finally, further evidence for the view that mitochondrially bound hexokinase can discriminate between intra- and extramitochondrial ATP comes from examining inhibition by the Glc-6-P analog, 1,5-anhydroglucitol-6-P (1,5-AnG6P). In the absence of oxidative phosphorylation and with extramitochondrial ATP as substrate, 1,5-AnG6P is a rather potent inhibitor, competitive *versus* ATP (Fig. 7) (Hashimoto and Wilson, 2000). In contrast, with ATP supplied by oxidative phosphorylation, 1,5-AnG6P is much less effective as an inhibitor. Clearly, ATP provided by oxidative phosphorylation is not equivalent to extramitochondrial ATP. Similar results have recently been reported using the mitochondrial hexokinase from bovine brain (de Cerqueira and Wilson, 2002).

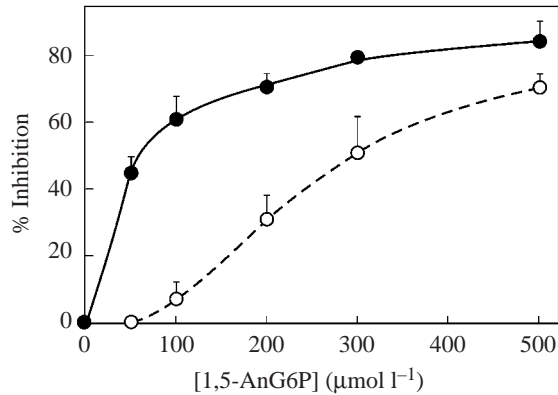


Fig. 7. Inhibition of mitochondrially bound hexokinase by the glucose-6-phosphate analog, 1,5-anhydroglucitol-6-P (1,5-AnG6P), with intramitochondrially generated (open circles) or extramitochondrial (filled circles) ATP as substrate. Reprinted with permission from Hashimoto and Wilson (2000).

In short, the present view is that, in the absence of oxidative phosphorylation, mitochondrial hexokinase can readily use extramitochondrial ATP, following classical Michaelis–Menten kinetics. During active oxidative phosphorylation, however, the mitochondrially bound enzyme is coupled to an intramitochondrial pool of ATP, with the rate of Glc phosphorylation closely correlated with the rate of oxidative phosphorylation. It seems difficult to believe that, in normal tissue, mitochondria are ever in a totally nonphosphorylating state. Changes in the rate? Yes, of course, depending on fluctuations in energy demand. But truly nonphosphorylating? Probably only under the most dire – and ultimately lethal – circumstances. It thus follows that, under normal conditions, the rate of Glc phosphorylation is closely coordinated with terminal oxidative stages of Glc metabolism occurring in the mitochondria, with associated production of ATP by oxidative phosphorylation. As previously noted (BeltrandelRio and Wilson, 1992a), such coordination may ensure introduction of Glc into glycolytic metabolism at a rate commensurate with terminal oxidative stages, avoiding production of neurotoxic lactate (Marie and Bralet, 1991) while ensuring net flux through the cytoplasmic and mitochondrial portions of the pathway at a rate adequate to meet energy demands (Fig. 8).

It is not known how this remarkable change in substrate specificity (intramitochondrial *versus* extramitochondrial ATP) is induced by oxidative phosphorylation, but it is clear that the conformation of the mitochondrially bound enzyme is affected by mitochondrial membrane potential as well as other factors related to mitochondrial function, indicating intimate interaction between the inner (across which the membrane potential exists) and outer (to which hexokinase is bound) membranes of this organelle (Hashimoto and Wilson, 2000). Conformational changes affecting regions of the molecule involved in binding of substrate ATP had previously been postulated (de Cerquiera and Wilson, 1998) to be responsible for changes in substrate specificity.

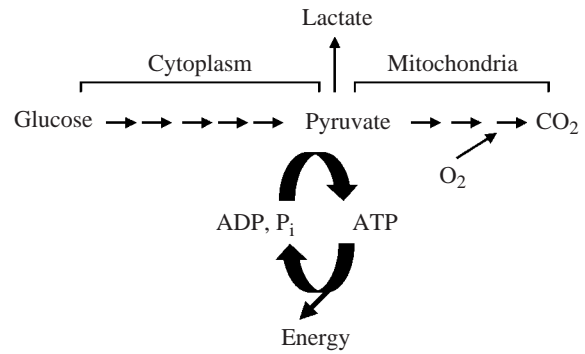


Fig. 8. Coordination of glycolytic and oxidative phases of glucose (Glc) metabolism. The rate of Glc phosphorylation by mitochondrially bound hexokinase, using intramitochondrially generated ATP as substrate, is correlated with the rate of oxidative phosphorylation. This mechanism is suggested to ensure coordination of Glc phosphorylation, the initial step in glycolytic metabolism, with terminal oxidative stages (tricarboxylic acid cycle, with associated electron transport and oxidative phosphorylation; bold curved arrows) occurring in the mitochondria, avoiding the buildup of potentially toxic lactate.

Type II isozyme

The Type II isozyme also includes a hydrophobic N-terminal sequence capable of targeting the hexokinase to mitochondria (Sui and Wilson, 1997). While association of the enzyme with the ‘particulate,’ presumably mitochondrial, fraction in homogenates of normal tissues has been reported (for references see Wilson, 1995), substantial amounts are also generally found in the ‘soluble’ fraction and recent immunolocalization studies (J. E. W., unpublished results) have clearly indicated the presence of non-mitochondrial Type II hexokinase in certain cell types in brain. The extent to which mitochondrially bound Type II hexokinase might be coupled to oxidative phosphorylation has not been, to our knowledge, examined with mitochondria isolated from normal tissues. This isozyme is also expressed at high levels in many tumors (Shinohara et al., 1994) but, in view of the aberrant Glc metabolism associated with tumors, it seems arguable whether the function of Type II hexokinase in tumors can be equated with that in normal tissues. However, experiments with mitochondrially bound Type II hexokinase from AS-30D hepatoma cells did indicate ‘preferred access’ to intramitochondrially generated ATP (Arora and Pedersen, 1988). While the study of Arora and Pedersen (1988) demonstrates the potential for such coupling, extrapolation of these results to the function of Type II hexokinase in normal tissues may be inappropriate. It would definitely be of interest to pursue similar studies with Type II hexokinase bound to mitochondria from normal tissues.

Type III isozyme

The Type III isozyme lacks the hydrophobic N-terminal sequence critical for targeting to mitochondria. Early reports generally indicated that this isozyme was found in the ‘soluble’

fraction of tissue homogenates, presumably indicating a cytoplasmic location, and recent immunolocalization studies have indicated that Type III hexokinase does exist in cytoplasmic regions of cerebellar Purkinje neurons (J. E. W., unpublished results). However, immunolocalization studies have also demonstrated that, at least in many tissues, the Type III isozyme is associated with the nuclear periphery (Preller and Wilson, 1992). The Type III isozyme lacks an obvious classical nuclear targeting sequence, and the structural feature that determines the perinuclear location has not been defined. Also undefined are the possible metabolic implications of such a subcellular location. Given the rather well established metabolic importance of the binding of Type I (and possibly Type II) hexokinase to mitochondria, it would seem reasonable to expect that association of Type III hexokinase with the nucleus may have similar metabolic significance.

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