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Review

Homologous and Heterologous Interactions between Hexokinase and Mitochondrial Porin: Evolutionary Implications

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Binding of the Type I isozyme of mammalian hexokinase to mitochondria is mediated by the porin present in the outer mitochondrial membrane. Type I hexokinase from rat brain is avidly bound by rat liver mitochondria while, under the same conditions, there is no significant binding to mitochondria from *S. cerevisiae*. Previously published work demonstrates the lack of significant interaction of yeast hexokinase with mitochondria from either liver or yeast. Thus, structural features required for the interaction of porin and hexokinase must have emerged during evolution of the mammalian forms of these proteins. *If* these structural features serve no functional role other than facilitating this interaction of hexokinase with mitochondria, it seems likely that they evolved in synchrony since operation of selective pressures on the hexokinase–mitochondrial interaction would require the simultaneous presence of hexokinase and porin capable of at least minimal interaction, and be responsive to changes in either partner that affected this interaction. Recent studies have indicated that a second type of binding site, which may or may not involve porin, is present on mammalian mitochondria. There are also reports of hexokinase binding to mitochondria in plant tissues, but the nature of the binding site remains undefined.

KEY WORDS: Hexokinase; binding to mitochondria; mitochondria, binding of hexokinase to; Porin; VDAC.

INTRODUCTION

Substantial amounts of the Type I isozyme of mammalian hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) are associated with mitochondria in several mammalian tissues, with resulting functional interaction between intramitochondrial oxidative phosphorylation and glucose phosphorylation by the mitochondrially bound hexokinase (see Wilson, 1985, 1995, for reviews). For example, Type I hexokinase bound to actively phosphorylating rat brain mitochondria appears to rely selectively on an intramitochondrial compartment of ATP, generated by oxidative phosphorylation, as a source of this substrate (de Cerqueira Cesar and Wilson, 1995). The physiological significance of this quasi-coupling of the initial step of glucose metabolism and terminal oxidative stages, occurring in the mitochondria, has been discussed extensively (Brdiczka, 1991; BeltrandelRio and Wilson, 1992; Wilson, 1995).

An outer mitochondrial membrane protein responsible for specific binding of hexokinase was first isolated by Felgner *et al.* (1979). This "hexokinase binding protein" was subsequently shown (Fiek *et al.*, 1982; Lindén *et al.*, 1982) to be identical to mitochondrial porin (also called VDAC, voltage-dependent anion channel), which forms the channel through which metabolites enter and exit the mitochondria. It seems likely that this provides a physical basis for metabolic interaction of hexokinase at the mitochon-

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drial surface with intramitochondrial oxidative phosphorylation, mediated by flux of ADP and ATP through the porin channel.

Transport of metabolites across the outer mitochondrial membrane is, of course, a required capability for normal mitochondrial function. Thus, porin is present in mitochondria from diverse sources. Song and Colombini (1996) have recently suggested a common folding pattern for mitochondrial porins; specifically, a pore formed by 13 transmembrane elements—12 amphiphilic β-strands and one α-helix—is proposed. However, at the level of primary structure, only limited conservation of sequence is seen, presumably in those regions critical for establishing the folding pattern. Although the basic structure of porin may be conserved across the biological spectrum, sequence differences may lead to different properties in the porins from different organisms—or even in different porins within the same organism (Blachly-Dyson *et al.*, 1993; Yu and Forte, 1996). Here we are concerned with the ability to bind hexokinase. If the latter capability is related to conserved structural features of porin, the mammalian Type I isozyme should be able to bind to mitochondria from diverse species, e.g., to mitochondria from *S. cerevisiae* and rat liver. On the other hand, if binding of Type I hexokinase is dependent on particular features within the sequence of porin, this might be expected to differ in mitochondrial porins from such widely divergent species.

HOMOLOGOUS AND HETEROLOGOUS INTERACTIONS OF YEAST HEXOKINASE WITH MITOCHONDRIA

Although the yeast and mammalian hexokinases do show extensive similarity in amino acid sequence, the yeast enzyme lacks the hydrophobic N-terminal segment that has been shown to be critical for binding of the Type I mammalian isozyme (Polakis and Wilson, 1985; Gelb *et al.*, 1992) to mitochondria. Thus, yeast hexokinase is inherently incapable of interacting with mitochondria in the same manner as the mammalian Type I hexokinase. In fact, several studies have demonstrated that yeast hexokinase does not bind to rat liver mitochondria (Mannella *et al.*, 1988; Nicolay *et al.*, 1990; Azoulay and Aflalo, 1996), while these same mitochondria avidly bind the mammalian Type I hexokinase (Felgner *et al.*, 1979; Nicolay *et al.*, 1990; Azoulay and Aflalo, 1996).

Does yeast hexokinase bind to yeast mitochondria? Subcellular fractionation (Kovác *et al.*, 1986) as well as immunolocalization studies (van Tuinen and Riezman, 1987) have indicated a lack of association of the endogenous yeast hexokinase with yeast mitochondria. Moreover, *in vitro* studies of Azoulay and Aflalo (1996) failed to demonstrate any binding of yeast hexokinase to yeast mitochondria.

There are, to our knowledge, only two reports to the contrary. Krause *et al.* (1986) incubated isolated outer membranes from yeast mitochondria with commercially obtained yeast hexokinase, and found "binding" of 0.008–0.010 units/mg membrane protein. This is far below the 4.1 units/mg membrane protein found with binding of the Type I isozyme to outer membranes from liver mitochondria (Felgner *et al.*, 1979). This difference becomes even more striking when one considers that the specific activity of the commercially-obtained hexokinase, nominally 450 u/mg, is several-fold higher than that of the Type I isozyme, ≈60 u/mg (Wilson, 1989); thus, in terms of stoichiometry, any binding of the yeast enzyme to yeast mitochondria must be several orders of magnitude less than that seen for mammalian hexokinase interacting with mitochondria of mammalian origin. Assuming that porin represents on the order of 10% of the protein in outer mitochondrial membranes (Freitag *et al.*, 1982; De Pinto *et al.*, 1987), the levels of binding mentioned above correspond to hexokinase:porin molar ratios of 10^{-4} and 0.2 for the yeast and Type I hexokinases, respectively. Admittedly these ratios must be taken as only crude estimates, but nonetheless they emphasize that any binding of the yeast enzyme is well below that seen with the mammalian enzyme.

Whether even this low level is truly "binding" may be questioned when one examines the protocol of Krause *et al.* (1986), in which 10 *units* of yeast hexokinase were incubated with 0.6 mg membrane protein. Thus, binding of 0.008–0.010 u/mg corresponds to finding 0.05–0.06% of the original activity in the pellet; this could easily be attributed to trapping of enzyme within the pellet, which was not washed prior to assay for "bound" activity. Similar concerns attend the study of Forte *et al.* (1987), who reported "binding" of 0.007 u of commercially obtained yeast hexokinase per mg outer membrane protein from yeast mitochondria.

In short, we believe there is no credible evidence for significant binding of yeast hexokinase to liver mitochondria *or* to yeast mitochondria.

HOMOLOGOUS AND HETEROLOGOUS INTERACTIONS OF MAMMALIAN TYPE I HEXOKINASE WITH MITOCHONDRIA

To our knowledge, there has been only one previous study comparing the binding of Type I hexokinase to yeast and mammalian (liver) mitochondria under identical conditions. Azoulay and Aflalo (1996) reported that, as expected, the rat Type I isozyme bound avidly to liver mitochondria but there was only a "weak association" of the Type I isozyme to mitochondria from *S. cerevisiae*.[2] Results from our own laboratory (Fig. 1) would indicate an even more striking difference in the ability of yeast and liver mitochondria to interact with the rat Type I isozyme—under the same conditions in which substantial amounts of the Type I hexokinase were bound to liver mitochondria, *no* binding to yeast mitochondria was detected, i.e., <5% of the hexokinase was found in the pelleted yeast mitochondria, equivalent to that found in the "pellet" in the absence of added mitochondria. As previously demonstrated (Felgner and Wilson, 1977), binding to liver mitochondria was markedly dependent on divalent cations; omission of $MgCl_2$ greatly diminished the binding of the Type I isozyme (Fig. 1). Blachly-Dyson *et al.* (1993) have also reported only "background" (which we interpret to mean "insignificant") levels of binding of the Type I isozyme to mitochondria from wild type *S. cerevisiae* or from a mutant strain *lacking* mitochondrial porin. All of these results lead to the conclusion that there is little, if any, interaction of the mammalian Type I isozyme with yeast mitochondria (and thus with yeast mitochondrial porin) under conditions in which the enzyme is avidly bound through homologous interactions with liver mitochondria.

It is evident from the above that binding of Type I hexokinase to mitochondria isolated from yeast expressing mutated forms of porin (Blachly-Dyson *et al.*, 1990, 1993) would provide a useful assay system for elucidation of particular residues within the porin sequence that are critical for binding of mammalian hexokinase, i.e., mutations conferring binding ability would readily be detected against a null background.

[2] Azoulay and Aflalo (1996) reported that substantial binding of the Type I isozyme to yeast mitochondria did occur when 25% dextran was added to the medium. This was attributed to "crowding", i.e., the concentration of hexokinase was effectively increased by the volume exclusion effect resulting from addition of the macromolecule. However, it remains to be determined whether this interaction was indeed analogous to that seen with mammalian mitochondria or an anomalous interaction induced by dextran.

EVOLUTIONARY IMPLICATIONS

A reasonable hypothesis emerging from these results would be that structural features conferring the ability to interact with the mammalian Type I isozyme have been acquired in the course of evolution of the mammalian porins. It is also evident that the structural features of hexokinase, required for binding to mitochondria and lacking in the yeast enzyme, were likely acquired in the course of evolution of the mammalian hexokinases (Wilson, 1995; Tsai and Wilson, 1996). Do these features, in either (or both) mitochondrial porin and hexokinase, have some function other than facilitating this interaction? If so, what might it be? If not, this implies a synergism during the evolutionary process. That is, if the sole purpose of the hydrophobic N-terminal sequence of Type I hexokinase is to facilitate binding to mitochondria, it is difficult to envisage how this structural feature would be acquired unless it was expressed in a physiological context in which its function could be subject to selective pressures. This would obviously imply the simultaneous presence of a mitochondrial porin capable of at least minimal interaction with the hexokinase. Thus, evolution of the molecular features on porin and on hexokinase, necessary for their interaction, would have occurred in synchrony. Selective pressures operating on the hexokinase–mitochondrial interaction would be responsive to changes, in either partner, that affected this interaction.

SOME ADDED COMPLEXITY: MORE THAN ONE TYPE OF BINDING SITE FOR HEXOKINASE ON MITOCHONDRIA

The role of mitochondrial porin in binding of Type I mammalian hexokinase is well established (Fiek *et al.*, 1982; Lindén *et al.*, 1982; Nakashima *et al.*, 1986) though not completely understood. Although a direct physical interaction between porin and hexokinase has not been demonstrated (e.g., by crosslinking), this remains the most straightforward interpretation of available results.

A hallmark of the binding of Type I hexokinase to porin purified from rat liver mitochondria (Felgner *et al.*, 1979) is that it is specifically sensitive to reversal

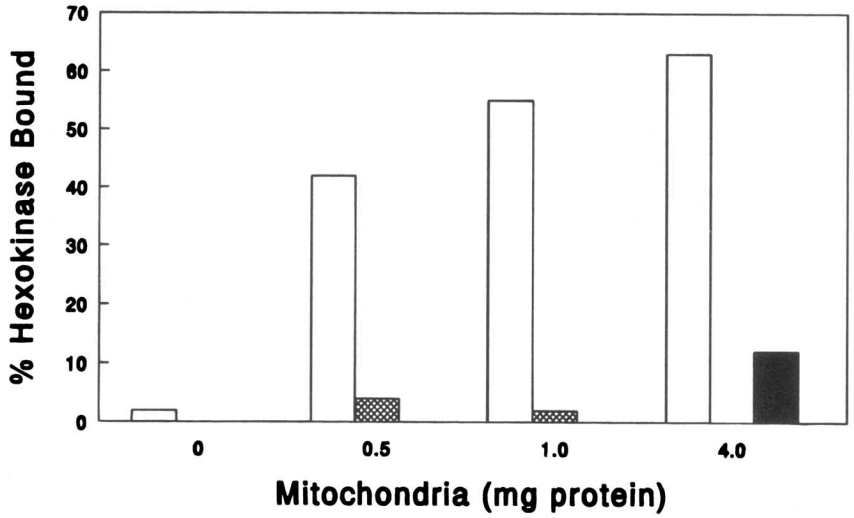

Fig. 1. Comparison of the binding of rat Type I hexokinase by rat liver and yeast mitochondria. Type I hexokinase was prepared from rat brain mitochondria by solubilization with glucose 6-phosphate; the procedure was essentially as previously described (Wilson, 1989) except that the buffer used was 0.25 M sucrose, 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, subsequently referred to as SH. Binding was assayed by incubating hexokinase (0.1–0.15 units in total volume of 0.5 ml) with the indicated amounts of mitochondria from rat liver (Sottocasa *et al.*, 1967) or *S. cerevisiae* (strain D273–10B, MATα) (Glick and Pon, 1995), suspended in SH containing 1.5 mM $MgCl_2$. Consistent with previous reports, both rat liver (Wilson and Felgner, 1977; Felgner *et al.*, 1979; Polakis and Wilson, 1985; Laterveer *et al.*, 1994) and yeast (Kovác *et al.*, 1986) mitochondria were devoid of endogenous bound hexokinase. After incubation for 30 min on ice, mitochondria were pelleted by microcentrifugation. Supernatants were assayed (Wilson, 1989) for unbound hexokinase, and then carefully removed with a constricted Pasteur pipette. Pellets were resuspended in 0.5 ml SH containing 0.5% (v/v) Triton X-100, then assayed to determine bound hexokinase activity. Bound activity is expressed as percent of the total activity (supernatant plus pellet); total activity recovered in supernatant plus pellet was ≥95% of initial activity added to the tube. For the experiment shown, total hexokinase activity present was 0.13 units. Open bars: results with no mitochondria or with the indicated amount of rat liver mitochondria. Crosshatched bars: mitochondria from *S. cerevisiae*. To illustrate the dependence on divalent cations, the filled bar shows binding to rat liver mitochondria in the absence of $MgCl_2$.

by glucose 6-phosphate. Binding of this ligand induces a conformational change in the enzyme which, among other things, results in release of the enzyme from its mitochondrial binding site (Wilson, 1985; Wilson and Chung, 1989). Binding of the enzyme to rat brain mitochondria, presumably to the porin therein, is also effectively and selectively reversed by incubation of the mitochondria with glucose 6-phosphate—and this, in fact, provides a convenient basis for purification of the Type I isozyme from rat brain (Wilson, 1989). It was, therefore, surprising to find a marked variation in the extent to which glucose 6-phosphate caused release of hexokinase bound to brain mitochondria from other species (Kabir and Wilson, 1993). To cite the extremes, approximately 90% of the enzyme is released by incubation of rat brain mitochondria with glucose 6-phosphate while, under the same conditions, only about 20% of the enzyme is released from human brain mitochondria. Kabir and Wilson (1993) were led to the conclusion that this is not due to any heterogeneity in the enzyme but rather, that there are two types of mitochondrial binding sites, which can coexist on a single mitochondrion (Kabir and Wilson, 1994). Binding of hexokinase to the first type of site is reversed in the presence of glucose 6-phosphate while hexokinase bound to the second type of site is not solubilized by, but *is* still inhibited by, this ligand. The relative proportions of the two types of sites vary among species; presumably this may have physiological significance, but this remains to be defined. While the first type of site is, based on previous studies, assumed to involve porin, it is not at all clear whether

this is also the case for the second type of site (from which hexokinase is not released by glucose 6-phosphate). Thus, the possibility that hexokinase might also bind to mitochondria in a manner not involving porin needs to be kept in mind.

BINDING OF HEXOKINASE TO MITOCHONDRIA IN PLANTS

By far the majority of the work on mitochondrially bound hexokinase has been done with mitochondria from animal tissues. This is probably, at least partially, by default. Thus, as discussed above, there appears to be no significant association of yeast hexokinase with mitochondria, homologous or heterologous. And, of course, prokaryotes are automatically excluded by virtue of their defining lack of intracellular organelles. But mitochondria do exist in plants and it is of more than passing interest that substantial portions, on the order of 30–50% of the total hexokinase activity, have been found to be associated with the mitochondrial fraction in homogenates of various plant tissues (Copeland and Tanner, 1988; Schnarrenberger, 1990; Galina *et al.*, 1995). The nature of the mitochondrial protein(s) involved in interaction with hexokinase is completely undefined, and there appear to have been no studies on the extent to which the bound enzyme is released by glucose 6-phosphate. Moreover, plant hexokinases generally are very poorly characterized and it is difficult to make any meaningful comparison with the studies that have been done with mammalian systems. In fact, it is only recently that the amino acid sequence has become available for any plant hexokinase, when the sequence of the hexokinase from *Arabidopsis thaliana* was deduced from the cloned cDNA (Dai *et al.*, 1995). Extensive sequence similarity with the hexokinases from yeast and mammalian sources (Wilson, 1995) confirms that the *A. thaliana* enzyme is indeed homologous. As with yeast hexokinase, the *A. thaliana* enzyme lacks the hydrophobic N-terminal segment known to be critical for binding of the mammalian Type I isozyme to mitochondria (Polakis and Wilson, 1985; Gelb *et al.*, 1992). Thus it may confidently be predicted that at least this plant enzyme is incapable of binding to mitochondria in the same manner as the mammalian Type I isozyme. Whether binding might occur in some other manner, to a site which may or may not involve porin, is unknown, as are the possible physiological consequences of such interaction. The extent to which the *A. thaliana* enzyme resembles the hexokinases thought to be associated with mitochondria in other plant systems (Copeland and Tanner, 1988; Schnarrenberger, 1990; Galina *et al.*, 1995) also remains to be determined. Has the ability to bind to mitochondria, to porin or otherwise, evolved independently in the plant kingdom?

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